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INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS (IJPBP)

Int. J. Plant Bas. Pharm.

e-ISSN: 2791-7509

This is an international peer-reviewed Open Access journal and published two issues per year

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Publisher

Kilis 7 Aralik University
(Publication No: 029)
Address:
Mehmet Sanli Mah. Dogan Gures Pasa Bul. No: 134
Kilis-TURKEY
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Volume: 2 Issue: 1 June, 2022

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An International Peer Reviewed Open Access Journal
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Journal Name:	International Journal of Plant Based Pharmaceuticals
Journal Abbreviation:	Int. J. Plant Bas. Pharm.
Scope & Subjects:	Researchers are cordially invited to submit their manuscripts to the International Journal of Plant Based Pharmaceuticals (IJPBP), an international and peer-reviewed open access journal devoted to the publish original research papers, review articles, short communications, letters to the editor/commentary and guest edited single topic issues in all areas of plant-based pharmaceuticals in English.
e-ISSN:	2791-7509
Publisher:	Kilis 7 Aralik University (No: 029)
Language:	English
Frequency:	Biannually (June and December)
Type of Publication:	Peer Review Double-Blinded
Publication Fee:	No Submission or Processing Charges
Access type:	Open Access
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e-ISSN: 2791-7509

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International Journal of Plant BasedPharmaceuticals (IJPBP) is a peer-reviewed open access journal for original research articles, review articles and short communications related to all aspects of plant based pharmaceuticals and pharmaceutical analysis. IJPBP was launched in 2021, and published biannually by Kilis 7 Aralik University (Kilis-TURKEY).

Current areas of interest include the following areas related to plant based pharmaceuticals, but are not limited to:

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- Quality control and methods of biotech drugs
- Action mechanism and their metabolisms in body
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- Tracer analysis in molecular pharmacology
- Quantitative analysis in biopharmaceutics
- Clinical laboratory and bioanalysis
- Analysis of pharmacological activity
- Analysis of drug toxicity
- Analytical chemistry techniques and methods
- New biochemistry methods for pharmaceutical analysis
- Rapid screening methods
- New analytical techniques and methods
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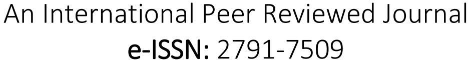

An International Peer Reviewed Journal
e-ISSN: 2791-7509

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Int. J. Plant Bas. Pharm. (2022) 2(1), 1-16

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS
https://ijpbp.com

RESEARCH ARTICLE OPEN ACCESS

Chemical composition and cholinesterase, tyrosinase, alpha-amylase and alpha-glucosidase inhibitory activity of the essential oil of *Salvia tomentosa*

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ARTICLE INFO

Article History:

Received: 10 October 2021
Revised: 25 October 2021
Accepted: 25 October 2021
Available online: 27 October 2021

Edited by: B. Tepe

Keywords:
Salvia tomentosa
Essential oil
Enzyme inhibitory activity
Molecular docking
ADMET
Drug likeness

ABSTRACT

The aim of this study was to determine the chemical composition of *Salvia tomentosa* (Miller) essential oil and to examine its inhibitory effect on acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase, α-amylase and α-glucosidase *in vitro*. In this study, the interaction between the main components of essential oil and the enzymes in question was analyzed through molecular docking analyses. The presence of 60 compounds representing 98.2% of the essential oil was determined. The major compounds of the oil were camphor (9.35%), γ-muurolene (8.37%), α-pinene (7.59%), α-caryophyllene (6.25%), viridiflorol (5.13), δ-cadinene (5.01%), and terpinene-4-ol (5.01 %). The oil exhibited higher inhibitory activity on BChE than on AChE. The BChE inhibitory activity of the oil was determined to be 16.48 mg GALAEs/g. The oil showed 47.13 mg KAEs/g inhibitory activity on tyrosinase. The inhibitory activities of the essential oil on α-glucosidase and α-amylase were determined as 703.29 and 694.75 mg ACEs/g, respectively. Based on docking binding energies, δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene were determined to be the most promising ligands showing the highest affinity (min. -6.90 kcal/mol; max. -8.40 kcal/mol) against α-amylase, AChE and BChE. However, all four ligands were found to exhibit low affinity (min. -5.50 kcal/mol; max. -5.90 kcal/mol) against tyrosinase. Considering *in silico* physicochemical properties, drug-like features (Lipinski's rule of 5) and intracellular targets, δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene possess hit features and do not show non-specific enzyme or protein affinity. Ligand binding assays (LBA) to be performed between the monoterpenes and enzymes in question may constitute the next step in confirming their competitive inhibitory capacity.
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1. Introduction

Studies on the use of cholinesterase inhibitors in the treatment of neurodegenerative diseases that cause weakening in cognitive functions such as Alzheimer's disease have been going on for decades. Galanthamine and physostigmine, which are acetylcholinesterase (AChE) inhibitors, can be used temporarily to alleviate the symptoms of the disease. However, these compounds are not suitable for long-term use due to their undesirable side effects. In addition to the compounds mentioned above, many compounds of plant origin can exhibit AChE inhibitory effects. Unfortunately, the effects of these phytochemicals are either not selective or remain limited (Perry and Howes, 2011). Phytochemicals can act on both AChE and butyrylcholinesterase (BChE). However, in the treatment of Alzheimer's disease, the focus is on the discovery of compounds with AChE inhibitory effects. In addition, it is suggested that BChE inhibitors may cause significant regression on the symptoms of the disease and reduce cognitive dysfunction (Li et al., 2008). Many studies have reported that cholinesterases can be inhibited by phytochemicals (Orhan et al., 2018; Orhan et al., 2017; Pinho et al., 2013; Politeo et al., 2018).

Products that reduce hyperpigmentation on the skin have become very popular in recent years, especially in the Asian markets (Pérez Gutierrez et al., 2006). For this reason, researchers began to carry out extensive research on compounds that are effective in

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e-ISSN: 2791-7509
doi:
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Please cite this article as: Kocer, M., Istifli, E.S., 2022. Chemical composition and cholinesterase, tyrosinase, alpha-amylase and alpha-glucosidase inhibitory activity of the essential oil of Salvia tomentosa. International Journal of Plant Based Pharmaceuticals, 2(1), 1-16.

lightening dark skin color due to excessive melanin accumulation (Ko et al., 2014; Murata et al., 2014; Tsai et al., 2013). The most critical enzyme involved in the skin pigmentation process is tyrosinase (Fukai et al., 2005). Researchers suggest that compounds with tyrosinase inhibitory effects can overcome skin darkening due to excessive melanin production. Today, there are some compounds with proven tyrosinase inhibitory effects such as hydroquinone, azelaic acid, kojic acid, and arbutin (Chang, 2009; Davis and Callender, 2010). However, it is known that these tyrosinase inhibitors exhibit some side effects such as mutagenicity, irritant and cytotoxicity on the body, and may even cause contact dermatitis or erythema (Lien et al., 2014). Therefore, experts agree that plant-derived compounds can be a natural tyrosinase inhibitory alternative. A large number of tyrosinase inhibitory-effective phytochemicals have been brought to the literature with studies carried out so far (Burlando et al., 2017; Chang, 2012).

Diabetes is an important health problem that affects the metabolism of organic macromolecules, especially carbohydrates, and causes deterioration in many other body functions (Mechchate et al., 2021b; Sacks, 1997). It is estimated that by 2030, the number of diabetic patients will approach 400 million worldwide (Wild et al., 2004). In these patients, some synthetic agents such as α-amylase/α-glucosidase inhibitors, biguanides and sulfonylureas are used to lower high blood sugar. However, long-term use of these drugs leads to various side effects such as hypoglycemia, nausea, dizziness, and excessive weight gain (Chaudhury et al., 2017; Edwin et al., 2006). Therefore, researchers sought to find safer alternatives to lower blood glucose levels (Es-Safi et al., 2021). Because plants have been used by local people in the treatment of diabetes since ancient times, plants are among the first resources that researchers refer to in search of anti-diabetic compounds (Mechchate et al., 2020; Mechchate et al., 2021a).

Salvia tomentosa (Miller) is one of the most consumed herbal teas. In addition, it has a wound-healing effect similar to iodine tincture. It contains significant amounts of secondary metabolites such as phenolics and terpenoids, which have antimicrobial (Askun et al., 2010; Haznedaroglu et al., 2001) and antioxidant (Erdogan-Orhan et al., 2010) effects. There are some studies on the chemical composition, antimicrobial and insecticidal activity of the essential oil obtained from this plant (Haznedaroglu et al., 2001; Tepe et al., 2005; Ulukanli et al., 2013). In this study, it was aimed to determine the chemical composition of the essential oil obtained from *S. tomentosa*, and to investigate its inhibitory activity on AChE, BChE, tyrosinase, α-amylase and α-glucosidase. In this study, the interaction between phytochemicals and enzymes, which are found in high amounts in the oil, was also examined through molecular docking and it was determined whether these compounds were responsible for the inhibitory activity.

2. Materials and methods

2.1. Plant material

The plant material (*S. tomentosa*) was collected from Osmaniye, Düziçi, Söğütlügöl village. After the plant material was dried in a cool environment without direct sunlight, it was subjected to water distillation for 3 hours using the Clevenger apparatus. Anhydrous sodium sulfate was added to the obtained essential oil to purify it from water, and it was kept at refrigerator temperature (+4 °C) until activity studies were carried out.

2.2. Determination of chemical composition of essential oil

The chemical composition of the essential oil was determined chromatographically by using GC-FID and GC-MS analysis. The analyzes of the oil were performed following the analytical conditions specified in the literature (Sarikurkcu et al., 2015).

2.3. Determination of cholinesterase inhibitory activity

Cholinesterase inhibitory activity test was performed in 96-well microplates using the Ellman method (Ellman et al., 1961). 125 µl of DTNB, 25 µl of ATCI or BTCI solutions were added to the microplate wells containing 50 µl of sample solution. Then, 25 µl of AChE or BChE solution prepared in tris-HCl buffer (pH 8.0) was added to this mixture. For each sample, a blank sample was prepared using tris-HCl buffer instead of the enzyme solution. All samples were incubated at 25 °C for 15 min and absorbance measurements of both blank and samples were made at 405 nm. The absorbance of the blank sample was subtracted from that of the sample, and the cholinesterase inhibitory activity was expressed as both inhibition (%) and galanthamine equivalents (µg GALAEs/g sample) (Ellman et al., 1961). Each measurement was made in triplicate and expressed as mean and standard deviation. Galanthamine was used as a positive control.

2.4. Determination of tyrosinase inhibitory activity

The tyrosinase inhibitory activity test was performed in 96-well microplates following the dopachrome method using L-DOPA (40 µl, 10 mM) as a substrate (Orhan et al., 2014). 100 µl of phosphate buffer (pH 6.8) and 40 µl of tyrosinase solution prepared in this buffer were added to the microplate wells containing 25 µl of sample solution. For each sample, a blank was prepared using phosphate buffer instead of enzyme solution. All samples were incubated for 15 min at 25 °C and absorbance measurements of both blank and sample were performed at 492 nm. The absorbance of the blank was subtracted from that of the sample and the tyrosinase inhibitory activity was calculated in terms of both inhibition (%) and kojic acid equivalent (mg KAEs/g sample) (Orhan et al., 2014). Each measurement was made in triplicate and expressed as mean and standard deviation. Kojic acid was used as a positive control.

2.5. Determination of α-amylase inhibitory activity

The α-amylase inhibitory activity test was performed in 96-well microplates using the Caraway-Somogyi I_2/KI method (Zengin et al., 2014). 50 µl of starch solution and 25 µl of α-amylase solution prepared in phosphate buffer (pH 6.9) were added to the microplate wells containing 25 µl of sample solution. A blank sample was prepared for each sample, using phosphate buffer instead of enzyme solution. All samples were incubated at 25 °C for 15 min and then 25 µl of HCl solution (1 M) was added to stop the reaction. Then, 100 µl of I_2/KI was added to all samples and absorbance measurements were performed at 630 nm. The absorbance of the blank was subtracted from that of the sample and the α-amylase inhibitory activity was expressed in terms of both inhibition (%) and acarbose equivalents (mg ACEs/g sample). Each measurement was made in triplicate and expressed as mean and standard deviation. Acarbose was used as a positive control.

2.6. Determination of α-glucosidase inhibitory activity

The inhibitory activity of the essential oil on *S. tomentosa* was determined using the method of Palanisamy et al. (2011). 50 µL of

PNPG, 50 µL of glutathione, and 50 µL of α-glucosidase in phosphate buffer (pH 6.8) were mixed in a 96-well microplate and 50 µL of sample solution was added onto this mixture. The mixture was incubated for 15 min at 37 °C. To prepare the blank, the same mixture without α-glucosidase solution was prepared. The reaction was terminated by adding 50 µL of 0.2 M sodium carbonate. The absorbance measurements were performed at 400 nm. The absorbance of the blank was subtracted from that of the sample and the α-glucosidase inhibitory activity was expressed as inhibition (%) and acarbose equivalents (mg ACEs/g sample).

2.7. Molecular docking analysis

To reveal the molecular scale contribution of the main essential oil components to the biological activity, *in silico* studies were performed following the method documented by Istifli et al. (2020). In this study, AMDock Vina program was used as the software for docking simulations. AMDock Vina is a graphical tool that facilitates user-friendly docking simulations that can be performed with Autodock Vina or AutoDock4. AMDock Vina uses various external programs (Open Babel, PDB2PQR, AutoLigand, ADT scripts) to prepare input structure files correctly and define an optimal search space. AMDock Vina also provides the option to use Autodock4Zn force field in molecular docking experiments with metalloproteins. Compatible with Windows or Linux, AMDock Vina can be downloaded from https://github.com/Valdes-Tresanco-MS. In addition, the UCSF Chimera 1.14 program used for addition of missing atoms in the protein side chains and assigning electrical charges before docking was downloaded from the web address https://www.cgl.ucsf.edu/chimera/download.html.

2.7.1. Protein and ligand preparation

In this study, the receptor 3D structures used in molecular docking simulations (human pancreatic α-amylase, human butyrylcholinesterase, human recombinant acetylcholinesterase and Bacillus megaterium tyrosinase) were retrieved from Protein Data Bank (PDB) with codes 1B2Y (resolution: 3.20 Å), 4BDS (resolution: 2.10 Å), 4EY6 (resolution: 2.40 Å) and 5I38 (resolution: 2.60 Å).

Before docking, inhibitors and heteroatoms (ex. water molecules) complexed with receptors were removed from the crystallographic structures using the Discovery Studio Visualizer v16 program. Calcium and chloride ions in the active site of the α-amylase enzyme and two copper ions in the active site of the tyrosinase enzyme were retained in complex with the catalytic site, since calcium and copper ions are important for these enzymes to perform their physiological functions (Aghajari et al., 2002; Solano, 2018). In general, metal binding affects the stability and catalytic properties of proteins. In addition, the missing atoms and electrical charges in the amino acid side chains of each protein were added using the UCSF Chimera 1.14 program (Pettersen et al., 2004). In our study, the crystallographic structures of α-pinene, camphor, terpinen-4-ol, α-caryophyllene, γ-muurolene, δ-cadinene and viridiflorol were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in sdf (structure data file) format and saved in pdb format after performing geometric optimization in the Avogadro program using the MMFF94 (Merck molecular force field) force field suitable for organic molecules (Hanwell et al., 2012).

2.7.2. Molecular docking study

Molecular docking is a bioinformatics method that allows the estimation of the optimal spatial orientation of ligand atoms in the catalytic cavity of a protein when two biomolecules (protein-ligand or protein-protein) react to form a stable complex under the laws of thermodynamics. In our study, molecular docking calculations of α-pinene, camphor, terpinen-4-ol, α-caryophyllene (α-humulene), γ-muurolene, δ-cadinene and viridiflorol against pancreatic α-amylase, acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and tyrosinase enzymes were carried out. Thus, the lowest energy (most favorable) conformations (pose) of the relevant small molecules in the catalytic sites of these enzymes were predicted and their binding free energies (kcal/mol) were calculated.

AMDock, a docking interface built on the AutoDock Vina algorithm, was implemented for molecular docking calculations of α-pinene, camphor, terpinen-4-ol, α-caryophyllene, γ-muurolene, δ-cadinene and viridiflorol against four enzymes (https://github.com/Valdes-Tresanco-MS/AMDock-win) (Trott and Olson, 2010; Valdes-Tresanco et al., 2020). Grid coordinates (search space) were adjusted to allow ligands to easily interact with the catalytic amino acid residues of these target proteins. In the Discovery Studio Visualizer v16 program, the active sites (groove or pocket) of the four different enzymes were determined by visual inspection of their amino acid residues with which the co-crystallized inhibitors interacted and the corresponding cartesian coordinates were recorded.

Such an approach for the determination of the catalytic site should be logical since it is based on the localization of the experimental inhibitor(s) on the protein molecule. Because the ligand with a similar or more negative binding free energy (binding affinity) compared to the inhibitor may inhibit the protein in question. On the basis of this approach, the grid coordinates (active site) of the target enzymes were adjusted as follows: a) 40 × 40 × 40 Å (x: 18.90, y: 5.79, z: 47) for the α-amylase; b) 82 × 56 × 54 Å (x: -9.94, y: -43.48, z: 30.29) for the AChE; c) 70 × 62 × 54 Å (x: 132.99, y: -116.01, z: 41.21) for the BChE and, d) 48 × 50 × 52 Å (x: 1.85, y: 101.73, z : 25.19) for the tyrosinase.

In the configuration settings prepared for molecular docking, the value of 'exhaustiveness' was set as '56' and the number of independent docking runs (number of poses) was set as '20'. In each docking iteration, all potential binding modes (conformations) of α-pinene, camphor, terpinen-4-ol, α-caryophyllene, γ-muurolene, δ-cadinene, and viridiflorol were clustered by AutoDock Vina, and the ligand conformations obtained against α-amylase, AChE, BChE and tyrosinase enzymes were ranked from the most negative to the least according to their binding free energies (ΔG°; kcal/mol). The best docking poses calculated by AMDock against protein targets of the seven ligands studied were visualized using the Discovery Studio Visualizer v16 program, and non-covalent chemical interactions were characterized at the molecular level.

2.8. Determination of 'relative binding capacity index' (RBCI) values of essential oil major components

RBCI values were calculated considering the affinities of the main components in the essential oil for all targets. For this purpose, the method developed by Istifli et al. (2020) was followed. Thus, 'hit' components showing higher affinity for all target structures were identified and the best poses (top-ranked conformations) of these components were presented.

2.9. Clarification of ADMET and drug-likeness profiles of 'hit' compounds, identification of possible intracellular targets

Identification of drug-likeness, absorption-distribution-metabolism-excretion and toxicity (ADMET) and intracellular target profiles of

promising 'hit' compounds in structure-based drug design (SBDD) studies is crucial to understand possible side effects of these bioactive molecules on the target organism. In this study, web-based SwissADME, pkCSM and SwissTargetPrediction tools were used to analyze such effects of the terpenoids in question (Daina et al., 2017; Daina et al., 2019; Delaney, 2004; Pires et al., 2015). In addition, the interaction probabilities of 'hit' molecules with various classes of intracellular targets were also given as maximum and minimum numerical values. ADMET and drug-likeness profiles and possible intracellular targets of the compounds identified as 'hit' as a result of RBCI analyzes were determined according to Istifli et al (2020).

3. Results and discussion

3.1. Chemical composition of the essential oil

The chemical composition of the essential oil was determined chromatographically by GC-FID and GC-MS analysis. The data obtained are given in Table 1.

According to the data in Table 1, the presence of 60 compounds representing 98.2% of the essential oil was determined as a result of the quantitative chromatographic analyzes performed. The major compounds of the oil were camphor (9.35%), γ-muurolene (8.37%), α-pinene (7.59%), α-caryophyllene (6.25%), viridiflorol (5.13), δ-cadinene (5.01%), and terpinene- 4-ol (5.01 %).

As a result of the literature research, some studies were found in which the chemical composition of *S. tomentosa* essential oil was investigated (Askun et al., 2010; Bardakci et al., 2019; Hanlidou et al., 2014; Haznedaroglu et al., 2001; Nagy et al., 1999; Özcan et al., 2002; Soltanbeigi and Sakartepe, 2020; Tepe et al., 2005; Ulubelen et al., 1981a, b; Ulukanli et al., 2013; Yilar et al., 2018). In these studies, the presence of α-pinene, β-pinene, 1,8-cineole, borneol, camphor, α-thujene, *cis*-thujone, camphene, α-caryophyllene, cyclofenchene, δ-cadinene, some diterpenoids and triterpenoids were reported as main components.

The essential oil data obtained from the present study are largely in agreement with the literature data. However, some of the major compounds (terpinen-4-ol, α-caryophyllene, γ-muurolene and viridiflorol) determined in the current study were not found as major compounds in previous studies with the essential oil of the plant in question. This difference is thought to be caused by factors such as differences in the area where the plant is collected, climate and soil structure, harvest time, etc.

3.2. Cholinesterase inhibitory activity potential of the essential oil

The data obtained from the AChE and BChE inhibitory activity tests of *S. tomentosa* essential oil are given in Table 2. The data in the table in question were presented in terms of both positive control (galanthamine) equivalent and IC_{50} (mg/ml).

The essential oil exhibited higher inhibitory activity on BChE than on AChE in terms of galanthamine equivalent. This situation is also reflected in the enzyme inhibitor activity data calculated in terms of IC_{50}. The BChE inhibitory activity of the essential oil was determined to be 16.48 mg GALAEs/g essential oil and 1.30 mg/ml in terms of galanthamine equivalent and IC_{50}, respectively. As can be seen from the data in the table, the essential oil exhibited quite low activity compared to galanthamine.

Table 1. Chemical composition of the essential oil of *S. tomentosa*

No	KI	Compound	Amount (%)
1	926	α-Thujene	0.16
2	935	α-Pinene	7.59
3	947	Camphene	1.66
4	974	β-Pinene	4.16
5	990	β-Myrcene	1.04
6	999	α-Phellandrene	0.40
7	1010	α-Terpinene	1.57
8	1028	Limonene	3.93
9	1035	*cis*-Ocimene	3.72
10	1050	γ-Terpinene	3.36
11	1061	*cis*-Sabinene hydrate	0.78
12	1065	*cis*-Linalool oxide (furanoid)	1.59
13	1074	*p*-cymenene	0.23
14	1079	Terpinolene	0.59
15	1083	Nonanal	0.13
16	1085	α-Pinene oxide	0.87
17	1097	*trans*-Sabinene hydrate	0.43
18	1099	Linalool	0.89
19	1105	*cis*-Thujone	0.43
20	1115	*trans*-Thujone	1.15
21	1125	α-Campholenal	0.68
22	1136	*trans*-Pinocarveol	1.24
23	1143	Camphor	9.35
24	1154	Borneol	4.05
25	1158	*cis*-Pinocamphone	0.21
26	1176	Terpinen-4-ol	5.01
27	1186	α-Terpineol	0.66
28	1286	Bornyl acetate	3.89
29	1330	α-Terpinyl acetate	0.21
30	1350	α-Cubebene	0.26
31	1370	α-Ylangene	0.29
32	1374	α-Copaene	0.18
33	1382	β-Bourbonene	0.37
34	1385	β-Cubebene	0.19
35	1439	Aromadendrene	0.23
36	1455	α-Caryophyllene	6.25
37	1477	γ-Muurolene	8.37
38	1481	α-Amorphene	0.94
39	1485	Germacrene D	1.27
40	1514	δ-Cadinene	0.72
41	1523	δ-Cadinene	5.01
42	1527	α-Cadinene	0.77
43	1532	α-Calacorene	0.30
44	1535	Germacrene B	0.19
45	1537	β-Elemol	0.36
46	1538	Selina-3.7(11)-diene	0.25
47	1547	β-Calacorene	1.14
48	1550	*(E)*-Nerolidol	0.13
49	1562	Palustrol	0.68
50	1568	Germecrene-D-4-ol	0.26
51	1571	Ledol	0.71
52	1575	(-)-Spathulenol	0.85
53	1593	Viridiflorol	5.13
54	1602	Guaiol	0.55
55	1639	τ-Cadinol	0.54
56	1646	α-Cadinol	0.50
57	1648	α-Muurolol	0.47
58	1670	Cadalene	0.56
59	1672	*epi*-β-Bisabolol	0.36
60	1687	α-Bisabolol	0.39
		Total	98.2

There is no study in the literature on the cholinesterase inhibitory activity of *S. tomentosa* essential oil. However, it is possible to refer to some literature data on the contribution of the oil major compounds to cholinesterase inhibitory activity. In a study investigating the inhibitory effect of camphor and α-pinene on human erythrocyte AChE, the inhibitory activity of α-pinene was found to be 0.63 mM, while camphor provided non-competitive reversible inhibition on the enzyme in question (Perry et al., 2000). This data reported for camphor has been confirmed by other researchers (Lopez et al., 2015; Savelev et al., 2003). In a study conducted by Ertas et al. (2014), it was determined that the essential oil obtained from *Lycopsis orientalis* contained 9.6% *tau*-muurolene (γ-muurolene isomer) as the major compound and

showed significant inhibitory activity on both AChE and BChE at a concentration of 200 µg/ml. In another study investigating the inhibitory activities of caryophyllene and terpinen-4-ol, the inhibitory activities of these terpenoids against BChE were determined to be 78.6 and 107.6 µg/ml in terms of IC_{50}, respectively (Bonesi et al., 2010). In the literature, no study was found that δ-cadinene and viridiflorol can contribute to cholinesterase inhibitory activity.

Table 2. Enzyme inhibitory activity potential of *S. tomentosa* essential oil

Enzyme	EC number	CAS number	Positive control equivalent activity	IC_{50} (mg/ml)	Galanthamine	Kojic acid	Acarbose
AChE[1]	3.1.1.7	9000-81-1	3.26 ± 0.47	1.35 ± 0.19	0.0043 ± 0.0003	-	-
BChE[1]	3.1.1.8	9001-08-5	16.48 ± 0.39	1.30 ± 0.03	0.0022 ± 0.0006[a]	-	-
Tyrosinase[2]	1.14.18.1	9002-10-2	47.13 ± 0.95	1.73 ± 0.03	-	0.08 ± 0.002	-
α-Amylase[3]	3.2.1.1	9000-90-2	694.75 ± 10.78	1.51 ± 0.02	-	-	1.05 ± 0.04
α-Glucosidase[3]	3.2.1.10	9001-42-7	703.29 ± 8.02	0.97 ± 0.01	-	-	0.68 ± 0.04

[1] mg GALAEs/g essential oil, [2] mg KAEs/g essential oil, [3] mg ACEs/g essential oil

3.3. Tyrosinase inhibitory activity potential of the essential oil

The data obtained from the tyrosinase inhibitory activity test of *S. tomentosa* essential oil are given in Table 2. The data in the table in question are presented in terms of both positive control (kojic acid) equivalents and IC_{50} (mg/ml).

As can be seen from Table 2, the essential oil of *S. tomentosa* showed 47.13 mg KAEs/g activity on tyrosinase in terms of kojic acid equivalent. The equivalent of this activity in terms of IC_{50} was determined as 1.73 mg/ml. In the same test system, kojic acid itself exhibited inhibitory activity on tyrosinase with an IC_{50} value of 0.08 mg/ml.

There are no reports in the literature regarding the tyrosinase inhibitory activity of *S. tomentosa* essential oil. However, there are some reports of tyrosinase inhibitory activities of essential oil major compounds themselves or essential oil samples containing these components in high amounts. In a study conducted by Ho (2010), *Alpinia speciosa* seed oil containing camphor, terpinen-4-ol and α-pinene as major compounds showed an inhibitory effect between 74-81% at 1000 ppm concentration on fungal tyrosinase. In another study conducted by Haliloglu (2017), it was stated that the essential oil of *Achillea sivasica* contained α-pinene and camphor as major compounds and showed significant anti-tyrosinase activity. There are also studies in the literature that caryophyllene inhibits melanogenesis, thus tyrosinase activity. In a study by Yang et al. (2015), it was reported that the aforementioned terpenoid inhibits melanogenesis by inhibiting MITF, TRP-1, TRP-2 and tyrosinase. There are also reports in the literature that various essential oils, including γ-muurolene, δ-cadinene, and viridiflorol as major compounds, show remarkable tyrosinase inhibitory activities (Elgamal et al., 2021; Salleh et al., 2015).

3.4. α-Amylase and α-glucosidase inhibitory activity potential of the essential oil

The data obtained from the α-amylase and α-glucosidase inhibitory activity tests of *S. tomentosa* essential oil are given in Table 2. The data in the table in question are presented in terms of both positive control (acarbose) equivalents and IC_{50} (mg/ml).

Although the inhibitory activity values on the enzymes were quite close to each other, according to the data in the table, *S. tomentosa* essential oil exhibited a slightly higher inhibitory activity on α-glucosidase than on α-amylase. However, in the calculations made in terms of IC_{50}, the α-glucosidase inhibitory activity of the oil was found to be approximately 30% higher than the α-amylase inhibitory activity. The inhibitory activities of the essential oil on α-glucosidase and α-amylase were determined as 703.29 and 694.75 mg ACEs/g essential oil in terms of acarbose equivalent, respectively. Acarbose, used as a positive control in the same test system, showed stronger activity on these enzymes than the essential oil. However, the α-amylase inhibitory activity of the oil was found to be significant, comparable to acarbose.

As a result of the literature search, no report was found that investigated the inhibitory effect of the extract and/or essential oil obtained from *S. tomentosa* on digestive enzymes. However, there are some reports that the major compounds of essential oil may contribute to the inhibitory activity on these enzymes. In a study by Ferrante et al. (2019), it was reported that *Artemisia santonicum* essential oil containing 36.6% camphor exhibited significant inhibitory activity on both digestive enzymes. Findings put forward by Jugreet et al. (2020) also support these data. In another study, *Hertia cheirifolia* essential oil, which contains high amounts of α-pinene as the major compound, was stated to be a potential α-glucosidase inhibitor (Majouli et al., 2016). In a molecular docking study conducted by Yang et al. (2019), it was stated that the binding score of α-pinene to α-amylase was high, while in the same study, it was stated that the binding scores were low for caryophyllene and cadinene. Although there are some reports in the literature that some plant species containing terpinen-4-ol as the main component exhibit remarkable α-amylase and α-glucosidase inhibitory activities (Bouyahya et al., 2020; Usman et al., 2020) viridiflorol is not mentioned. No report has been found regarding its effectiveness on the enzymes in question.

3.5. Molecular docking results of essential oil major compounds

Molecular docking is a widely used method in structure-based drug design (SBDD) due to its ability to predict the binding conformation of small molecules on respective target proteins with a high degree of precision (Ballante, 2018; Dos Santos et al., 2018; Meng et al., 2011; Wang et al., 2020). Molecular docking programs, the first known algorithms of which were written in 1980s, have now become an essential tool in drug discovery (Lohning et al., 2017; Lopez-Vallejo et al., 2011). Using this method, important intermolecular biochemical reactions such as the binding conformations of ligands to their respective receptors and interactions that stabilize the ligand-receptor system can be easily analyzed and visualized (Huang and Zou, 2010). In addition, molecular docking algorithms perform quantitative estimations of receptor-ligand binding energies, enabling the 'docked' conformations of the simulated ligands to be ranked, taking into account their binding free energies (Ferreira et al., 2015; Huang and Zou, 2010; Lopez-Vallejo et al., 2011).
In this study, molecular interactions between target proteins (α-amylase, AChE, BChE, and tyrosinase) and 7 terpenoids (δ-cadinene, viridiflorol, γ-muurolene, α-caryophyllene, terpinene-4-ol, camphor,

and α-pinene) were investigated via molecular docking simulations. Subsequently, the binding free energy (binding affinity) of each ligand was predicted, and the stabilizing non-bonded interactions were characterized by visualizing top-ranked ligand-receptor conformations. However, as a result of the RBCI analysis, the interactions of only 4 compounds (δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene) determined as 'hit' were given (Tables 4, 5, 6 and 7). The binding free energy (ΔG°, kcal/mol) and inhibition constant (Ki, µM) of these four 'hit' phytochemicals for which molecular docking simulations were performed are given in Table 3.

Table 3. Binding free energy and inhibition constant of 'hit' terpenoids determined in molecular docking

Molecule	Binding free energy (kcal/mol)				Inhibition constant (µM)			
	α-Amylase	AChE	BChE	Tyrosinase	α-Amylase	AChE	BChE	Tyrosinase
δ-Cadinene	-7.10	-8.40	-7.50	-5.90	6.25	0.69	3.18	47.35
Viridiflorol	-7.50	-7.80	-7.70	-5.70	3.18	1.92	2.27	66.36
γ-Muurolene	-6.90	-8.00	-7.50	-5.80	8.76	1.37	3.18	56.05
α-Caryophyllene	-7.40	-8.30	-7.10	-5.50	3.77	1.37	6.25	93

The top-ranked conformation of δ-cadinene in the binding cavity of the human pancreatic α-amylase enzyme and the types of amino acid molecular interactions are given in Figure 1A and Table 4. While δ-cadinene formed alkyl interactions with Leu161 and Leu164 residues of human pancreatic α-amylase enzyme and pi-alkyl interactions with Trp58, Tyr61 and His298 residues, it formed a pi-sigma interaction with Tyr61 (Table 4). All the chemical bonds induced by δ-cadinene in the catalytic pocket of α-amylase enzyme are hydrophobic type (Table 4). δ-cadinene binds with high affinity to the binding cavity of the pancreatic α-amylase enzyme (-7.10 kcal/mol, Table 3).

Table 4. Amino acid molecular interactions between human pancreatic α-amylase and 'hit' terpenoids and types of formed chemical interactions[1]

Molecule	Classical H-bond	Van der Waals	Non-classical H-bond (C-H, Pi-Donor)	Hydrophobic		Electrostatic	Miscellaneous (Lone pair/Pi-sulphur)
				π-π interaction	Mixed π/Alkyl		
δ-Cadinene	-	-	-	-	Trp58, Tyr61, Leu161, Leu164, His298	-	-
Viridiflorol	-	-	-	-	Trp57, Trp58, Tyr61, Leu161, Leu164, His304	-	-
γ-Muurolene	-	-	-	-	Trp58, Tyr61, Leu161, Leu164, His298	-	-
α-Caryophyllene	-	-	-	-	Trp57, Trp58, Tyr61, Leu164, His304	-	-

[1] Asp197, Glu233 and Asp300 are active site residues that play direct role in the catalytic function of the human pancreatic α-amylase.

The types of amino acid molecular interactions and top-ranked conformation of δ-cadinene in the catalytic pocket of human AChE enzyme are given in Figure 1B and Table 5. As a result of the docking simulation, δ-cadinene formed pi-alkyl interactions with AChE's Trp83, Tyr121, Phe288, Phe329, His438 and Tyr440 residues, and pi-sigma bonds with the Tyr328. The chemical bonds induced by δ-cadinene in the binding cavity of the AChE are all hydrophobic type (Table 5). δ-cadinene binds to the catalytic pocket of the human AChE enzyme with a very favorable affinity (-8.40 kcal/mol, Table 3).

In a molecular simulation study performed by Hussein et al. (2019), it was found that the bicyclic sesquiterpene δ-cadinene, present in the total extract of the *Commiphora myrrha* (Nees) plant, bound to the human AChE with a very high affinity of -9.2 kcal/mol, and formed hydrophobic contacts with Trp83, Phe330 and His439 residues localized in the catalytic triad of the enzyme (Hussein et al., 2019). In another *in silico* study, δ-cadinene exhibited a binding affinity of -6.77 kcal/mol to AChE, the target protein of Alzheimer's disease, and formed pi-alkyl bonds with polar aliphatic residues Trp286 and His447, and hydrophobic contacts with hydrophobic residues Tyr124 and Phe295 (da Silva Barbosa et al., 2020). Although the results of our study show some differences from the above-mentioned two *in silico* studies in terms of the amino acid residues to which δ-cadinene binds, δ-cadinene generally forms hydrophobic contacts with residues bearing cyclic side chains in AChE, and therefore, our results are in agreement with the other two *in silico* studies in this respect. Furthermore, the wide active site (active gorge) of AChE displays high variability, and the X-ray crystallographic structures obtained to date proved that the aromatic residues (Trp286 and Tyr337) that fall within the P (peripheral)- and A (acylation)-sites of the enzyme demonstrates high mobility in their ligand binding patterns (Rosenberry et al., 2017).

The top-ranked conformation of δ-cadinene in the catalytic cavity of the human BChE enzyme is given in Figure 1C. While δ-cadinene formed pi-alkyl contacts with Trp79, Tyr329, Trp425, His433 and Tyr435 residues of human BChE enzyme, it formed alkyl bonds with Ala325 and Met432 residues as well as some additional pi-sigma bonds with Tyr79 residue (Table 6). Notably, both the Ala325 and Tyr329 residues are located in the active site of the BChE enzyme, and the Tyr329 residue is located in the A (acylation)-site of the active site pocket (Rosenberry et al., 2017). As a result, δ-cadinene entered an energetically favorable (exergonic) binding reaction at the active site of the human BChE enzyme and formed non-bonded interactions preferably with hydrophobic amino acids.

Since the crystal structure of the human tyrosinase enzyme has not been clarified yet, the molecular docking simulations of the tyrosinase enzyme in this study were performed on the tyrosinase

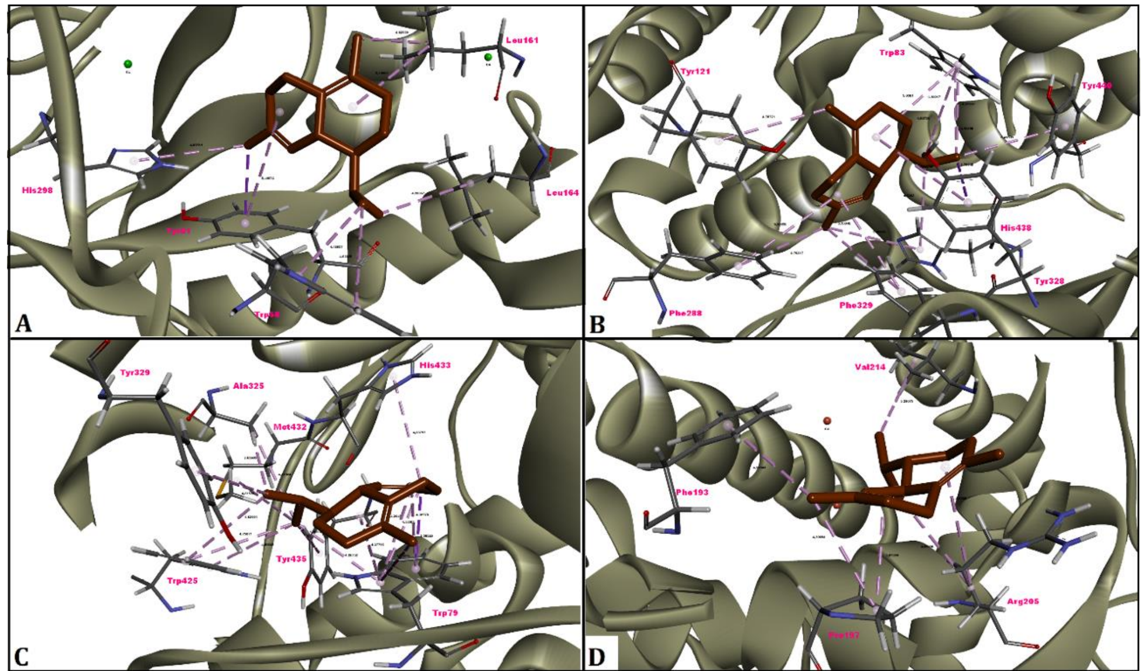


Figure 1. Top-ranked conformations of δ-cadinene (A- α-amylase, B- AChE, C- BChE, D- Tyrosinase)

enzyme (PDB ID: 5I38) isolated from Bacillus megaterium (Noh et al., 2020). The top-ranked conformation of δ-cadinene in the active site of the B. megaterium tyrosinase enzyme is given in Figure 1D. δ-cadinene formed alkyl contacts with the Pro197, Arg205 and Val214 residues and pi-alkyl bonds with the Phe193 residue of *B. megaterium* tyrosinase (Table 7). In *B. megaterium* tyrosinase, Met61 and Met184 residues play a catalytic role in the transfer of

Table 5. Amino acid molecular interactions between human AChE and 'hit' terpenoids and types of formed chemical interactions[1]

Molecule	Classical H-bond	Van der Waals	Non-classical H-bond (C-H, Pi-Donor)	Hydrophobic		Electrostatic	Miscellaneous (Lone pair/Pi-sulphur)
				π-π interaction	Mixed π/Alkyl		
δ-Cadinene	-	-	-	-	Trp83, Tyr121, Phe288, Tyr328, Phe329, His438, Tyr440	-	-
Viridiflorol	-	-	His438	-	Trp83, Tyr121, Phe288, Tyr328, Phe329, His438	-	-
γ-Muurolene	-	-	-	-	Trp83, Tyr121, Phe288, Tyr328, His438, Tyr440	-	-
α-Caryophyllene	-	-	-	-	Trp83, Tyr328, Phe329, His438	-	-

[1] Tyr72, Asp74, Trp86, Tyr121, Tyr124, Tyr133, Glu202, Ser203, Ser229, Trp236, Trp286, Phe295, Phe297, Phe288, Glu334, Tyr337, Phe338, Tyr341, His440 and His447 are directly involved in the catalytic function of the human AChE.

Table 6. Amino acid molecular interactions between human BChE and 'hit' terpenoids and types of formed chemical interactions[1]

Molecule	Classical H-bond	Van der Waals	Non-classical H-bond (C-H, Pi-Donor)	Hydrophobic		Electrostatic	Miscellaneous (Lone pair/Pi-sulphur)
				π-π interaction	Mixed π/Alkyl		
δ-Cadinene	-	-	-	-	Trp79, Ala325, Tyr329, Trp425, Met432, His433, Tyr435	-	-
Viridiflorol	-	-	-	-	Trp79, Phe326, His433	-	-
γ-Muurolene	-	-	-	-	Trp79, Ala325, Phe326, Met432, His433, Tyr435	-	-
α-Caryophyllene	-	-	-	-	Trp79, Ala325, Tyr329, Trp425	-	-

[1]Active site residues that play a direct role in the catalytic function of human BChE (Asn68, Asp70, Trp82, Gln119, Tyr128, Glu197, Ser198, Ser224, Trp231, Ala277, Leu286, Val288, Glu(Ala)325, Ala328, Phe(Tyr)329, Tyr332, Phe398, His438). The residues given in parentheses (Ala325, Tyr329) are silent mutations that occur as a result of polymorphism in the DNA molecule, however, have no effect on the catalytic function of the enzyme.

copper ions to the flexible His60 residue, thereby positioning copper ions in the active site. *B. megaterium* tyrosinase enzyme (PDB ID: 5I38) used in docking calculations in this study shows some differences in terms of active site residues reported in the literature

(Noh et al., 2020). For instance, histidine at position 60 has changed to alanine, methionine at position 61 to phenylalanine, and methionine at position 184 to asparagine. It can be envisioned that these substitutions could be due to the polymorphism in bacterial

Table 7. Amino acid molecular interactions and types of chemical interactions between B. megaterium tyrosinase and 'hit' terpenoids 1

Molecule	Classical H-bond	Van der Waals	Non-classical H-bond (C-H, Pi-Donor)	Hydrophobic		Electrostatic	Miscellaneous (Lone pair/Pi-sulphur)
				π-π interaction	Mixed π/Alkyl		
δ-Cadinene	-	-	-	-	Phe193, Pro197, Arg205, Val214	-	-
Viridiflorol	Gly209	Gly212	-	-	Phe120, Ala151	-	-
γ-Muurolene	-	-	-	-	Pro197, His204, Arg205, Val213, Val214, Ala217	-	-
α-Caryophyllene	-	-	-	-	Arg161, Val164, Leu165, Val236, Ile284	-	-

[1] His60, Met61 and Met184 are active site residues that play direct role in the catalytic function of the B. megaterium tyrosinase.

DNA at the molecular level, but the altered amino acid sequence is a silent mutation that does not adversely affect the catalytic function of the enzyme. In this study, although δ-cadinene did not form chemical contacts with Ala60, Phe61 and Asn184 residues of the tyrosinase enzyme, the amino acids it interacts with (Phe193, Pro197, Arg205 and Val214) are positionally close to Ala60 and Phe61, however, due to the low binding energy (-5.90 kcal/mol) it can be said that the possibility of a competitive inhibition is low. Consistent with this study, Ak et al. (2021) reported a low GOLD fitness score (38.92) of δ-cadinene against the tyrosinase enzyme isolated from the fungus (PDB ID: 2Y9X). Taken together, the results from both studies indicate that δ-cadinene is not a favorable competitive inhibitor for the tyrosinase enzyme.

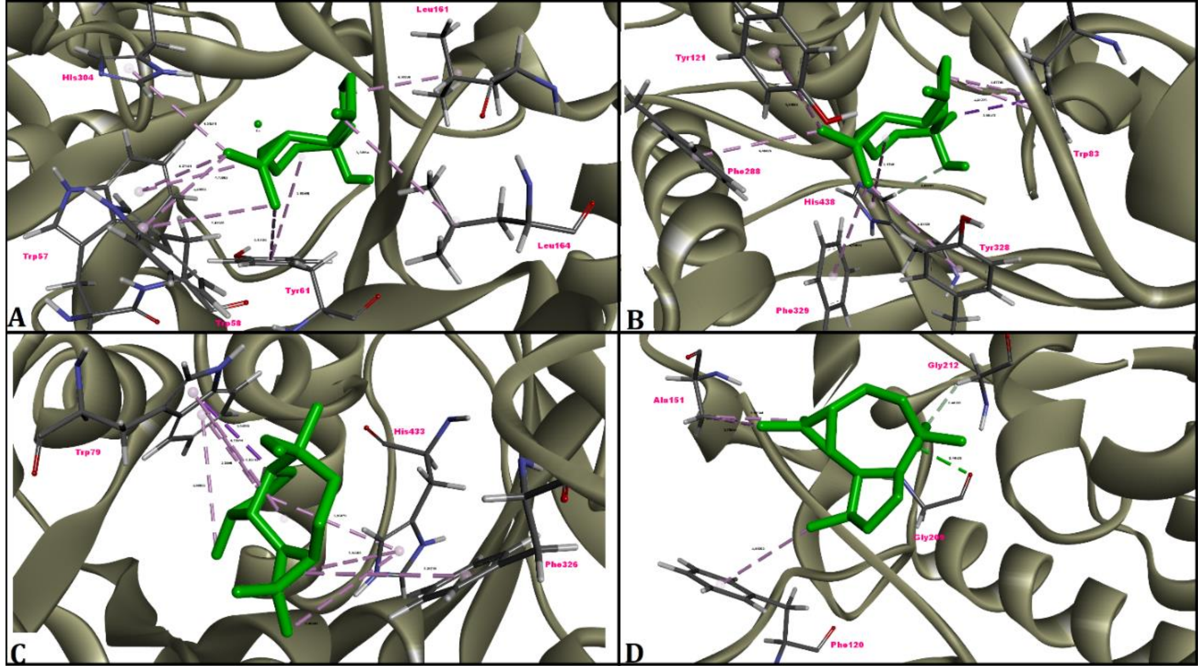


Figure 2. Top-ranked conformations of viridiflorol (A- α-amylase, B- AChE, C- BChE, D- Tyrosinase)

The top-ranked conformation of viridiflorol in the binding pocket of the human pancreatic α-amylase enzyme is shown in Figure 2A. Viridiflorol formed hydrophobic pi-alkyl contacts and alkyl bonds with Leu161 and Leu164 with Trp57, Trp58, Tyr61 and His304 residues of the human pancreatic α-amylase enzyme (Table 4). Viridiflorol exhibited a very favorable binding with the α-amylase enzyme (-7.50 kcal/mol, Table 3) and was localized in the vicinity of the active site residues (Asp197, Glu233, Asp300) reported in the literature (Brayer et al., 1995). The top-ranked conformation of viridiflorol in the inhibitor binding cavity of the human AChE enzyme is given in Figure 2B. Viridiflorol formed pi-alkyl contacts with the Trp83, Tyr121, Phe288, Tyr328, Phe329 and His438 residues of AChE, a pi-sigma bond with Trp83, and a carbon-hydrogen bond with His438 (Table 5). The affinity of the hydrophobic viridiflorol molecule against the active pocket of the human AChE enzyme was found to be quite favorable (-7.80 kcal/mol, Table 3). The top-ranked conformation of viridiflorol in the inhibitor binding pocket of the human BChE enzyme is given in Figure 2C. Viridiflorol formed pi-alkyl bonds and a pi-sigma bond with the Trp79, Phe326 and His433 residues of BChE (Table 6). Moreover, viridiflorol exhibited an energetically favorable binding (-7.70 kcal/mol) with BChE and displayed a higher binding affinity than AChE (Table 3). The top-ranked conformation of viridiflorol in the active site of the *B. megaterium* tyrosinase is shown in Figure 2D. Viridiflorol formed alkyl and pi-alkyl bonds with Phe120 and Ala151 residues, classical hydrogen bonds with Gly209, and carbon-hydrogen bonds with Gly212 residues of *B. megaterium* tyrosinase (Table 7). The affinity of viridiflorol against the active site of the *B. megaterium* tyrosinase enzyme is energetically less favorable (-5.70 kcal/mol, Table 3). Consistent with these results, Ak et al. (2021) reported that

viridiflorol exhibited a low GOLD fitness score (33.71) against the fungal tyrosinase (PDB ID: 2Y9X). The top-ranked conformation of γ-muurolene in the binding pocket of the human pancreatic α-amylase enzyme is shown in Figure 3A. γ-Muurolene formed pi-alkyl bonds with the Trp58, Tyr61 and His298 residues of the human α-amylase enzyme, alkyl bonds with Leu161 and Leu164, and additional pi-sigma bonds with Tyr61 (Table 4). The affinity of γ-muurolene against the active site of α-amylase is moderately strong (-6.90 kcal/mol, Table 3). However, this docking binding score of the same ligand is more favorable than the binding energy (-5.59 kcal/mol) determined by Yang et al. (2019) against α-amylase. The

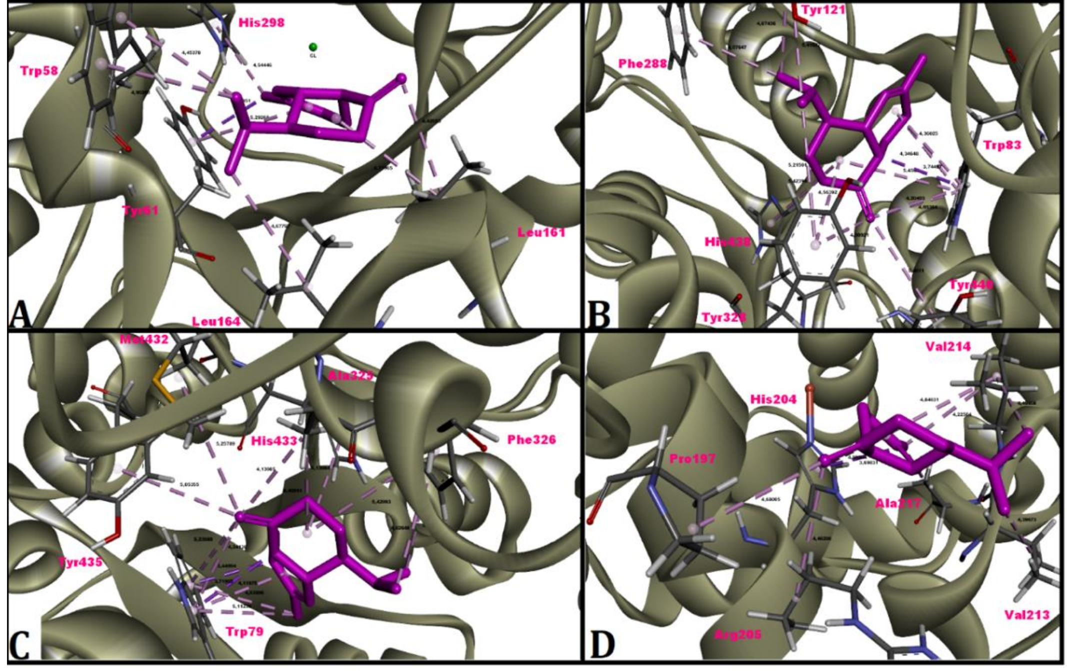


Figure 3. Top-ranked conformations of γ-muurolene (A- α-amylase, B- AChE, C- BChE, D- Tyrosinase)

better binding affinity scored by AutoDock Vina program compared with the score found by Yang et al. (2019) shows that AutoDock Vina has a more optimized scoring function than the AutoDock 4.2, and thus can identify energetically more favorable binding conformations in the active site of the α-amylase. The top-ranked conformation of γ-muurolene in the inhibitor binding cavity of the human AChE enzyme is given in Figure 3B. The interaction of γ-muurolene with the catalytic pocket of AChE is energetically very favorable (-8.00 kcal/mol, Table 3). While γ-muurolene formed pi-alkyl contacts with AChE residues Trp83, Tyr121, Phe288, Tyr328, His438 and Tyr440, it also formed pi-sigma bonds with Trp83 (Table 5). The top-ranked conformation of γ-muurolene in the binding pocket of human BChE enzyme is given in Figure 3C. γ-muurolene formed pi-alkyl bonds with Trp79, Phe326, His433 and Tyr435 residues of the BChE enzyme, pi-sigma bonds with Trp79, as well as alkyl bonds with Ala325 and Met432 (Table 6). The interaction of γ-muurolene with the active site of the BChE enzyme is energetically very favorable (-7.50 kcal/mol, Table 3). The top-ranked conformation of γ-muurolene in the active site of *B. megaterium* tyrosinase enzyme is shown in Figure 3D. γ-Muurolene formed alkyl bonds with Pro197, Arg205, Val213, Val214 and Ala217 residues of *B. megaterium* tyrosinase, and pi-alkyl and pi-sigma bonds with His204 (Table 7). The docking-binding affinity of γ-muurolene for *B. megaterium* tyrosinase is energetically not very favorable (-5.80 kcal/mol, Table 3). Sinan et al. (2021), reported that γ-muurolene exhibited a low GOLD fitness score (40.31) against tyrosinase enzyme, confirming the result of our study, in their chemodiversity and biological activity research.

The top-ranked conformation of α-caryophyllene in the active site of the human pancreatic α-amylase enzyme is given in Figure 4A. Alpha-caryophyllene formed pi-alkyl bonds with the Trp57, Trp58, Tyr61 and His304 residues in the active site of the enzyme as well as alkyl bonds with Leu164 (Table 4). The interaction of α-caryophyllene with the active site of the pancreatic α-amylase is energetically very favorable (-7.40 kcal/mol, Table 3). The top-ranked conformation of α-caryophyllene in the active site of the human AChE enzyme is given in Figure 4B. α-Caryophyllene formed pi-alkyl contacts with Trp83, Tyr328, Phe329 and His438 residues of the enzyme as well as a pi-sigma contact with Tyr328 (Table 5). The interaction of alpha-caryophyllene with residues in the active site of the AChE enzyme is energetically highly favorable (-8.30 kcal/mol, Table 3). Consistent with our docking results, Karimi et al. (2021) reported that α-caryophyllene in the herbal extract of *Cannabis sativa* L. showed a binding affinity of -9.00 kcal/mol with the AChE and formed hydrophobic contacts with the Asp71, Trp83 and Tyr120.

The top-ranked conformation of α-caryophyllene in the active site of the human BChE enzyme is shown in Figure 4C. Alpha-caryophyllene formed pi-alkyl bonds with Trp79, Tyr329 and Trp425 residues of BChE as well as alkyl bonds with the Ala325 (Table 6). The interaction of α-caryophyllene with the active site of the BChE enzyme is energetically favorable (-7.10 kcal/mol, Table 3). In support of our results, Karimi et al. (2021) found that α-caryophyllene in the herbal extract of *C. sativa* plant showed a favorable binding affinity of -7.70 kcal/mol for BChE active site, and formed hydrophobic interactions with residues His433, Asp67 and Try329 in the peripheral anionic site (PAS), as well as Trp79, Ala325 and Phe326 in the acylation site (AS).

In molecular docking studies, it may be necessary to examine the binding poses of ligands which display favorable binding energetics (high affinity) and to check whether the binding event takes place in

the correct cavity (active site) of the enzyme. Because, although a ligand shows a highly negative binding free energy, the binding event can sometimes occur in a location outside the active site of the enzyme that has multiple binding sites. Therefore, to examine

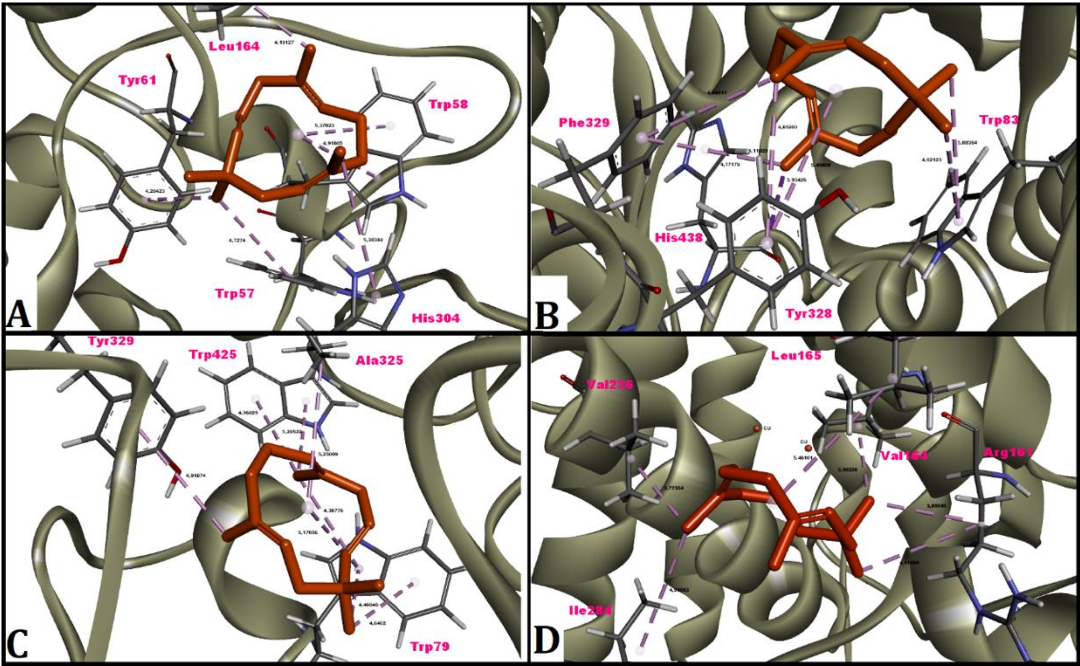


Figure 4. Top-ranked conformations of α-caryophyllene (A- α-amylase, B- AChE, C- BChE, D- Tyrosinase)

this issue, the top-ranked poses of the four 'hit' ligands were superposed on the active site with the binding pose of the co-crystallized inhibitors on the hydrophobicity surfaces of α-amylase, AChE, BChE and tyrosinase (Figures 5-8). Delta-cadinene, viridiflorol, γ-muurolene and α-caryophyllene were all found to be colocalized with acarbose in the active site of human pancreatic α-amylase enzyme. Thus, the docking scoring function produced a 'true positive' result for the α-amylase enzyme (Figure 5A, B, C, D). Delta-

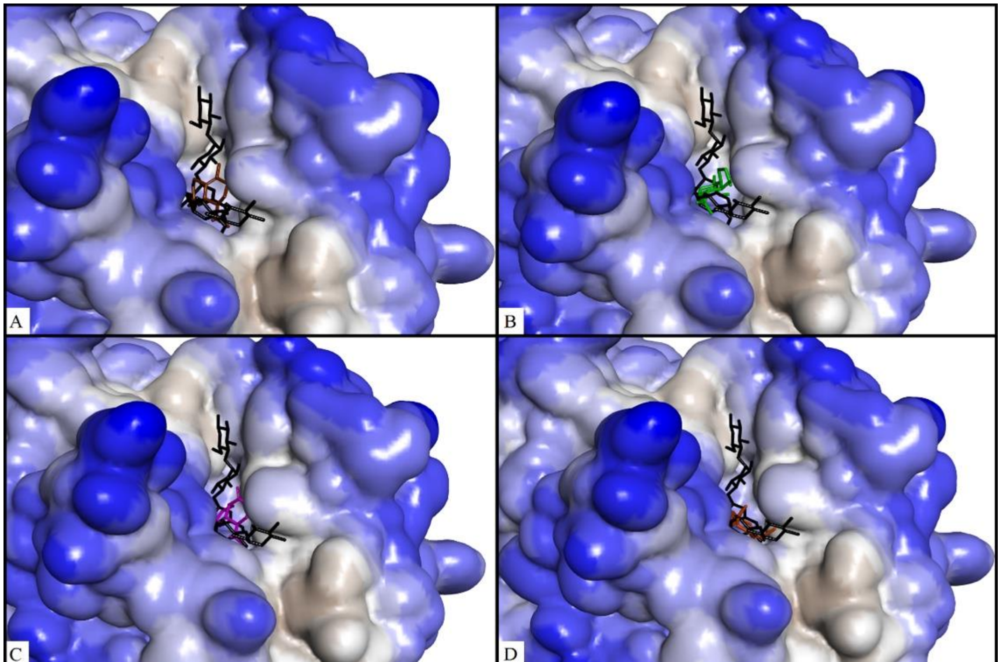


Figure 5. Superposed images of co-crystallized inhibitor (acarbose, black) with δ-cadinene (A), viridiflorol (B), γ-muurolene (C), and α-caryophyllene (D) ligands in the active site of human pancreatic alpha-amylase

cadinene, viridiflorol, γ-muurolene and α-caryophyllene colocalized with galanthamine at the active site of the human AChE enzyme. Thus, the docking scoring function produced a 'true positive' result for the AChE (Figure 6A, B, C, D). Delta-cadinene, viridiflorol, γ-muurolene and α-caryophyllene were determined to be colocalized with the inhibitor tacrine in the active site of the human BChE

enzyme and the ligand conformations produced by the program was 'true positive' (Figure 7A, B, C, D). In the case of tyrosinase, δ-cadinene (A) and γ-muurolene (C) are localized around the active site close to the inhibitor (but not fully within the active site), while viridiflorol (B) and α-caryophyllene (D) are localized outside the active site of *B. megaterium* tyrosinase, in a region distant from kojic acid (red arrows) (Figure 8A, B, C, D). The obtained poses clearly explain the weak binding affinities given for the tyrosinase enzyme in Table 3. The active site of the tyrosinase is not capable of harboring δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene due to topological limitations. Therefore, our docking poses verify the experimental results, since it was determined that the tyrosinase inhibitory activity of *S. tomentosa* essential oil was lower in terms of IC_{50} (1.73 ± 0.03 mg/mL) compared to other enzymes (AChE, BChE and α-amylase). Therefore, it is suggested that a ligand capable of competitively inhibiting the tyrosinase enzyme should not have higher molecular weight and volume values than kojic acid due to the topological restraints in the catalytic region.

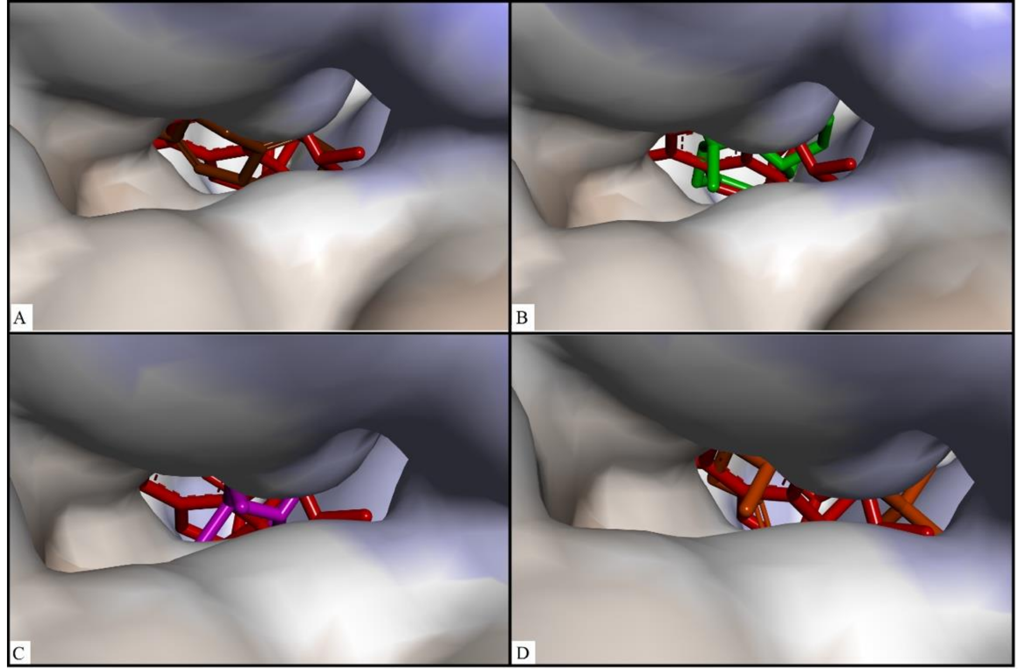


Figure 6. Superposed images of co-crystallized inhibitor (galanthamine, orange) with δ-cadinene (A), viridiflorol (B), γ-muurolene (C), and α-caryophyllene (D) ligands in the active site of human AChE

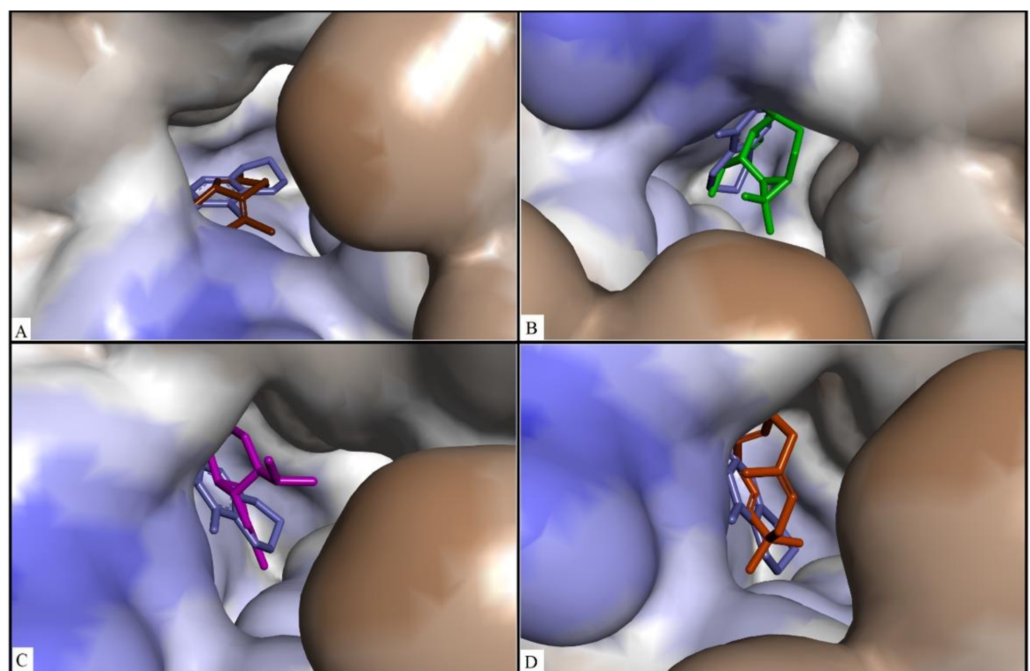


Figure 7. Superposed images of co-crystallized inhibitor (tacrine, grey) with δ-cadinene (A), viridiflorol (B), γ-muurolene (C), and α-caryophyllene (D) ligands in the active site of human BChE

In this study, the relative binding capacity index (RBCI), which allows statistical ranking of the activity potentials of phytochemicals by using the binding free energy values obtained from molecular docking calculations, was used (Figure 9 and Table 8) (Istifli et al., 2020). As a result of the RBCI analysis, phytochemicals such as δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene were determined to be 'hit' components. Top-ranked poses of human pancreatic α-amylase, human AChE, human BChE and *B. megaterium* tyrosinase enzymes and δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene are given in Figures 1, 2, 3 and 4, respectively. RBCI analysis also revealed that terpinene-4-ol, camphor and α-pinene were the ligands with the weakest affinity for these 4 different enzyme targets (Figure 9).

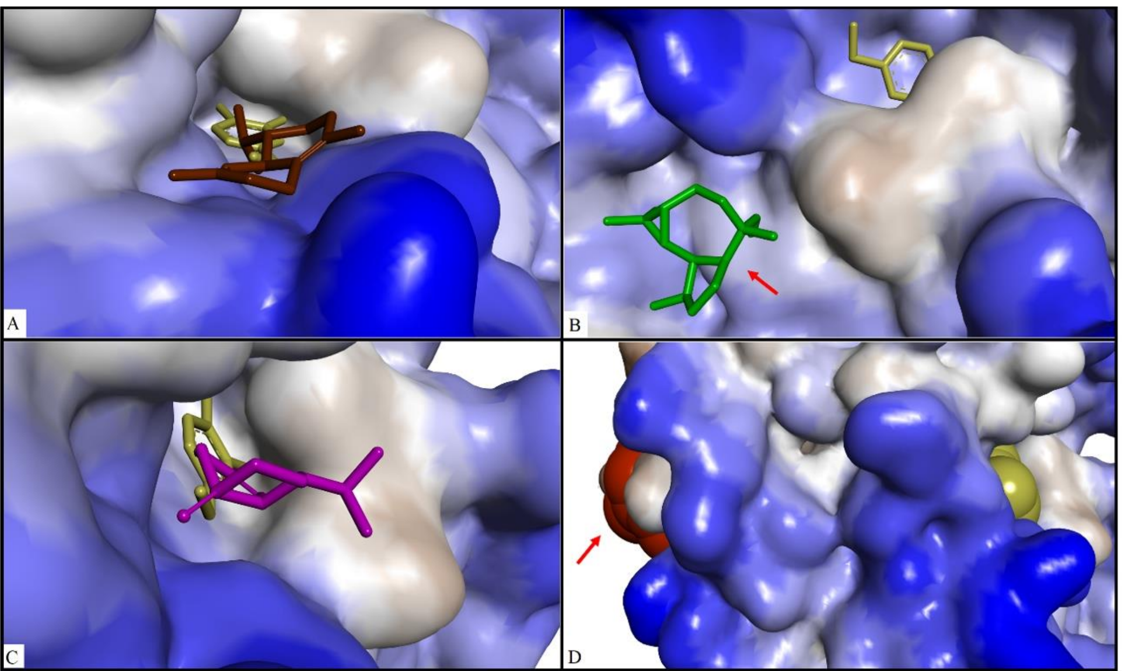


Figure 8. Superposed images of co-crystallized inhibitor (kojic acid, yellow) with δ-cadinene (A), viridiflorol (B), γ-muurolene (C), and α-caryophyllene (D) in the active site of B. megaterium tyrosinase

3.6. ADMET and drug-likeness features of hit compounds

In this study, ADMET and drug-likeness properties of δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene were calculated by entering the SMILES (simplified molecular input line entry specification) characters of 'hit' ligands into the search engines of online pkCSM (http://biosig.unimelb.edu.au/pkcsm/) and SwissADME (http://www.swissadme.ch/) servers. Considering ADMET properties, none of the 'hit' ligands show AMES toxicity or hepatotoxicity, nor are they a substrate for P-glycoprotein (P-gp). Rat LD_{50} values range from 1.540 to 1.776 mol/kg and are in an acceptable non-toxic range. On the other hand, blood-brain barrier (BBB) permeability is only detected for the viridiflorol ligand, and all ligands inhibit at least CYP2C19 or CYP2C9 (Table 9). In addition, although viridiflorol exhibits a lower absorption (92.814%) than other ligands in terms of intestinal absorption, it exhibits a higher advantage as a drug candidate due to its low total clearance (0.817 ml/kg). A low total clearance rate indicates that viridiflorol will be excreted more slowly from the body and will therefore require longer dosing intervals in a possible drug administration (Table 9). According to Lipinski's rule of 5, all four 'hit' ligands were showed drug-like properties (Table 10). However, viridiflorol exhibits a more favorable drug-like profile since it does not exceed the MLOGP > 4.15 limit that is violated by the other four ligands. As a result, the 'hit' ligands δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene, in general, show good pharmacokinetic activity and low toxicity (da Silva Barbosa et al., 2020; Hussein et al., 2019; Schepetkin et al., 2021). Cytochrome P450 (CYP) inhibition, on the other hand, is a common handicap observed in all ligands, and may have adverse effects on the drug metabolism of the host organism.

3.7. Identification of possible intracellular targets of hit compounds

In this study, the most likely intracellular macromolecular targets of hit phytochemicals were documented using the SwissTargetPrediction (http://www.swisstargetprediction.ch) web server.

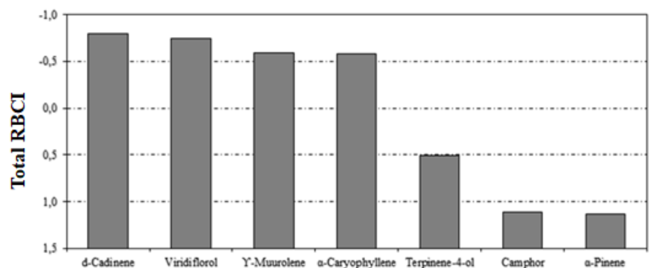


Figure 9. Relative binding capacity index of terpenoids (RBCI)

According to Figure 10A, nuclear receptors (22.9%), various enzymes (20.5%) and family A G protein-coupled receptors (10.8%) constitute the major group of the most likely intracellular targets of δ-cadinene. However, the probability of interaction of δ-cadinene with these proteins was found to be between 0.042 and 0.052 (Table 11), which is not statistically significant. According to Figure 10B, family A G protein-coupled receptors (21%), various enzymes (19%), and extracellular secretory proteins (11%) constitute the most likely intracellular target group of viridiflorol. However, the probability of interaction of viridiflorol with these proteins was found to be between 0.060 and 0.346 (Table 11), which is not

statistically significant. According to Figure 10C, various enzymes (21%), nuclear receptors (21%), and family A G protein-coupled receptors (9%) constitute the most likely intracellular target group of γ-muurolene. However, the probability of interaction of γ-muurolene with these proteins was found to be between 0.042-0.090 (Table 11), which is not statistically significant. According to Figure 10D, family A G protein-coupled receptors (21.4%), nuclear receptors (19%) and various enzymes (19%) constitute the most likely intracellular target group of α-caryophyllene. However, the probability of interaction of α-caryophyllene with these proteins was found to be 0.042 (Table 11), indicating a statistically insignificant outcome.

Table 8. RBCI values of terpenoids

Component	RBCI
δ-cadinene	-0.8
Viridiflorol	-0.7
γ-muurolene	-0.6
α-caryophyllene	-0.6
Terpinene-4-ol	0.5
Camphor	1.1
α-pinene	1.1

4. Conclusions

In this study, the characterization of the chemical composition of *S. tomentosa* essential oil, the α-amylase, α-glucosidase, AChE, BChE and tyrosinase inhibitory activity assays of the dominant compounds, and the possible molecular interactions of the active sites of these enzymes with the major compounds were investigated. Considering the IC_{50} values, it was determined that *S. tomentosa* essential oil showed relatively higher inhibitory activity (IC_{50}= 0.97 mg/mL – 1.35 mg/mL) on the α-glucosidase, BChE and AChE enzymes. On the other hand, α-amylase and tyrosinase inhibitory capacity (IC_{50}= 1.51 mg/mL – 1.73 mg/mL) of *S. tomentosa* essential oil was found to be lower. Molecular docking studies have brought further atomistic perspective to our study in terms of explaining the molecular-scale interactions between the dominant compounds of *S. tomentosa* essential oil and α-amylase, α-glucosidase, AChE, BChE and tyrosinase as well as the binding mode of ligands. In relative agreement with the experimental inhibition study, the dominant compounds of *S. tomentosa* essential oil (δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene) displayed high binding affinity (-6.90 – -8.40 kcal/mol) to AChE, BChE and α-amylase and good inhibition constant (Ki = 0.69 µM – 8.76 µM). On the other hand, the affinities of these 'hit' compounds to tyrosinase were not found to be energetically highly favorable (-5.50 – -5.90 kcal/mol; Ki = 47 µM - 93 µM). Considering their localization around the active site, it was determined that the 'hit' ligands were not suitable for entry into the active site of the tyrosinase enzyme. This finding partially explains why *S. tomentosa* essential oil failed to inhibit the tyrosinase enzyme experimentally. Therefore, docking studies have also revealed that the size of the ligands and the enzyme active sites should be compatible with each other in terms of volume. Considering *in silico* physicochemical properties, drug-likeness (Lipinski's rule of 5) and intracellular targets, δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene showed 'hit' molecule properties and they did not show non-specific enzyme or protein affinity. However, possible CYP450 inhibitory activities calculated by *in silico* analyzes (especially CYP2C19 and CYP2C9) should also be confirmed experimentally and, if necessary, CYP450 affinities should be reduced by molecular modification so as not to adversely affect the biological activities of the components. In this study, possible AChE, BChE and α-amylase inhibitors and their molecular interaction patterns in the active sites of these targets are proposed. In the light of the results obtained from this study, it was concluded that the dominant interaction pattern of δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene with AChE, BChE and α-amylase is via hydrophobic contacts and the compatibility between ligand size and active site volume are important factors in enzyme inhibition. Ligand binding tests (LBT) between isolated δ-cadinene, viridiflorol, γ-muurolene and α-caryophyllene and AChE, BChE and α-amylase would constitute the next step in confirming the competitive inhibitory capacity of these proposed 'hit' ligands.

Table 9. Certain ADMET properties of hit terpenoids

Ligand	BBB permeability[1,*]	Intestinal absorbtion (%)	P-gp substrate[2,*]	Cytochrome P inhibition[3,*]	Total clearance (ml/kg)	AMES Toxicity[4]	Hepatotoxicity[4]	Rat LD_{50} (mol/kg)[4]
δ-cadinene	No	96.128	No	Yes (CYP2C19, CYP2C9)	1.182	No	No	1.552
Viridiflorol	Yes	92.814	No	Yes (CYP2C19)	0.817	No	No	1.615
γ-muurolene	No	96.475	No	Yes (CYP2C19, CYP2C9)	1.188	No	No	1.540
α-caryophyllene	No	94.682	No	Yes (CYP2C19)	1.282	No	No	1.766

[1] BBB: Blood-Brain Barrier, [2] P-gp: P-glycoprotein substrate, [3] CYP: Cytochrome P, [4] http://biosig.unimelb.edu.au/pkcsm/prediction, [*]https://www.swissadme.ch

Table 10. Drug-likeness properties of docked hit terpenoids

Ligand	Number of rotatable bonds	TPSA[1]	Consensus Log P	Log S (ESOL[2])	Drug-likeness (Lipinski's rule of 5)
δ-cadinene	1	0.00	4.12	-3.43	Yes; 1 violation (MLOGP>4.15)
Viridiflorol	0	20.23	3.42	-3.57	Yes; 0 violation
γ-muurolene	1	0.00	4.18	-3.76	Yes; 1 violation (MLOGP>4.15)
α-caryophyllene	0	0.00	4.26	-3.97	Yes; 1 violation (MLOGP>4.15)

[1] TPSA: Topological polar surface area ($Å^2$), [2] ESOL: Estimated water solubility [(Non-soluble < -10 < Poor < -6 < Moderately < -4 < Soluble < -2 Good < 0 < Highly soluble), according to Delaney, J.S. (2004)]. Data source: http://www.swissadme.ch/index.php

Table 11. Probabilities of interaction of hit terpenoids with their putative intracellular targets

Ligand	Probability of interaction with intracellular proteins*	
	Max.	Min.
δ-cadinene	0.052	0.042
Viridiflorol	0.346	0.060
γ-muurolene	0.090	0.042
α-caryophyllene	0.042	0.042

* Values between 0-1 are the probability values that the intracellular protein is the target of the presumed bioactive molecule. A probability value equal to or very close to '1' usually indicates that the molecule under investigation is a known bioactive agent. Source: http://www.swisstargetprediction.ch/help_results.php

Acknowledgments

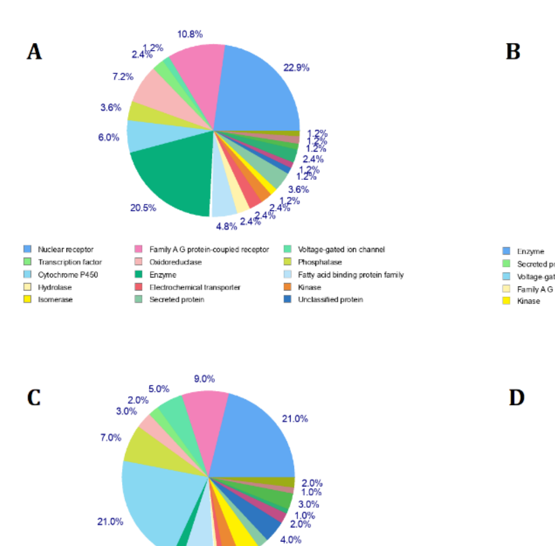
The data presented here is basically originated from Mr. Mustafa KOCER's M. Sc. thesis. The authors would like to thank to the Research Council of Kilis 7 Aralik University (project number: 2128LTP1).

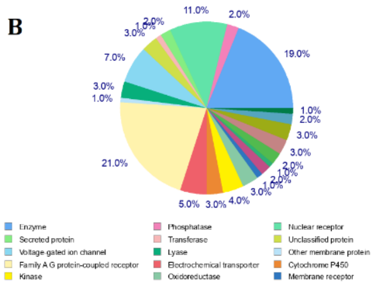
Conflict of interest

The author confirms that there are no known conflicts of interest.

CRediT authorship contribution statement

Mustafa Kocer: Conceptualization, Data curation, Investigation, Methodology, Writing, Review & Editing
Erman Salih Istifli: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing, Review & Editing





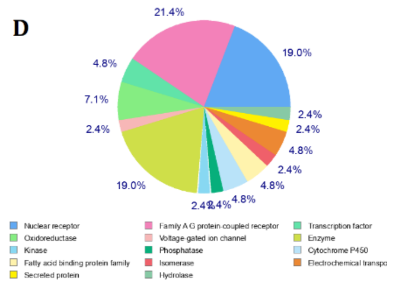


Figure 10. The most likely intracellular macromolecular targets predicted for A - δ-cadinene B - Viridiflorol, C - γ-muurolene and D - α-caryophyllene

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Supplementary File

None.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 17-24

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

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RESEARCH ARTICLE

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Effect of genotype and extraction method on polyphenols content, phenolic acids, and flavonoids of olive leaves (*Olea europaea* L. subsp. *europaea*)

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ARTICLE INFO

Article History:

Received: 14 September 2021
Revised: 27 October 2021
Accepted: 28 October 2021
Available online: 28 October 2021

Edited by: B. Tepe

Keywords:
Polyphenols
Cultivated olive leaves
Wild olive leaves
Genotype
Method of extraction

ABSTRACT

Polyphenol's extraction varied according to various factors. In this study, the effect of genotype and method of polyphenols extraction were investigated using leaves of two cultivated and two wild olive varieties and four hydromethanolic extraction methods. Quantitatively, significant differences were observed according to the extraction method, the genotype, and the interaction genotype-method of extraction. The heat reflux extraction showed the highest polyphenols content in wild olive leaves having an amount of 841.17 mg GAE/100 g DM. The qualitative phytochemical examination using high performance liquid chromatography (HPLC) of olive leaves showed some significant differences of phenolic compounds between genotypes. For the same oleaster genotype, the extraction method seemed to influence qualitatively the polyphenols profiles. The quinic acid was the dominant phenolic acid and the luteolin-7-*O*-glucoside was the major flavonoid observed in wild olive leaves having, respectively, 618.24 and 3211.44 mg/kg DM. The quinic acid has an amount of 400.15 and 275.39 mg/kg and the luteolin-7-*O*-glucoside has an amount of 2059.62 and 1214.49 mg/kg in cultivars leaves. The extraction by Soxhlet of wild olive leaves showed the highest quinic acid (1085.80 mg/kg DM) and luteolin-7-*O*-glucoside (3720.15 mg/kg DM) amounts. The hydromethanolic extraction assisted by Soxhlet of wild olive leaves constituted the optimal method to obtain high polyphenols contents enriched with phenolic acids and flavonoids.

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1. Introduction

Plants are natural source of molecules used in therapeutic, pharmaceutic and cosmetology fields. Currently, about 25-30% of all drugs are derived from natural products (Boldi, 2004). Phenolic compounds are known as secondary plant metabolites and are the most important phytochemicals due to their bioactive functions.

Olive tree (*Olea europaea* L. subsp. *europaea*) includes two botanical varieties, the cultivated olive tree (variety *europaea*) grouped olive cultivars and wild olive trees or oleasters (variety *sylvestris*) including natural oleasters and feral forms (Besnard et al., 2001; Hannachi et al., 2008). *Olea europaea* L. is an exceptional species and was widely studied for their organs benefits that are rich in phenolic compounds (Ben Salah et al., 2012; Hannachi et al., 2013; Hannachi et al., 2019). The olive leaves have an important antioxidant activity and are richer in bioactive compounds compared to other parts of olive tree (Hannachi et al., 2020). In addition, phenolic compounds of olive leaves have beneficial effects on health such as antihypertensive, hypoglobulin, hypocholesterolemic, cardioprotective and anti-inflammatory properties. The olive leaves properties are mostly attributed to their polyphenols (Ryan et al., 2002; Vermerris and Nicholson, 2006).

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e-ISSN: 2791-7509
doi:
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Please cite this article as: Guebebia, S., Othman, K.B., Yahia, Y., Romdhane, M., Elfalleh, W., Hannachi, H., 2022. Effect of genotype and extraction method on polyphenols content, phenolic acids, and flavonoids of olive leaves (Olea europaea L. subsp. europaea). International Journal of Plant Based Pharmaceuticals, 2(1), 17-24.

The phenolic compounds extraction is the first and the important step in the isolation and identification of phenolic compounds (Bucić-Kojić et al., 2007). Several extraction methods were used as solid-liquid extraction that is an old operation by using water or an organic solvent. Many studies have shown that the highest yield was obtained using a co-solvent as mixture of ethanol or methanol with water (Tsakona et al., 2012; Miguel et al., 2010). Olive is one of the most investigated plant all around the world as well as this topic about extraction influence applied on different plant materials (Hannachi et al., 2019). Conventional and modern extraction methods have been used to extract biomolecules from plant material (Liazid et al., 2007; Hannachi et al., 2019; Yahia et al., 2020). Several factors can affect the phenolic compounds profile such geographical origin, genotype, and extraction process (Vinhaet al., 2005; Papoti et al., 2009). The chemotaxonomy is a plant classification based on chemical constituents (Singh, 2016). The plant classification based on secondary metabolites were used for several species as the genus *Aquilaria* (Andary et al., 2019), *Propolus* (Abdellatif et al., 2019), *Solanum lycopersicum* L. (Siracusa et al., 2012) and Olive cultivars (Ben Mohamed et al., 2018).

The aim of this study was to compare four extraction methods on olive leaves using two cultivars and two oleasters, highlighting the effect of genotype, the extraction method, and the method-genotype interaction on phenolics contents and profile using HPLC analysis.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade methanol, methanol solvents and hydrochloric acid were obtained from Lab-Scan. Folin–Ciocalteu reagent and aluminum chloride were obtained from Loba Chemie. Gallic acid, Rutin, Trolox (6-hydroxy2,5,7,8-tetramethylchroman-2-carboxylic acid), DDPH (2,2-diphényl-1-picrylhydrazyl), ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] and catechin were supplied by Sigma Chemical Co. (USA). Vanillin and sodium carbonate were purchased from Chemi-Pharama.

2.2. Plant materials

Leaves from four Tunisian genotypes of olive (*Olea europaea* L.) including two cultivated olive trees called 'Chemlali' and 'Zarrazi' cultivated in the Gabes region (variety *europaea*) and two wild olive trees or oleasters (variety *sylvestris*) originating from natural ecosystems at Tunisia southern (Tounine and Toujene) were harvested and collected. The olive leaves were washed, and air-dried, then grounded to a fine powder using a crusher (type FW135, 200Mic).

2.3. Methods of extraction

Polyphenols extraction from olive leaves was conducted with 70% methanol using four techniques of extraction: Soxhlet extraction (SOE), heat reflux extraction (HRE), maceration (MAC) and ultrasonic assisted extraction (UAE). The extraction procedures were repeated three times. All obtained extracts were filtered. Then, the filtrates were centrifuged at 4000 tr/min for 20 min. The extracts were recovered and stored at 4°C in the dark for analyses. Dried powdered olive leaves (5 g) were extracted with 100 mL of 70% methanol: i) by maceration (MAC) for 24 h at 25°C under agitation, ii) by Soxhlet (SOE) for 4h at 70°C, iii) by heat reflux extraction (HRE) for 1h at 70°C, and iv) by ultrasonic assisted extraction (UAE) for 40 min at 25°C (Hannachi et al., 20019; Yahia et al., 2020).

2.4. Total polyphenols content (TPC)

TPC was determined by Folin Ciocalteu method. Folin reagent (0.5 mL) was added to 0.1 ml of olive leaves extract. After 5 min, 4 ml of Na_2CO_3 (1M) was added. The mixture was then leaved for 90 min in the dark at room temperature. The absorbance was recorded at 765 nm using a T60 UV-Spectrophotometer. A calibration curve was prepared with a gallic acid solution having a concentration ranged between 0 and 500 µg/ml. Results were expressed as milligram of gallic acid equivalent (GAE) per 100 grams of dry matter (mg GAE/100g DM) (Hannachi et al., 20019; Yahia et al., 2020).

2.5. Total flavonoids content (TFC)

Olive leaves extract (1 ml) was added to 1 ml of aluminum solution $AlCl_3$ (10%). The mixture was incubated at room temperature for 30 min and then, the absorbance was measured at 430 nm using a T60 UV-Spectrophotometer. The average data were interpolated in a rutin calibration curve with a concentration ranging between 0 and 250 µg/ml. The TFC was expressed as milligram of rutin equivalent per 100 grams of dry matter (mg RE/100g DM) (Elfalleh et al., 2009).

2.6. Condensed tannins content (CTC)

The condensed tannins content was determined by the vanillin method in acid medium. Olive leaves extract (250 µl) was added to 1500 µl of vanillin/methanol solution (4%). Then, 750 µl of 37% hydrochloric acid was added. The mixture was incubated at room temperature for 15 min and the absorbance was measured at 500 nm using a T60 UV-Spectrophotometer. The average data were interpolated in a catechin calibration curve with a concentration ranging from 0 to1000 µg/ml. Result was expressed as milligram of catechin equivalent per 100 grams of dry matter (mg CE/100g DM) (Elfalleh et al., 2009).

2.7. High performance liquid chromatography analysis

The olive leaves of oleaster from Tounine natural ecosystem were used to extract phenolic compounds by four different extraction methods to determine the effect of the extraction methods on the quality of phenolic compounds. For other parts, phenolic compounds of the four studied olive leaves were extracted by maceration process to evaluate the effect of genotype on the quality of phenolic compounds.

An Agilent HPLC (high performance liquid chromatography) system with DiscoVery BIO Wide Pore C18-5 binary pump with LC-20ADXR type pump A and LC-20ADXR type B pump was used to identify phenolic compounds. The column has a length of 250 mm and an internal diameter of 4.0 mm (Vinha et al., 2002). The total flow of the mobile phase was 0.4000 ml/min and the pressure varied from 0% to 10%. Elution was carried out at a maximum temperature of 75°C for 35 min. The mass spectrometer (MS) was coupled with a high-speed liquid chromatography system to identify the phenolic compounds. The peaks of phenolic compounds were identified by comparing their retention time with those of the standards and verifying their characteristic spectrum (λ = 163 to 717 nm). Results were expressed as milligram per kg dry matter (mg/kg DM).

2.8. Antioxidant activities

2.8.1. DPPH· radical scavenging activity

Olive leaves extract (20 µl) was mixed with 180 µl of 1,1-diphenyl-2-picrylhydrazyl (DPPH·) methanolic solution (0.2 mM). The mixture was shaken and left for 30 min in darkness at 25°C. The presence of an antioxidant donator of hydrogen, the DPPH. Radical was reduced in 2,2-diphényl-1-picrylhydrazine (DPPH-H). The absorbance of the solution was measured at 517 nm (Elfalleh et al., 2009). A calibration curve was done using Trolox. The antioxidant activity was recorded as equivalent antioxidant capacity expressed as mg equivalent Trolox per 100 g dry matter (mg ET/100 g DM).

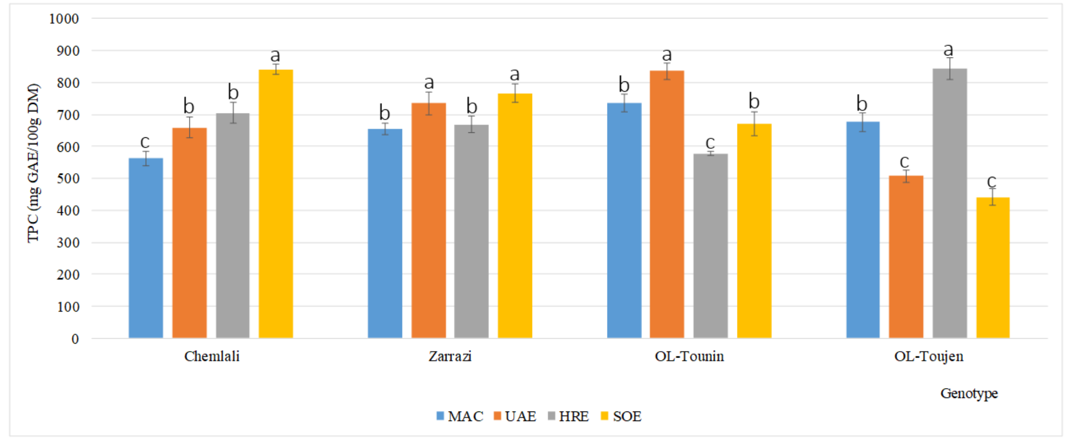

Figure 1. Total polyphenols content (TPC) of olive leaves according to genotype and extraction method (GAE: gallic acid equivalent, DM: dry matter; MAC: maceration extraction; UAE: ultrasonic assisted extraction; HRE: heat reflux extraction; SOE: Soxhlet extraction, different letters for each genotype showed significant difference at $p < 0.05$)

2.8.2. ABTS⁺ radical scavenging activity

Le radical monocation $ABTS^{+}\cdot$ [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] was generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of hydrogen-donating from an antioxidant (Re et al., 1999). The $ABTS^{+}\cdot$ radical generated by mixing 7 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) and allowing the mixture in the dark at room temperature for 16 h. Before usage, the $ABTS^{+}\cdot$ solution was diluted with ethanol to get an absorbance of 0.700 ± 0.02 at 734 nm. Then, a quantity of 20 µl of the olive leaves extract was added to a 180 µl of $ABTS^{+}\cdot$ solution. The absorbance was measured at 734 nm after 5 min of incubation (Elfalleh et al., 2011). The curve absorbance reduction at 734 nm function of Trolox concentration (mM) were used to determine the ABTS radical scavenging activity. Results were expressed as mg ET/100 g DM.

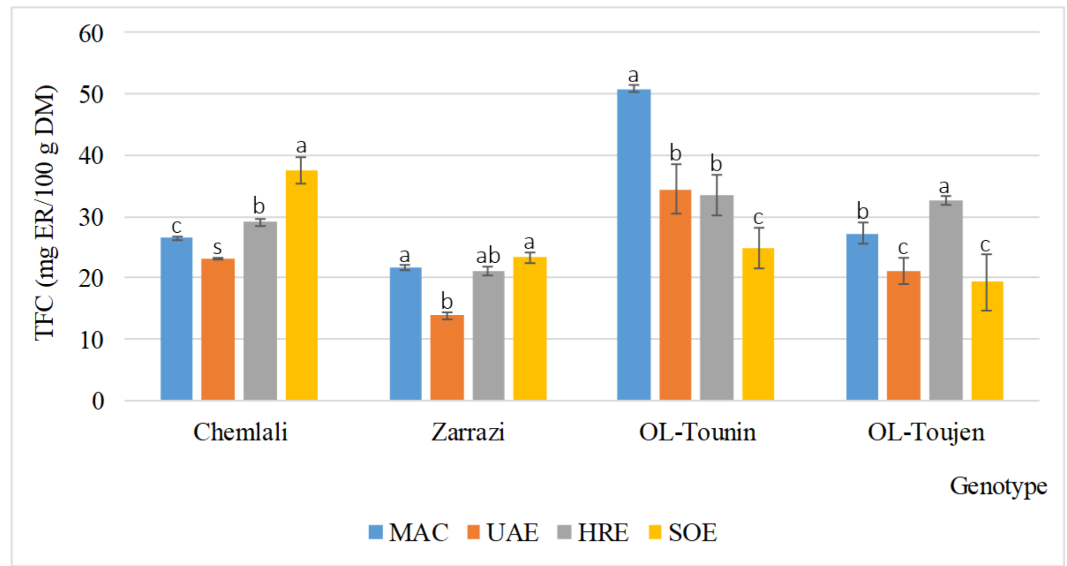

Figure 2. Total flavonoids content (TFC) of olive leaves according to genotype and extraction method (RE: rutin quivalent, DM: dry matter, MAC: maceration extraction, UAE: ultrasonic assisted extraction, HRE: heat reflux extraction, SOE: Soxhlet extraction, different letters for each genotype showed significant difference at $p < 0.05$)

2.9. Statistical analyses

Statistical analyses were performed using the XLSTAT software (www.xlstat.com). All analyzes were conducted in triplicate and the data were presented as an average ± standard deviation. The influence of the genotype, the extraction method and their possible interactions were evaluated by analysis of the variance (ANOVA) and Duncan's multiple range. An effect was considered significant when $p < 0.05$. Principal component analysis (PCA) was conducted on obtained data to show the distribution of genotypes and extraction methods based on polyphenols, flavonoids, condensed tannins, phenolic acids content and antioxidant activities.

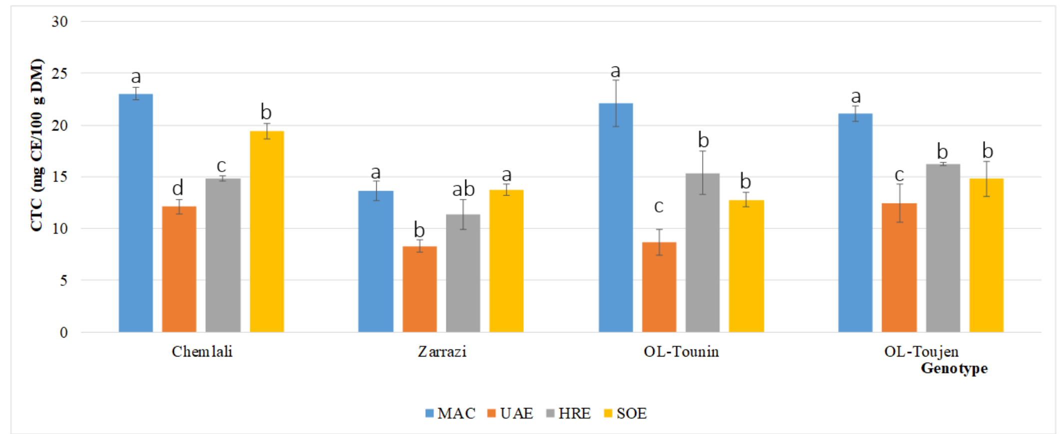

Figure 3. Condensed tannin content (CTC) of olive leaves according to genotype and extraction method (CE: catechin equivalent, DM: dry matter, MAC: maceration extraction, UAE: ultrasonic assisted extraction, HRE: heat reflux extraction, SOE: Soxhlet extraction, different letters for each genotype showed significant difference at $p < 0.05$)

3. Results and discussion

3.1. Total polyphenols contents (TPC)

The oleaster OL-Toujene showed the highest TPC obtained by heat reflux extraction (843.17 mg GAE/100 g DM) and the lowest one (441.01 mg GAE/100 g DM) obtained by Soxhlet. The TPC varied from 653.42 to 841.17 mg GAE/100 g DM and from 441.01 to 843.17 mg GAE/100 g DM of leaves from cultivars and oleasters, respectively. ANOVA showed that genotype, method of extraction and genotype-method interaction have a significant effect on TPC (Figure 1).

Extraction by maceration of oleaster OL-Tounine leaves showed the highest TFC (50.85 mg RE/100g DM), followed by a Soxhlet extraction of Chemlali leaves (37.48 mg RE/100g DM). Ultrasonic assisted extraction of Zarrazi olive leaves yielded the lowest TFC (13.87 mg RE/100g DM). ANOVA showed significant effect of genotype, extraction method and genotype-extraction method interaction (Figure 2).

The CTC obtained from Chemlali leaves (23.03 mg CE/100g DM) using maceration were higher than other extraction methods. The condensed tannins content obtained by ultrasonic assisted extraction (Zarrazi leaves) showed the lowest content (8.32 mg CE/100g DM). ANOVA showed a significant effect of genotype, extraction method and genotype-extraction method interaction on the condensed tannins contents (Figure 3).

Results showed that the genotype, method of extraction and the genotype-method of extraction interaction influenced the polyphenols, flavonoids, and condensed tannins contents.

It has been reported that the extractions method affected the polyphenols yields extracted from plants (Yahia et al., 2020). Results showed that polyphenols content increased at high temperature. Indeed, the highest TPC were obtained by using Soxhlet and heat reflux extractions. High temperature during promotes the diffusion and solubility of the extracted substances. It has been noted that Soxhlet extraction provided high yields of polyphenols among conventional techniques (Tsakona et al., 2012; Al-Bandak and Oreopoulou, 2007; Horžić et al., 2009). Conventional method of extraction is usually conducted at high temperature such as Soxhlet and reflux or under longer time of extraction as maceration. New extraction method has been developed as ultrasonic and microwave assisted extractions to avoid energy and solvent consumption (Hannachi et al., 20019; Yahia et al., 2020). However, in this study the ultrasonic assisted extraction showed lower polyphenols, flavonoids and condensed tannins contents as noted previously (Da Porto et al., 2013). Based on literature, the ultrasonic assisted polyphenols extraction is more efficient for polyphenols extraction (Nayak et al., 2015; Yahia et al., 2020). These differences would be explained by genetic factors.

3.2. Influence of the genotype on phenolic profile

The phenolic compounds identification of HPLC analysis were conducted by using 33 available standards.

Quinic acid is the major phenolic acid, varying from 275.39 to 618.24 mg/kg DM, followed by 4,5-di-*O*-cafeoylquinic acid varying from 7.39 to 32.26 mg/kg DM. Gallic acid was detected only in leaves extract of Zarrazi cultivar. In contrast, caffeic acid was absent only in Chemlali leaves extract. 1,3-di-*O*-caffeoyquinic acid was detected only in oleaster OL-Tounine only and *trans*-ferulic acid was detected in the both Zarrazi and oleaster OL-Toujene leaves extracts.

Luteolin-7-*O*-glucoside was the predominant flavonoids (from 1214.49 to 3211.44 mg/kg DM), followed by quercetrin (quercetin-3-*O*-rhamnose) (from 767.45 to 1574.63 mg/kg DM). The *(+)*-catechin was identified only in the Chemlali leave extract. The genotype significantly influenced the polyphenols compounds

reflecting by analysis of variance followed by Duncan's multiple range (Table 1).

The PCA analysis showed that the oleaster Tounine was distinguishable for other genotype and was closely with SOX extraction method (Figure 4).

Table 1. Identification of phenolic compounds by HPLC of olive leaves according to genotypes

Phenolic compounds (mg/kg extract)	Brut formula	[M-H] m/z	RT (min)	Cultivars leaves		Oleaster leaves	
				Chemlali	Zarrazi	Tounine	Toujene
Quinic acid	$C_7H_{12}O_6$	191	2.048	400.15 ± 4.31^{b}	275.39 ± 27.73^{c}	618.24 ± 6.22^{a}	394.71 ± 18.26^{b}
Gallic acid	$C_7H_6O_5$	169	3.926	nd	7.43 ± 0.07	nd	nd
Chlorogenic acid	$C_{16}H_{18}O_9$	353	9.882	12.02 ± 0.99^{b}	6.75 ± 0.41^{b}	28.42 ± 0.48^{a}	11.35 ± 3.55^{b}
Catechin (+)	$C_{15}H_{14}O_6$	289	11.059	5.54 ± 0.03	nd	nd	nd
4-*O*-caffeoylquinic acid	$C_{16}H_{18}O_9$	353	12.533	12.6 ± 0.04^{b}	7.39 ± 0.52^{c}	32.26 ± 1.05^{a}	12.83 ± 0.98^{b}
Caffeic acid	$C_9H_8O_4$	179	14.528	nd	14.2 ± 2.52	3.28 ± 0.23	8.60 ± 1.64
1,3-di-*O*-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	515	20.367	nd	nd	10.44 ± 0.42	nd
p-Coumaric acid	$C_9H_8O_3$	163	20.90	5.12 ± 0.65^{c}	115.14 ± 3.11^{a}	22.15 ± 1.77^{b}	29.66 ± 7.16^{b}
Salviolinic acid	$C_{36}H_{30}O_{16}$	717	28.946	11.8 ± 2.12	nd	nd	nd
trans-Ferulic acid	$C_{10}H_{10}O_4$	193	23.142	nd	17.14 ± 8.82	nd	32.68 ± 6.99
Rutin	$C_{27}H_{30}O_{16}$	609	23.798	825.77 ± 0.81^{c}	531.24 ± 17.17^{d}	2612.59 ± 30.5^{a}	1342.01 ± 0.25^{b}
Luteolin-7-*O*-glucoside	$C_{21}H_{20}O_{11}$	447	24.440	2059.62 ± 3.22^{c}	1214.49 ± 13.99^{d}	2905.97 ± 16.76^{b}	3211.44 ± 4.02^{a}
Naringin	$C_{27}H_{32}O_{14}$	579	25.855	104.07 ± 1.71^{a}	27.95 ± 1.06^{b}	94.95 ± 0.52^{a}	100.10 ± 9.25^{a}
Apigenin-7-*O*-glucoside	$C_{21}H_{20}O_{10}$	431	26.711	135.95 ± 1.15^{b}	28.54 ± 0.91^{d}	123.98 ± 0.4^{c}	315.67 ± 0.27^{a}
Quercetin (quercetin-3-*O*-rhamnose)	$C_{21}H_{20}O_{11}$	447	26.806	1108.18 ± 22.5^{b}	767.45 ± 15.68^{c}	1536.5 ± 2.12^{a}	1574.63 ± 15.68^{a}
4,5-di-*O*-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	515	26.811	122.33 ± 0.07^{b}	40.08 ± 0.06^{d}	139.94 ± 1.81^{a}	104.93 ± 0.78^{c}
Quercetin	$C_{15}H_{10}O_7$	301	31.714	33.96 ± 1.75^{b}	4.29 ± 0.01^{c}	168.12 ± 3.25^{a}	38.03 ± 1.00^{b}
Kaempferol	$C_{15}H_{10}O_6$	285	31.762	34.27 ± 0.66^{c}	33.79 ± 0.21^{c}	53.44 ± 0.82^{b}	90.01 ± 0.04^{a}
Naringenin	$C_{15}H_{12}O_5$	271	33.730	2.71 ± 0.38^{b}	1.00 ± 0.04^{c}	2.7 ± 0.01^{b}	4.54 ± 0.31^{a}
Apigenin	$C_{15}H_{10}O_5$	269	34.364	1.78 ± 0.37^{c}	0.61 ± 0.07^{d}	6.04 ± 0.14^{b}	23.09 ± 0.01^{a}

RT: retention time, nd: not detected, different letters in the same line showed significant difference at $p < 0.05$

The genotype influenced the phenolic composition of olive leaves extracts. It has been reported that quantitative differences in the phenolic profile were observed between the two Tunisian cultivars Chamlali and Neb Jmel (Brahmi et al., 2013). Results showed differences between the studied cultivars and oleasters. Furthermore, the phenolic profiles depended to the varieties sylvestris and europaea of *Olea europaea* subsp. *europaea*. The variations in phenolic profile can be related to the olive-growing geographical area. It was noted also that the levels of polyphenols in fennel seeds (*Foeniculum vulgarae* Mill.) were affected by their provenances (Bettaieb-Rebey et al., 2011). Moreover, the phenolic composition can vary between the organs of the same plant.

Table 2. Identification of phenolic compounds by HPLC of wild olive leaves (OL-Tounine) according to the extraction methods

Phenolic compounds (mg/kg extract)	Brut formula	[M-H] m/z	RT (min)	Maceration Extraction	Soxhlet extraction	Heat reflux extraction	Ultrasonic extraction
Quinic acid	$C_7H_{12}O_6$	191	2.101	633.61 ± 1.35^{a}	1085.80 ± 0.42^{c}	745.65 ± 0.49^{d}	778.96 ± 0.06^{b}
Chlorogenic acid	$C_{16}H_{18}O_9$	353	9.817	28.27 ± 0.64	nd	nd	nd
4-*O*- Caffeoylquinic acid	$C_{16}H_{18}O_9$	353	3.630	28.11 ± 0.39^{a}	71.83 ± 1.09^{b}	49.55 ± 0.64^{d}	40.82 ± 0.25^{c}
Caffeic acid	$C_9H_8O_4$	179	14.959	6.05 ± 0.58^{a}	13.75 ± 0.35^{ab}	12.66 ± 0.48^{c}	10.99 ± 0.01^{b}
p-Coumaric acid	$C_9H_8O_3$	163	21.312	25.94 ± 0.45^{a}	50.83 ± 0.83^{b}	43.52 ± 0.68^{d}	39.79 ± 0.3^{c}
trans-Ferulic acid	$C_{10}H_{10}O_4$	193	23.537	nd	25.61 ± 0.80^{a}	20.78 ± 0.31^{b}	18.90 ± 0.14^{c}
Rutin	$C_{27}H_{30}O_{16}$	609	24.327	2261.39 ± 1.10^{a}	2582.89 ± 0.90^{c}	2178.54 ± 2.06^{b}	1381.96 ± 0.06^{d}
Luteolin-7- *O*-glucoside	$C_{21}H_{20}O_{11}$	447	25.095	2459.50 ± 1.47^{a}	3720.15 ± 0.69^{a}	3499.67 ± 0.47^{a}	2769.50 ± 0.71^{a}
Quercetin (quercetin-3- *O*-rhamnose)	$C_{21}H_{20}O_{11}$	447	27.402	1581.39 ± 1.48^{a}	1800.05 ± 0.33^{c}	1525.67 ± 0.64^{b}	882.11 ± 1.15^{d}
Naringin	$C_{27}H_{32}O_{14}$	579	26.431	93.83 ± 0.83^{b}	82.83 ± 0.30^{c}	76.89 ± 0.52^{a}	58.16 ± 0.45^{d}
Apigenin -7- *O*-glucoside	$C_{21}H_{20}O_{10}$	431	27.332	123.00 ± 0.39^{a}	170.78 ± 0.90^{ab}	169.05 ± 0.58^{c}	167.25 ± 0.64^{b}
4,5-di-*O*- caffeoylquinic acid	$C_{25}H_{24}O_{12}$	515	26.803	143.42 ± 0.71	nd	nd	nd
trans Cinnamic	$C_9H_8O_2$	147	32.214	nd	2.46 ± 0.57	1.31 ± 0.36	2.26 ± 0.31
Quercetin	$C_{15}H_{10}O_7$	301	32.538	154.05 ± 0.83^{a}	324.28 ± 0.34^{c}	102.83 ± 0.83^{b}	63.94 ± 0.33^{d}
Kaempferol	$C_{15}H_{10}O_6$	285	32.460	56.00 ± 0.64^{a}	137.89 ± 0.65^{b}	104.94 ± 0.96^{d}	76.70 ± 0.71^{c}
Naringenin	$C_{15}H_{12}O_5$	271	34.239	2.55 ± 0.38^{a}	2.65 ± 0.21^{a}	2.33 ± 0.35^{a}	2.26 ± 0.28^{a}
Apigenin	$C_{15}H_{10}O_5$	269	34.975	6.66 ± 0.25^{a}	19.48 ± 0.40^{ab}	18.00 ± 1.15^{c}	15.34 ± 0.45^{d}
Cirsiliol	$C_{17}H_{14}O_7$	329	35.793	nd	14.47 ± 0.49^{a}	11.14 ± 0.17^{b}	9.03 ± 0.19^{c}
Cirsilineol	$C_{18}H_{16}O_7$	283	38.949	nd	1.92 ± 0.03^{a}	1.19 ± 0.01^{b}	0.82 ± 0.01^{c}

RT: retention time; nd: not detected, ET: Equivalent Trolox, DM: dry matter, different letters showed significant difference at $p < 0.05$

3.3. Influence of the extraction method on phenolic profile

Four extraction methods were used to extract polyphenols from oleaster OL-Tounine leaves (Table 2). 15 phenolic compounds were identified in extract prepared by maceration and 17 phenolic compounds were identified using Soxhlet, heat reflux and UAE. Ferulic and *trans*-cinnamic acids were not obtained by maceration. However, the 4,5-di-*O*-caffeoylquinic and chlorogenic acids were obtained only in the extract obtained by maceration. Quinic acid was the major phenolic acid of the oleaster OL-Tounine leaves having an amount of 1085.80 mg/kg DM (Soxhlet), followed by 4,5-di-*O*-caffeoylquinic (143.42 mg/kg DM) (maceration). *trans*-Cinnamic acid was the minor phenolic acid detected by all extraction methods excepting maceration.

For flavonoids, the same compounds profile was obtained by four extraction methods, except cirsiliol and cirsilineol, which were not obtained by maceration extraction. Luteolin-7-*O*-glucoside was the major flavonoid followed by rutin having an amount of 3720.15 and 2582.80 mg/kg DM, respectively, obtained by Soxhlet (Table 2).

The PCA showed that the UAE was more related to the composition of cultivars Zarrazi and Chemlali et the oleaster OL-Toujène. However, the oleater OL-Tounine was more related to the Soxhlet extraction (Figure 4).

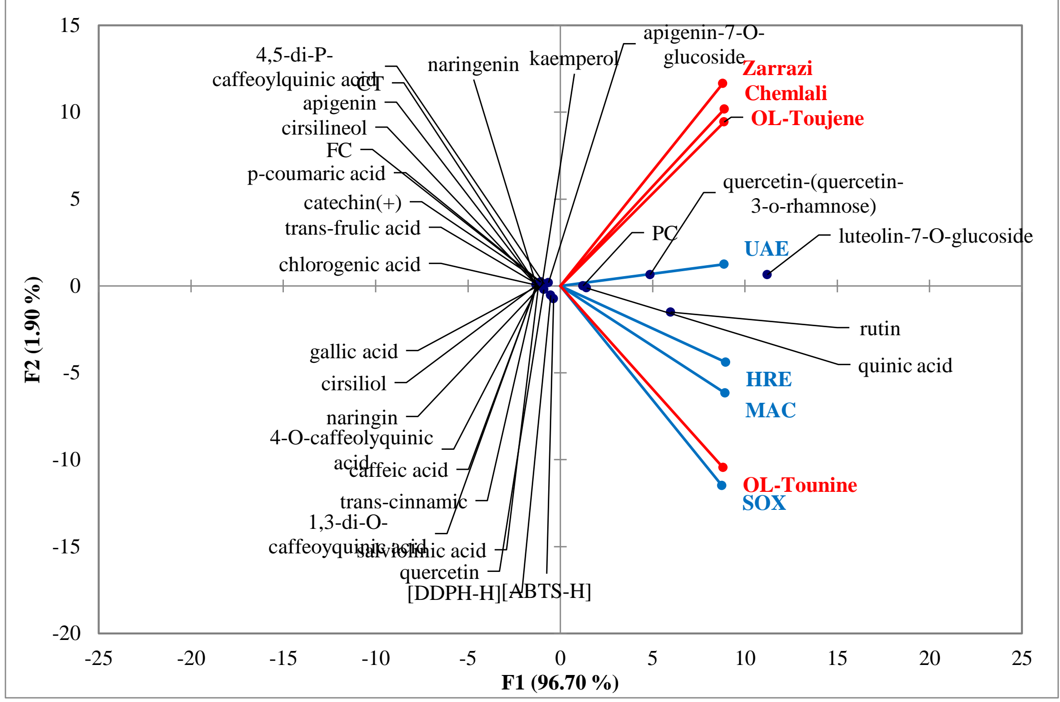

Figure 4. Principal Component analysis based on phenolics compounds and antioxidant activities of olive genotypes using four extraction methods (UAE: ultrasound asisted extraction, HRE: heat reflux extraction, MAC: maceration method, SOX: Soxhlet extraction: PC: polyphenols content, FC: flavonoids content, CT: condensed tannins content)

Extraction by Soxhlet provided extracts richer in phenolic compounds compared to extracts obtained by heat reflux, ultrasonic assisted extraction, and maceration. The effect of extraction methods on flavonoids stability form plants has been previously studied (Trusheva et al., 2007; Biesaga et al., 2011). Significant decomposition of myrcetin, kaempferol and quercetin was noted by using ultrasonic and microwave assisted extraction from maize (Cui et al., 2008). Gourguillon et al. (2016) indicated that the choice of extraction method influenced the extraction of dicafeoylquinic acids in halophytes.

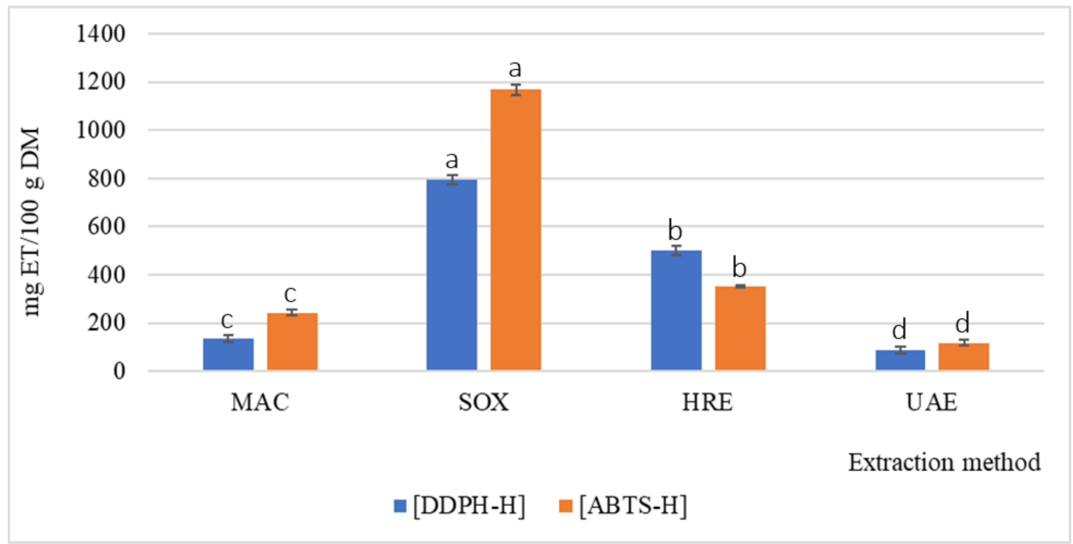

Figure 5. Antioxidant activities of oleaster leave extracts according extraction method (different letters for extraction method showed significant difference at $p < 0.05$; ET: equivalent trolox, UAE: ultrasound asisted extraction, HRE: heat reflux extraction, MAC: maceration method, SOX: Soxhlet extraction)

The qualitatively analysis by HPLC showed the identification of 9 phenolic acids and 11 flavonoids according to the availability of standards. Some phenolic compounds were showed high levels. Although, olive leaves were considered richer in phenolic

compounds compared to olive oil and fruits (Lalas et al., 2011; Hannachi et al., 2020).

The extraction method of olive leaves has a quantitative and qualitative influences on phenolic compounds. Results of HPLC analysis confirm the presence of phenolic acids and flavonoids in the various extracts of olive leaves recording the effect of the extraction method used and the genotype on certain compounds.

3.4. Influence of the extraction methods on antioxidant activities

Results showed that all olive extracts have an antioxidant activity using the DPPH and ABTS assays. The extraction method influent significantly the antioxidant activities. The extract obtained by SOX method showed the highest antioxidant activity by DPPH (793.60 mg ET/100 g DM) and ABTS (1168.00 mg ET/100 g DM) followed by the extracts obtained by HRE, and by MAC (Figure 5).

4. Conclusions

Olive leaves are considered as sustainable source of natural antioxidants and phenolic compounds. Four extraction methods were compared to evaluate quantitatively and qualitatively phenolic compounds of olive leaves (*Olea europaea* var. *sylvestris* and *Olea europaea* var. *europaea*). The polyphenols content varied according to the genotype, the extraction method, and their interaction. However, extracts of oleasters leaves obtained by Soxhlet extraction and by heat reflux extraction gave high polyphenols content. Qualitatively, genotype and extraction method influenced the phenolic compounds profile. Results showed the richness of *Olea europea* var. *sylvestris* in phenolics representing a new potential source of bioactive molecules.

Acknowledgments

None.

Conflict of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Salma Guebebia: Experimental analyses, Writing original draft
Khadija Ben Othman: Supervision on experimental analyses, Methodology, Investigation
Yassine Yahia: Supervision on high performance liquid chromatography analyses
Mehrez Romdhane: Conceptualization, Methodology, Supervision
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Supplementary File

None.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 25-29

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

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RESEARCH ARTICLE

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Chemical constituents of the essential oil isolated from seed of black pepper, *Piper nigrum* L., (Piperaceae)

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ARTICLE INFO

Article History:

Received: 28 November 2021
Revised: 01 December 2021
Accepted: 01 December 2021
Available online: 01 December 2021

Edited by: B. Tepe

Keywords:
Analysis
Black pepper
Constituent
Volatile compound

ABSTRACT

Black pepper (*Piper nigrum* L.) is a flowering plant from family Piperaceae. In this research, chemical analysis of the essential oil isolated from this important tropical medicinal plant was performed. For this, seed of this plant was provided, dried under shadow (25 °C), powdered, it's essential oil isolated by Clevenger apparatus, and analyzed by Gas Chromatography related to Mass Spectroscopy device (Agilent 7890). The results showed that there were valuable compounds in its essential oil, total numbers of them reach to 89. Total identification time was estimated to be 38.143 min. Also, nineteen compounds contained more than 1% of total volume; among them, *trans*-caryophyllene bicyclo (peak 44, 19.512 min, 36.43%), L-limonene cyclohexene (peak 13, 7.193 min, 6.75%), 3-carene (peak 9, 6.838 min, 4.97%), cyclohexene, 1-methyl-4-(5-methyl) (peak 60, 22.081 min, 4.93%), and 2-beta-pinene bicyclo (peak 5, 6.220 min, 4.18%) were five dominant constituents, respectively. Moreover, thirty-seven compounds contained lower and equal with 0.1% of total volume and named as the least constituents. In conclusion, this medicinal plant has important secondary compounds which could seriously be focused on them in medical, pharmacology, and toxicology.

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1. Introduction

Black pepper (*Piper nigrum* L.) is a tropical medicinal plant belongs to family Piperaceae, cultivated for its fruit known as a peppercorn which is usually used as an important spice (Jaramillo et al., 2001). The fruit is drupe (stone fruit) which when fresh and fully mature is about 5 mm in diameter, dark red, and contains a stone which encloses a single pepper seed. Black pepper is native to present-day Kerala, a state on the southwestern coast of India, and is extensively cultivated there and the other tropical regions of the world (Govindarajan, 1980). Ground, dried, and cooked peppercorns have been used since antiquity, both for flavor and as a traditional medicine. Black pepper is the world's most traded spice and is one of the most common spices added to cuisines around the world (Nirmala Menon, 2000). Its spiciness is due to the chemical compound piperine which is a different kind of spicy from the capsaicin characteristic of chili peppers. The pepper is a perennial woody plant growing up to 4 m in height on supporting trees, poles or trellises. It is a spreading vine, rooting readily where trailing stems touch the ground. Its leaves are alternate, entire, with 5-10 cm long and 3-6 cm width. The flowers are small, produced on pendulous spikes 4-8 cm long at the leaf nodes, the spikes lengthening up to 7-15 cm as the fruit matures (Samsam Shariat, 2007). A single stem bears 20-30 fruiting spikes. The harvest begins as soon as one or two fruits at base of the spikes begin to turn red, and before the fruit is fully mature, and still hard; if allowed to ripen completely, the fruits lose pungency and ultimately fall off and are lost. The spikes are collected and spread out to dry in sun and then the peppercorns are stripped off the spike (Dini, 2005).

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e-ISSN: 2791-7509
doi:
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Essential oils are volatile and aromatic compounds in plants mostly contain terpenes and terpenoids in their chemical structure. Identifying of these compounds and understanding their roles are very important issues in plant science (Isman, 2000; Isman et al., 2008). Essential oils, known as secondary metabolites, are mainly abundant in Myrtaceae, Lauraceae, Lamiaceae, and Asteraceae families. These compounds mainly have contact, fumigant, repellent, and antifeedant effects and are one of the main components in defense mechanisms of plants against the herbivores (Bakkali et al., 2008; Rafiee-Dastjerdi et al., 2013; Asadi et al., 2018,

Please cite this article as: Asadi, M., 2022. Chemical constituents of the essential oil isolated from seed of black pepper, Piper nigrum L., (Piperaceae). International Journal of Plant Based Pharmaceuticals, 2(1), 25-29.

2019). About the isolation method, volatile and aromatic products that are isolated by disstillation process called as the essential oil (Asadi et al., 2019). The essential oils could be used in different aspects due to their important medicinal features. These compounds are different in each plant species and also in each geographical region; therefore, it is not possible to expect same compounds in one plant species from different locations; although, the similarities might be available between their constituets. Therefore, major aim of this study was to identify secondary compounds in the seed of *P. nigrum* as a basic research for their using in future.

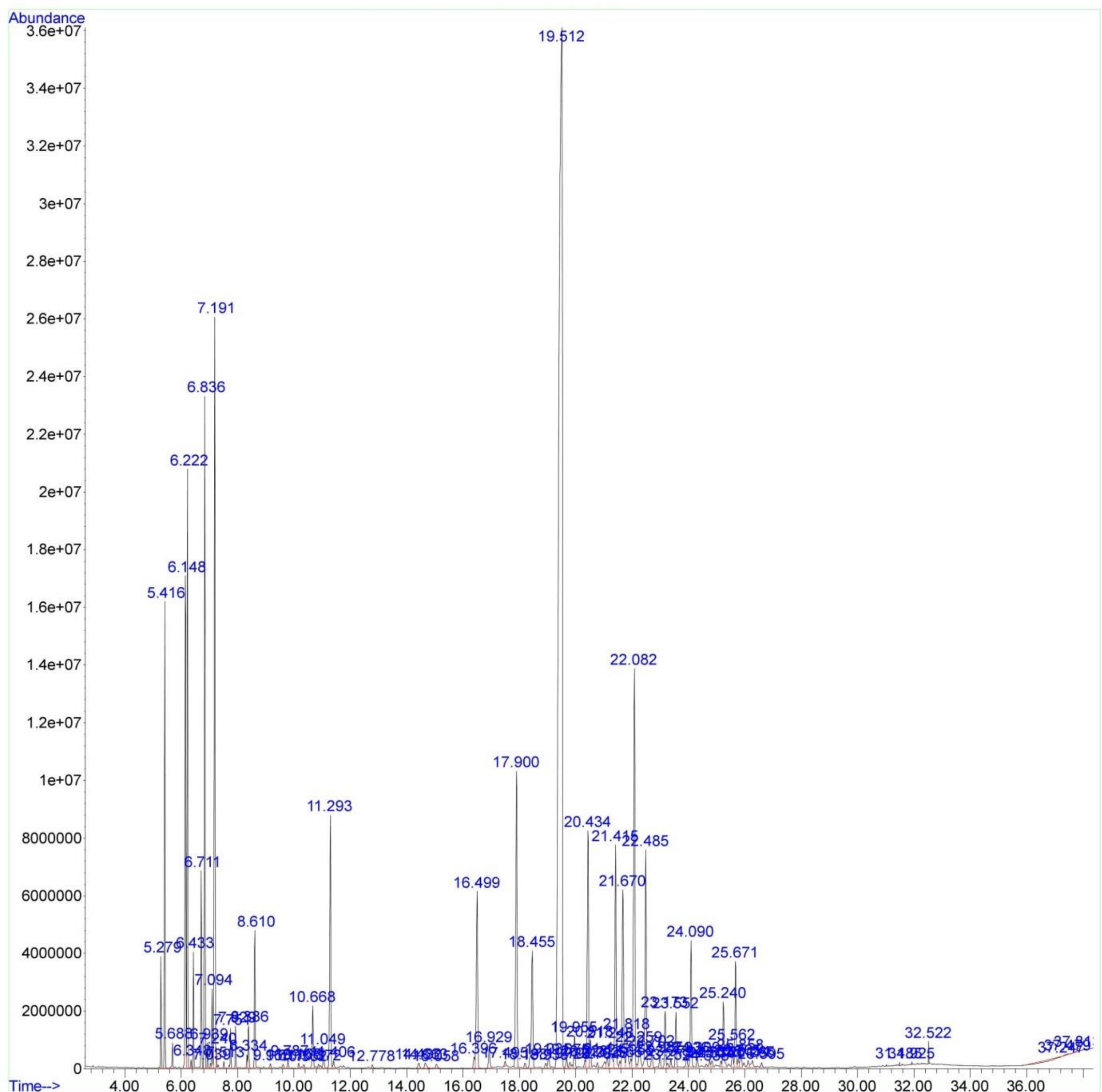

Figure 1. Chromatogram of total compounds in the essential oil of *P. nigrum*

2. Materials and methods

2.1. Identification

Identification of black pepper seed specimen was done by sending of it to Razi University Herbarium (RUHK), Kermanshah, Iran (Code: 1118).

2.2. Isolation of essential oil

P. nigrum seed was provided from medicinal plants stores in Kermanshah province, Iran, during 2018. After complete drying of the specimen in the temperature about 25 °C, it was transferred to the laboratory and essential oil isolated. For this, the seed of plant was powdered. Then, 30 g of its powder was added with 300 ml of the deionized water in 1 liter balloon of the Clevenger apparatus (Babaee Ghaghelestany et al., 2020). After three hours, its essential oil was separated as pale green layer above the water. In order to remove the water for purifying the essential oil, Na_2SO_4 (Sodium Sulfate) compound was used (Asadi et al., 2019). Finally, the purified essential oil was stored in special microtubes (2 ml) covered with aluminum foil in the refrigerator (about 4 °C) until GC-MS analysis (Negahban et al., 2007; Samsam Shariat, 2007; Shiva Parsia and Valizadegan, 2015; Asadi et al., 2018; Babaee Ghaghelestany et al., 2020).

2.3. Gas Chromatography-Mass Spectroscopy (GC-MS)

Chemical compounds in the isolated essential oil of *P. nigrum* were identified by using chromatographic device related to mass spectroscopy (GC-MS: Agilent 7980, the USA) in the central laboratory, University of Mohaghegh Ardabili, Ardabil, Iran. The device was able to inject samples with dilute split splitless inlet (SSI) ability with mass spectroscopy detector (MSD) to quantitatively and qualitatively recognize of samples. The detector was also equipped with ionization system and four-coupled single analyzer (SQA) (Babaee Ghaghelestany et al., 2020). After injecting of the essential oil by Hamilton syringe, different compounds were detected based on their molar mass in different times and their chromatogram was also drawn by the analyzer device (Figure 1).

Table 1. Total compounds in *P. nigrum* essential oil with their retention time and percentage of total

Peak	Compound	Retention time (min)	Percentage of total
1	Thujene	5.276	0.65
2	1R-alpha-Pinene	5.413	2.93
3	Camphene bicyclo [2.2.1] heptan	5.688	0.17
4	Sabinene bicyclo [3.1.0] hexane	6.146	3.81
5	2-beta-Pinene bicyclo	6.220	4.18
6	3-Octanone (cas) eak	6.346	0.05
7	beta-Myrcene 1, 6-octadiene	6.432	0.70
8	1-Phellandrene	6.712	1.28
9	3-Carene	6.838	4.97
10	alpha-Terpinene para-menth	6.941	0.15
11	alpha-Terpinene 1, 3-cyclohexene	7.038	0.03
12	Benzene, 1-methyl-2-(1-methylethyl)	7.096	0.56
13	L-Limonene cyclohexene	7.193	6.75
14	1, 8-Cineole 2-oxabicyclo	7.239	0.10
15	1, 3, 6-Octatriene, 3, 7-dimethy	7.513	0.05
16	1, 4-Cyclohexadiene, 1-methyl-4	7.748	0.26
17	*cis*-Sabinenehydrate	7.931	0.29
18	alpha-Terpinolene cyclohexe	8.332	0.09
19	(+)-4-Carene	8.389	0.34
20	1, 6-Octadien-3-ol, 3, 7-dimethyl	8.612	1.03
21	gamma-Terpinene 1, 4-cyclohexen	9.161	0.05
22	beta-Phellandrene cyclohexen	9.619	0.05
23	Bicyclo [2.2.1] heptan-2-one	9.785	0.08
24	*p*-Mentha-1, 5-dien-8-ol	10.168	0.05
25	Isoborneol	10.351	0.03
26	3-Cyclohexen-1-ol, 4-methyl-1	10.666	0.62
27	Benzenemethanol	10.872	0.04
28	Beta fenchyl alcohol	11.050	0.20
29	Estragole	11.290	2.65
30	Benzene, butyl	11.404	0.08
31	2-Cyclohexen-1-one, 2-methyl-5	12.778	0.04
32	Bicyclo [2.2.1] heptan-2-ol	14.431	0.09
33	Phenol, 5-methyl-2-(1-methylethyl)	14.660	0.10
34	Phenol, 2-methyl-5-(1-methylethyl)	15.061	0.06
35	1, 3-Cyclohexadiene, 1-methyl-4	16.394	0.14
36	Cyclohexene, 4-ethenyl-4-methyl	16.497	2.32
37	alpha-Cubebene	16.932	0.29
38	gamma-Cadinene naphthalene	17.492	0.05
39	alpha-Copaene tricyclo	17.899	3.71
40	Cyclohexane, 1-ethenyl-1-methyl	18.196	0.06
41	beta-Elemene	18.454	1.63
42	Benzene, 1, 2-dimethoxy-4	18.934	0.07
43	1H-Cycloprop *[E]* azulene	19.037	0.16
44	*trans*-Caryophyllene bicyclo	19.512	34.63
45	Germacrene-D	19.673	0.12
46	gamma-Elemene cyclohexane	19.793	0.07
47	Azulene, 1, 2, 3, 4, 5, 6, 7, 8-octahyd	19.953	0.37
48	alpha-Cubebene 1h-cyclopent	20.331	0.10
49	alpha-Humulene	20.434	2.85
50	*trans*-beta-Farnesene	20.519	0.30
51	gamma-Gurjunene azulene	20.765	0.06
52	6, 10, 11-Tetramethyl-tricyclo	21.029	0.11
53	alpha-Amorphene	21.114	0.15
54	Germacrene D 1, 6-cyclodecadie	21.246	0.45
55	Eudesma-4(14), 11-diene	21.418	2.61
56	Isoledene	21.567	0.09
57	alpha-Selinene	21.669	2.23
58	Naphthalene, 1, 2, 4a, 5, 6, 8a	21.818	0.57
59	Germacrene A (CAS)	21.956	0.12
60	Cyclohexene, 1-methyl-4-(5-methyl)	22.081	4.93
61	Naphthalene, 1, 2, 3, 4, 4a, 5, 6, 8a	22.259	0.38
62	delta-Cadinene naphthalene	22.482	2.25
63	Cadina-1, 4-diene	22.699	0.23
64	*cis*-alpha-Bisabolene	22.986	0.14
65	Cyclohexanemethanol, 4-ethenyl	23.174	0.60
66	Caryophyllene oxide	23.260	0.06
67	gamma-Elemene	23.375	0.10
68	1, 6, 10-Dodecatrien-3-ol, 3, 7, 11	23.552	0.56
69	1H-Cycloprop[E]azulen-7-ol	23.935	0.13
70	*(-)*-Caryophyllene oxide	24.090	1.40
71	*(-)*-Dehydroaromadendrene	24.307	0.13
72	Cyclohexene, 6-ethenyl-6-methyl	24.633	0.05
73	*cis*-Z-alpha-Bisabolene epoxide	24.748	0.09
74	beta-Selinene naphthalene	24.834	0.09
75	Epizonarene	25.148	0.07
76	7-Tetracyclo [6.2.1.0 (3.8) 0 (3.9)]	25.240	0.80
77	Bicyclo [4.4.0] dec-1-ene	25.560	0.27
78	1-Naphthalenol	25.669	1.11
79	beta-Eudesmol 2-naphthalene	25.766	0.10
80	beta-Panasinsene	25.858	0.22
81	*ar*-Tumerone	26.115	0.10
82	Cyclohexane, 1, 5-diethenyl-3	26.264	0.16
83	1H-Benzocycloheptene	26.596	0.07
84	Oxirane, hexadecyl	31.483	0.02
85	Eicosane	31.923	0.01
86	4H-1, 3, 5-Thiadiazin-4-one	32.524	0.08
87	Silicone grease, siliconfett	37.245	0.31
88	Gibberellin A3 gibb-3-ene	37.479	0.02
89	Silane, 1, 4-phenylenebis[trimethyl]	37.811	0.03

3. Results and discussion

The chromatogram of available chemical compounds in *P. nigrum* seed essential oil by GC-MS is shown in Figure 1. In the chromatogram, longitudinal axis (X) contained retention time and transverse axis (Y) was the amount of each compound. According to the chromatogram, compounds with higher and lower peaks indicated high and low values in the essential oil, respectively.

Table 2. Nineteen dominant constituents in *P. nigrum* essential oil

Peak	Compound	Retention time (min)	Percentage of total
44	*trans*-Caryophyllene bicyclo	19.512	34.63
13	L-Limonene cyclohexene	7.193	6.75
9	3-Carene	6.838	4.97
60	Cyclohexene, 1-methyl-4-(5-methyl)	22.081	4.93
5	2-beta-Pinene bicyclo	6.220	4.18
4	Sabinene bicyclo [3.1.0] hexane	6.146	3.81
39	alpha-Copaene tricyclo	17.899	3.71
2	1R-alpha-Pinene	5.413	2.93
49	alpha-Humulene	20.434	2.85
29	Estragole	11.290	2.65
55	Eudesma-4(14), 11-diene	21.418	2.61
36	Cyclohexene, 4-ethenyl-4-methyl	16.497	2.32
62	delta-Cadinene naphthalene	22.482	2.25
57	alpha-Selinene	21.669	2.23
41	beta Elemene	18.454	1.63
70	*(-)*-Caryophyllene oxide	24.090	1.40
8	1-Phellandrene	6.712	1.28
78	1-Naphthalenol	25.669	1.11
20	1, 6-Octadien-3-ol, 3, 7-dimethyl	8.612	1.03
	Total volume		87.27

The results showed that *P. nigrum* essential oil contained 89 different chemical compounds (Table 1) with a total retention time about 38.143 min. The retention time in first and last compounds (thujene and silane, 1, 4-phenylenebis [trimethy l]) were determined as 5.276 and 37.811 min, respectively. As shown in Table 2, among the identified compounds, nineteen compounds contained more than 1% of total volume and known as the dominant compounds. By comparing of the percentage for each compound on total volume in

the essential oil, it was found that *trans*-caryophyllene bicyclo at peak of 44 (19.512 min and 34.63%) (Table 2) and Eicosane at peak 85 (31.923 min and 0.01%) (Table 3) were the highest and lowest constituents in the essential oil, respectively. Approximately, it can be said that nineteen major compounds have been identified by the detector in middle peaks; In contrast, the least compounds of the essential oil have been distinguished in initial and last peaks. Looking closely at the composition of the essential oil, there were seventy different compounds with percentages of lower and equal with 1% in total volume which totally contained 13.03% of the essential oil; nevertheless, nineteen dominant compounds make up 87.27% of total volume, indicating that most of the essential oil volume was occupied by these nineteen compounds (Table 2).

Table 3. Thirty-seven constituents in *P.nigrum* essential oil that had the lowest percentage (≤0.1 in total)

Peak	Compound	Retention time (min)	Percentage of total
14	1, 8-Cineole 2-oxabicyclo	7.239	0.10
33	Phenol, 5-methyl-2-(1-methylethyl)	14.660	0.10
48	alpha-Cubebene 1h-cyclopent	20.331	0.10
67	gamma-Elemene	23.375	0.10
79	beta-Eudesmol 2-naphthalene	25.766	0.10
81	*ar*-Tumerone	26.115	0.10
18	alpha-Terpinolene cyclohexe	8.332	0.09
32	Bicyclo [2.2.1] heptan-2-ol	14.431	0.09
56	Isoledene	21.567	0.09
73	*cis*-Z-alpha-Bisabolene epoxide	24.748	0.09
74	beta-Selinene naphthalene	24.834	0.09
23	Bicyclo [2.2.1] heptan-2-one	9.785	0.08
30	Benzene, butyl	11.404	0.08
86	4H-1,3,5-Thiadiazin-4-one	32.524	0.08
42	Benzene, 1, 2-dimethoxy-4	18.934	0.07
46	gamma-Elemene cyclohexane	19.793	0.07
75	Epizonarene	25.148	0.07
83	1H-Benzocycloheptene	26.596	0.07
34	Phenol, 2-methyl-5-(1-methylethyl)	15.061	0.06
40	Cyclohexane, 1-ethenyl-1-methyl	18.196	0.06
51	gamma-Gurjunene azulene	20.765	0.06
66	Caryophyllene oxide	23.260	0.06
6	3-Octanone (cas) eak	6.346	0.05
15	1, 3, 6-Octatriene, 3, 7-dimethy	7.513	0.05
21	gamma-Terpinene 1, 4-cyclohexen	9.161	0.05
22	beta-Phellandrene cyclohexen	9.619	0.05
24	*p*-Mentha-1, 5-dien-8-ol	10.168	0.05
38	gamma-Cadinene naphthalene	17.492	0.05
72	Cyclohexene, 6-ethenyl-6-methyl	24.633	0.05
27	Benzenemethanol	10.872	0.04
31	2-Cyclohexen-1-one, 2-methyl-5	12.778	0.04
11	alpha-Terpinene 1, 3-cyclohexene	7.038	0.03
25	Isoborneol	10.351	0.03
89	Silane, 1, 4-phenylenebis[trimethyl]	37.811	0.03
84	Oxirane, hexadecyl	31.483	0.02
88	Gibberellin A3 gibb-3-ene	37.479	0.02
85	Eicosane	31.923	0.01

Due to high importance of *P. nigrum* in traditional and modern medical, different researches have been performed on this valuable medicinal plant worldwide that we will briefly review them. Sumathykutty et al. (1999) studied the essential oil composition from some *Piper* species by capillary GC and GC-MS methods and stated that elemol (11.5%) and beta-caryophyllene (13%) were the highest constituents of leaf essential oils from *P. nigrum* and *P. attenuatum*, respectively. Moreover, beta-cubebene (10%) and cubebol (23.6%) were the major constituent of *P. attenuatum* and *P. cubeba* berry essential oils. In another study, Sasidharan and Menon (2010) studied the chemical composition of *P. nigrum* and concluded that the main compounds of its leaf essential oil were alpha-bisabolol (24.3%), alpha-cubebene (20%), elemol (15%), bisabolene (15%), and alpha-guaiene (15%) that was completely different with my results about the seed essential oil. Parts of plants differ in terms of essential oil constituents and this is the main reason for different results in these researches. Singh et al. (2013) studied the chemical properties of *P. nigrum* essential oil and concluded that there were 40 different components approximately contained 97.7% of the essential oil while beta-caryophyllene (16.0%), sabinene (12.6%), limonene (11.9%), and torreyol (9.3%) were the major components. Plants from different geographical areas have different constituents; so, we can not expect similar compounds in different regions. For this reason, the plant essential oils science is very interesting and complex. Generally, climate is one of the most affecting factors in changing of secondary metabolites from plants. Bagheri et al. (2014) studied *P. nigrum* essential oil extracted by supercritical carbon dioxide (SC-CO_2) technique and stated that the main components isolated by this method under optimal conditions were beta-caryophyllene (25.38%), limonene (15.64%), sabinene (13.63%), 3-carene (9.34%), beta-pinene (7.27%), and alpha-pinene (4.25%). This technique is new compared to the hydro-distillation method which could be developed in future. Jeena et al. (2014) studied black pepper (*P. nigrum*) essential oil and stated that that its main constituents were caryophyllene (23.98%) and limonene (14.36%). Another subject that must be mentioned is the collection conditions, type of plant part, and analyzer device which are very effective in changing the results of plant essential oils. Morshed et al. (2017) studied the physicochemical features of isolated essential oil from *P. nigrum* cultivated in Chittagong from Bangladesh and concluded that major components of its essential oil were caryophyllene (19.12%), limonene (9.74%), and camphene (8.44%) which is different with my results. One of the main reasons for this difference is the geographical changes between Iran and Bangladesh, which are responsible for variable secondary compounds in this plant from two regions.

4. Conclusions

The medicinal plants are considered as god-given natural resources; so, identification, classification, and study of their chemical properties are very important issues in plant science. Author of this article hopes that next researchers will take more steps to identify the compounds of the other medicinal plants belonging to different species and determine their properties for usage in medical, pharmacology, and toxicology.

Acknowledgments

None.

Conflict of interest

The author confirms that there is no known conflict of interest.

CRediT authorship contribution statement

Mohammad Asadi: M. Asadi performed all parts of the article alone.

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Supplementary File

None.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 30-36

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS
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RESEARCH ARTICLE

OPEN ACCESS

Chemical content of the aerial parts essential oil from rosemary, *Rosmarinus officinalis* L. (Lamiaceae) samples collected from Kermanshah province in the west of Iran

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ARTICLE INFO

Article History:

Received: 03 December 2021
Revised: 15 December 2021
Accepted: 17 December 2021
Available online: 17 December 2021

Edited by: B. Tepe

Keywords:
Essential oil
Constituents
Rosemary

ABSTRACT

Rosemary (*Rosmarinus officinalis* L.) is one of the evergreen and fragrant medicinal plants belonging to Lamiaceae (Labiatae). In the present research, the chemical content of the essential oil isolated from its aerial parts (leaf and flower) was investigated. Accordingly, the mature plant was collected in its natural habitats from Kermanshah province in the west of Iran, dried gradually under the shadow (25 ˚C), pulverized, its essential oil isolated, and finally analyzed by Gas Chromatography-Mass Spectroscopy. The results showed that the important compounds were available in the essential oil of this medicinal plant; the number of them reached 147. Run (retention) time for total compounds was determined as 38.358 min. Also, 27 compounds had > 1% share in total volume; among them, alpha-pinene, (-)-bicyclo (peak 4, 5.448 min, 7.50%), bicyclo [2.2.1] heptan-2-one (peak 29, 9.848 min, 5.92%), bicyclo [3.1.1] hept-3-en-2-one (peak 40, 11.771 min, 5.48%), 1,8-cineole 2-oxabicyclo (peak 17, 7.256 min, 4.60%), and acetic acid 1,7,7-trimethyl (peak 45, 14.506 min, 4.29%) were five major constituents, respectively. Moreover, 56 different compounds contained ≤ 0.1% share in total volume and were distinguished as minor compounds. Accordingly, this fragrant medicinal plant has important essential oil constituents which could be considered in medical, pharmacology, and toxicology.

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1. Introduction

Rosmarinus officinalis L. (Figure 1) is a significant medicinal plant from the family Lamiaceae. This perennial plant is woody with fragrant leaves and many volatile compounds, which are originally native for the Mediterranean regions; but also grow in cold climates (Wang et al., 2008). The height of the plant can reach 1.5 m. The leaves are evergreen with 2–4 cm height and 2–5 mm width, above green and below white, with dense, short, and woolly hairs. The plant flowering occurs during the spring and summer seasons under the temperate climates; but can be in constant bloom in warm climates. This plant has white, pink, purple, and deep blue flowers. Rosemary has a suitable tendency to flowering outside its regular season. This plant has a high tolerance to water shortage cause surviving under severe lack of water for long periods (Pintore et al., 2002). Its seed is often difficult to start due to low germination percentage and for this has slow growth relatively; but, can live for 30 years. Rosemary is cultivated in some parts of Iran as an ornamental plant (Dini, 2005).

There are fundamental differences between essential oil and extract, which is necessary to know them. The aromatic compounds obtained by the hydro-distillation method, isolated by volatile mechanisms, are called essential oil. In comparison, when the substances in plant cells are isolated by solvents (water or organic compounds), it is called extract. In general, the extract is a solution that contains all valuable substances inside the cells (tannins, mucilage, vitamins, and minerals); but, the essential oil commonly contains terpene and terpenoids. On the other hand, the extract may have different solvents such as oil, alcohol, and water (Jahanshahi, 2016). Identifying these constituents and understanding their impacts are important aspects of plant science (Isman, 2000; Isman et al., 2008). Essential oils are common in families Myrtaceae, Lauraceae, Lamiaceae, and Asteraceae that rosemary also belongs to Lamiaceae. These compounds mainly have fumigant, repellent, and antifeedant effects and are one of the main

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e-ISSN: 2791-7509
doi:
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Please cite this article as: Asadi, M., 2022. Chemical content of the aerial parts essential oil from rosemary, Rosmarinus officinalis L. (Lamiaceae) samples collected from Kermanshah province in west of Iran. International Journal of Plant Based Pharmaceuticals, 2(1), 30-36.

components in the defense mechanisms of plants against the herbivores for centuries (Bakkali et al., 2008; Rafiee-Dastjerdi et al., 2013; Asadi et al., 2018, 2019, 2022). This research aimed to identify essential oil constituents in the aerial parts of *R. officinalis* as a basic research for its application in the future.

Figure 1. Mature plant of *R. officinalis*

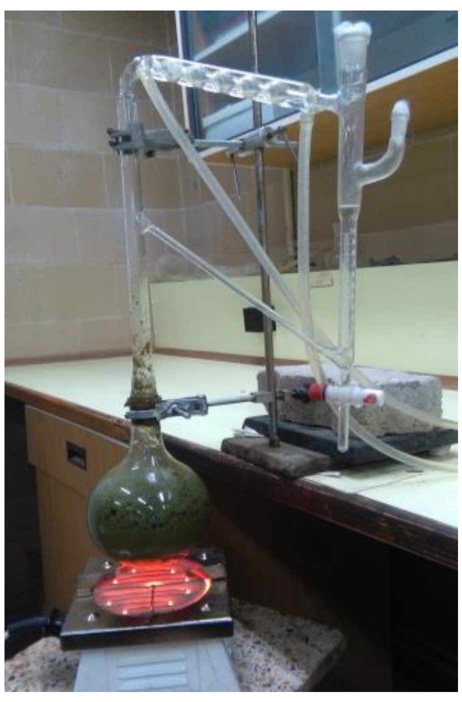

Figure 2. Clevenger apparatus for isolation of *R. officinalis* essential oil

2. Materials and methods

2.1. Identification of species

Identification of rosemary species was performed by sending its complete specimen to one of the botany experts in Razi University Herbarium (RUHK), Kermanshah, Iran. Accordingly, the species of *R. officinalis* L. was confirmed, and code 1918 was assigned.

2.2. Essential oil isolation

Mature plants of *R. officinalis* were collected from their natural habitats in different regions of Kermanshah province in the west of Iran, during 2018. Then, the specimens were dried at 25 °C (shadow), moved to the laboratory, and its essential oil was isolated. In the first stage, aerial parts of the plant were pulverized with a grinder, then 50 g of its plant powder was mixed with 500 ml of the

distilled water inside the balloon of the Clevenger apparatus (volume 1 liter) (Figure 2) (Babaee Ghaghelestany et al., 2020). After four hours, the essential oil was given as a green layer. To remove essential oil's water and its purifying, sodium sulfate (Na_2SO_4) was applied (Asadi et al., 2019). Finally, the purified essential oil was maintained inside the microtubes (2 ml) covered with aluminum tape in a refrigerator (4 °C) until chemical analysis (Negahban et al., 2007; Samsam Shariat, 2007; Parsia Aref, 2015; Asadi et al., 2018; Babaee Ghaghelestany et al., 2020).

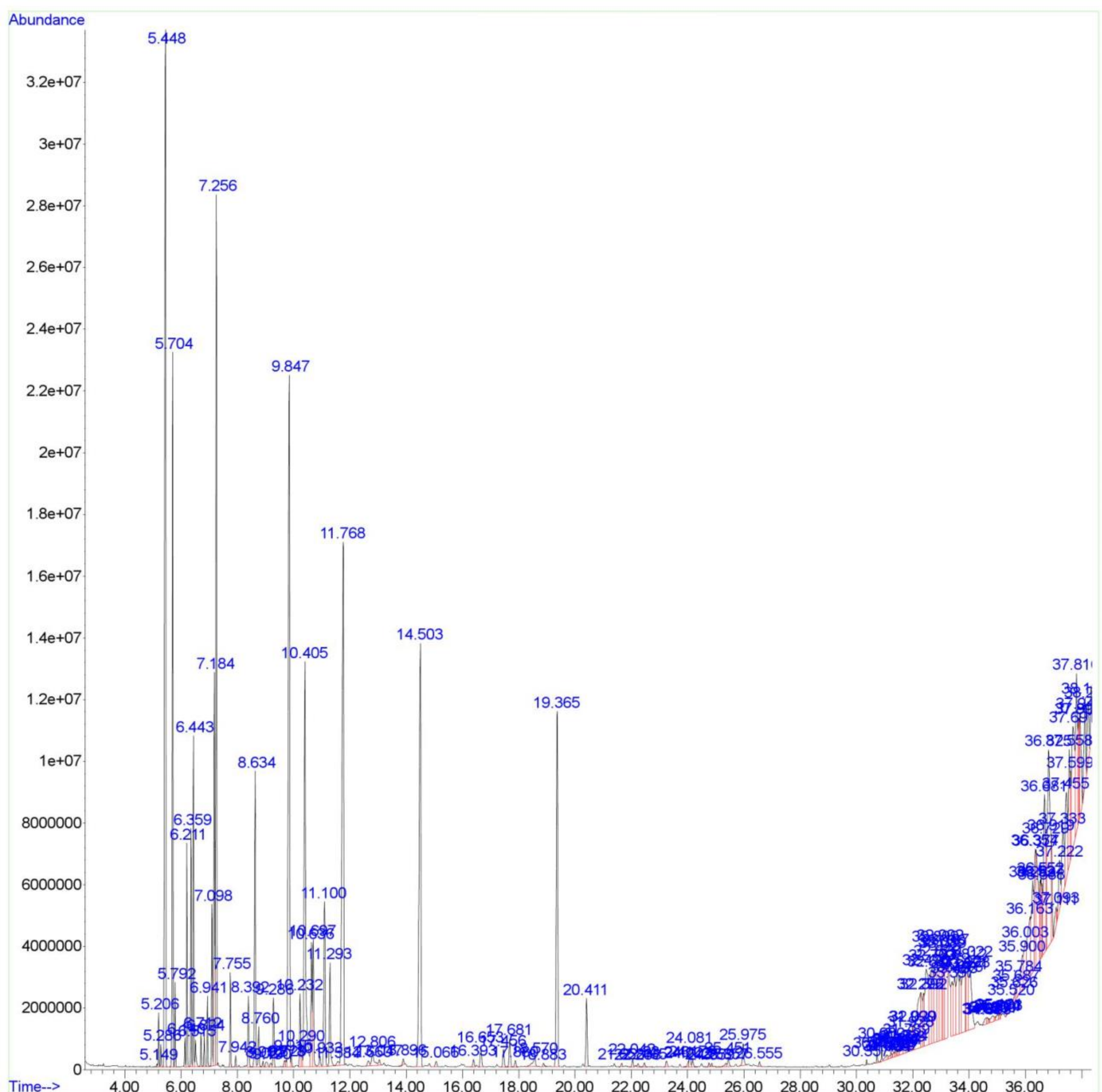

Figure 3. Chromatogram of total compounds in *R. officinalis* essential oil

2.3. Analyzer device (GC-MS)

A gas chromatography-mass spectroscopy device in the central laboratory of the University of Mohaghegh Ardabili, Ardabil, Iran, was applied to determine chemical content in the essential oil of *R. officinalis* (Agilent 7980B, the USA). This device could dilute split splitless inlet as its main feature and mass spectroscopy detector to quantitative and qualitative samples' recognition. The ionization system and four-coupled single analyzer were also convenient features of the detector (Babaee Ghaghelestany et al., 2020). After injecting pure essential oil by Hamilton syringe, compounds at different times were gradually detected based on their molar mass. Finally, the analyzer device exhibited the chromatogram (Figure 3).

3. Results and discussion

The chromatogram of available chemical constituents in *R. officinalis* essential oil by GC-MS is shown in Figure 3. In the chromatogram, longitudinal (X) and latitudinal (Y) axes showed run (running) time and amount of each compound, respectively. According to the chromatogram, compounds with higher peaks had maximum values and lower values being at minimum.

The results showed that *R. officinalis* essential oil had 147 chemical compounds (Table 1) with a total run time of 38.358 min. The run time in first and last compounds (1,3,6-octatriene,3,7-dimethyl, and alpha-terpinolene cyclohexene) were 5.150 and 38.229 min, respec-

Table 1. All compounds in *R. officinalis* essential oil with their run time and percentage

Peak	Compound	Run time (min)	Percentage
1	1,3,6-Octatriene,3,7-dimethyl	5.150	0.01
2	Tricyclene tricyclo	5.208	0.21
3	Thujene	5.288	0.10
4	alpha-Pinene, (-)-bicyclo	5.448	7.50
5	Camphene bicyclo [2.2.1] heptan	5.705	3.20
6	Bicyclo [3.1.0] hex-3-en-2-ol	5.791	0.32
7	Sabinene bicyclo [3.1.0] hexane	6.140	0.11
8	(-)-Beta-Pinene 6,6-dimethyl	6.209	0.90
9	3-Octanone	6.358	0.99
10	beta-Myrcene 1,6-octadiene	6.444	1.34
11	3-Octanol (cas) n-octan-3-ol	6.512	0.16
12	L-Phellandrene 1,3-cyclohexad	6.712	0.15
13	3-Carene	6.827	0.13
14	(+)-2-Carene	6.941	0.21
15	Benzene, 1-methyl-4-(1-methylethyl)	7.096	0.73
16	DL-Limonene cyclohexene	7.182	2.17
17	1,8-Cineole 2-oxabicyclo	7.256	4.60
18	gamma-Terpinene 1,4-cyclohexene	7.754	0.39
19	Copaene tricyclo	7.943	0.05
20	(+)-4-Carene	8.395	0.41
21	Linalool L	8.635	1.65
22	Filifolone	8.761	0.22
23	1,3-Cyclopentadiene	8.910	0.04
24	D-Fenchyl alcohol bicyclo	9.030	0.07
25	1-(1'-Ethoxyethyl) bicyclo [2.1.1]	9.190	0.04
26	Chrysanthenone bicyclo [3.1.1]	9.287	0.39
27	*trans*-Pinocarveol bicyclo	9.676	0.05
28	Bicyclo [3.1.1] hept-3-en-2-ol	9.728	0.04
29	Bicyclo [2.2.1] heptan-2-one	9.848	5.92
30	*exo*-Methyl camphenilol	9.917	0.07
31	Bicyclo [3.1.1] heptan-3-one	10.231	0.43
32	Bicyclo [2.2.1] heptan-3-one	10.289	0.12
33	Borneol L	10.403	3.80
34	Pinocarvone 6,6-dimethyl-2	10.638	0.82
35	3-Cyclohexen-1-ol, 4-methyl	10.695	0.79
36	*p*-Cymen-8-ol	10.935	0.08
37	alpha-Terpineol	11.101	1.27
38	Estragole	11.290	0.70
39	Bicyclo [3.1.1] hept-2-ene-2-ethan	11.582	0.06
40	Bicyclo [3.1.1] hept-3-en-2-one	11.771	5.48
41	Naphthalene, decahydro-2-methyl	12.658	0.06
42	2-Cyclohexen-1-one, 2-methyl-5	12.806	0.21
43	1,4-Cyclooctadiene	13.047	0.06
44	2-Cyclohexen-1-one, 3-methyl-6	13.894	0.03
45	Acetic Acid, 1,7,7-trimethyl	14.506	4.29
46	Phenol, 2-methyl-5-(1-methylethyl)	15.067	0.05
47	alpha-Terpinolene cyclohexene	16.394	0.06
48	2,4-Cycloheptadien-1-one	16.651	0.17
49	Bicyclo [4.1.0] heptan-3-ol	17.458	0.13
50	alpha-Copaene tricyclo	17.681	0.22
51	Copaene	17.882	0.05
52	Bicyclo [3.1.1] hept-2-en-6-one	18.568	0.16
53	Benzene, 1,2-Dimethoxy-4	18.883	0.03
54	Caryophyllene	19.364	2.68
55	alpha-Humulene	20.411	0.50
56	alpha-Selinene	21.658	0.03
57	Cyclohexene, 1-methyl-4-(5-methyl)	22.042	0.06
58	Benzene, isocyanato	22.236	0.03
59	Naphthalene, 1,2,3,5,6,8a	22.465	0.04
60	1 Tricyclo [6.4.0.0 (3,7)] dodecane	23.243	0.05
61	Bicyclo [3.2.0] hept-2-ene-6-one	24.021	0.04
62	Caryophyllene oxide	24.079	0.14
63	2-(4a, 8-Dimethyl-2,3,4,4a,5,6)	24.176	0.06
64	2-Oxabicyclo [9.1.0] dodeca-3,7-D	24.760	0.03
65	beta-Myrcene	24.857	0.02
66	*trans-Z*-alpha-Bisabolene epoxide	25.355	0.04
67	10,10-Dimethyl-2,6-dimethylenebi	25.452	0.08
68	Cyclooctane, 1,5-dimethyl-2	25.973	0.19
69	di-epi-alpha-Cedrene-(I)	26.556	0.03
70	Pyrimidine, 5-methyl-2	30.356	0.02
71	Farnesyl acetone	30.728	0.06
72	1,2-Diphenyl tetramethyl disilane	30.899	0.08
73	Isoterpinolene cyclohexene	31.065	0.06
74	4-[2-(Adamantan-1-yloxy)]-ethylamide	31.105	0.03
75	Cyclopentanone, 2-(1-adamantyl)	31.191	0.02
76	1,3-Dioxan-2,2-dimethyl-4	31.225	0.03
77	Tricyclo [3.3.1.1 (3,7)] decane	31.271	0.02
78	1-Dodecanone, 2-(imidazol-1-yl)	31.328	0.04
79	*trans*-Sabinene hydrate	31.391	0.04
80	(6e, 9z)-6-(Dimethyl phenylsilyl)	31.414	0.03
81	1,2-bis (Dimethyl phenylsilyl) ethane	31.449	0.03
82	1-Cyclopropene-1-pentanol	31.506	0.04
83	Thymyl acetate 2-isopropyl-5	31.632	0.08
84	2-Iodoadamantane	31.723	0.11
85	1-Epoxy-2-methyl-3-isobutenyl-1	31.792	0.09
86	N-[2-(Adamantan-1-yloxy)-ethyl]	31.929	0.27
87	(+)-2,2,3-Trimethylcyclopent-3	31.992	0.19
88	*p*-Mentha-1(7), 8(10)-dien-9-ol	32.026	0.10
89	*m*-Anisic Acid, morpholide	32.278	1.44
90	Ethanone, 2-hydroxy-1	32.290	0.50
91	2-N-Butyladamantane	32.393	0.40
92	Cyclononasiloxane, octadecamethyl	32.478	1.57
93	1-Adamantaneethanol tricyclo	32.650	0.92
94	1 Hexasiloxane, tetradecamethyl	32.702	0.86
95	Iron, monocarbonyl-(1, 3-butadien)	32.810	1.33
96	Cordycepin	32.879	0.75
97	2-Butanone, 4-(dimethyl phenylsil)	32.993	1.18
98	Hexasiloxane, tetradecamethyl	33.033	0.44
99	Heptasiloxane, hexadecamethyl	33.091	0.75
100	Isoindole-1,3-dione	33.188	1.42
101	Cyclodecasiloxane, eicosamethyl	33.400	0.67
102	(3r*, 4s*)-4-(4-Methoxyphenyl)	33.486	0.68
103	Pulegone	33.611	0.98
104	Methyl 4-(1-hydroxycyclohex-2-en)	33.674	0.58
105	*n*-1-Adamantyl-*p*-nitrobenzalimine	33.812	1.17
106	N, N'-Ethylenebis [3-methoxy-N)	33.869	0.18
107	[Dimethyl-(3-trimethylsilanyloxyl)]	33.926	0.68
108	Anhydro 5-hydroxy-3-piperonyl	34.023	1.25
109	1,4-Cyclohexadiene, 1,3,6-tris	34.601	0.08
110	*cis*-Ocimene	34.647	0.04
111	3-(4-Morpholino)bicyclo	34.681	0.02
112	3-Methyl-1-phenyltriazene	34.847	0.10
113	beta-Selinene naphthalene	34.899	0.02
114	*trans*-Decalin, 2-methyl	35.002	0.06
115	Methyl isopropyl disulphide	35.076	0.03
116	*trans*-verbenol bicyclo [3.1.1]	35.099	0.03
117	2-[(4-Isopropyl-phenylcarbamoyl)]	35.523	0.15
118	beta-Phellandrene cyclohexene	35.626	0.08
119	Bornyl acetate	35.688	0.13
120	3,4-Methylenedioxy-*n*-ethyl	35.786	0.27
121	*Z*-Jasmone	35.900	0.39
122	Etracosamethyl cyclododecasiloxan	36.003	0.60
123	Acetamide, 2-(adamantan-1-yl)	36.163	1.01
124	6-Phenyl-3, 5-dithioxo-2,3,4,5	36.261	1.31
125	3-(4-Chlorophenyl)-4,6-dimethoxy	36.352	0.89
126	*o*-Anisic acid, 2-adamantyl ester	36.375	1.71
127	delta-Cadinene naphthalene	36.530	0.46
128	L-Valine, n-(*o*-anisoyl), methyl	36.553	0.40
129	Pentasiloxane, dodecamethyl	36.587	0.44
130	1,1,1,5,7,7,7-Heptamethyl-3	36.678	1.71
131	Trichloroacetic acid, 1-adamanty	36.730	0.54
132	Bistrimethylsilyl *n*-acetyl	36.827	3.20
133	1,2-Benzisothiazole-3-propanoic	36.919	0.69
134	3,6-Dioxa-2,4,5,7-tetrasilaoctan	37.090	0.20
135	Linalool 1,6-octadien-3-ol	37.113	0.05
136	3,5,6,8,9-Tetramethoxy-2-methylpep	37.222	0.58
137	1h-Pyrazole, 4,5-dihydro-5	37.331	0.82
138	Bicyclo [4.3.0] nona-3,7-diene	37.457	1.12
139	Octadecamethyl cyclononasiloxane	37.560	0.95
140	1-Amino-1-*o*-chlorophenyl-2	37.600	0.48
141	1,3-Xylyl-15-crown-4,2,3-pinan	37.691	2.03
142	5,6,8,9-Tetramethoxy-2-methyl	37.817	1.69
143	*n*-Adamantan-1-ylmethyl-4	37.891	0.52
144	9-Borabicyclo [3.3.1] nonane	37.909	0.31
145	4beta-Acetoxy-1beta	37.943	0.90
146	Adamantane-1-carboxamide	38.120	0.82
147	alpha-Terpinolene cyclohexexene	38.229	0.62

tively. According to Table 2, twenty-seven compounds occupied more than 1% of essential oil volume which was distinguished as dominant components; among them, alpha-pinene, (-)-bicyclo in peak 4 (5.448 min with 7.50%), bicyclo [2.2.1] heptan-2-one in peak 29 (9.848 min with 5.92%), bicyclo [3.1.1] hept-3-en-2-one in peak 40 (11.771 min with 5.48%), 1,8-cineole 2-oxabicyclo in peak 17 (7.256 min with 4.60%), and acetic acid 1,7,7-trimethyl in peak 45 (14.506 min with 4.29%) were five major of them, respectively (Table 2). By comparing percentage for each compound on total

volume, it was found that 1,3,6-octatriene,3,7-dimethyl in peak 1 (5.150 min with 0.01%) given in Table 3 were minor constituent in the essential oil. It can be approximately said that the detector has distinguished twenty-seven major compounds in primary to final peaks; moreover, the minor components were observed in the middle. With closely looking at constituents of the essential oil, there were 120 different compounds with shares of ≤ 1% in total volume, which altogether contained 32.96% of the essential oil volume; nevertheless, twenty-seven dominant compounds occupied 67.04% of total volume, indicating most of the essential oil volume was filled by them (Table 2).

Table 2. Twenty-seven dominant compounds in the essential oil of *R. officinalis*

Peak	Compound	Run time (min)	Percentage
4	alpha-Pinene, (-)-bicyclo	5.448	7.50
29	Bicyclo [2.2.1] heptan-2-one	9.848	5.92
40	Bicyclo [3.1.1] hept-3-en-2-one	11.771	5.48
17	1,8-Cineole 2-oxabicyclo	7.256	4.60
45	Acetic acid, 1,7,7-trimethyl	14.506	4.29
33	Borneol L	10.403	3.80
5	Camphene bicyclo [2.2.1] heptan	5.705	3.20
132	Bistrimethylsilyl *n*-acetyl	36.827	3.20
54	Caryophyllene	19.364	2.68
16	DL-Limonene cyclohexene	7.182	2.17
141	1,3-Xylyl-15-crown-4,2,3-pinan	37.691	2.03
126	*o*-Anisic acid, 2-adamantyl ester	36.375	1.71
130	1,1,1,5,7,7,7-Heptamethyl-3	36.678	1.71
142	5,6,8,9-Tetramethoxy-2-methyl	37.817	1.69
21	Linalool L	8.635	1.65
92	Cyclononasiloxane, octadecamethyl	32.478	1.57
89	*m*-Anisic acid, morpholide	32.278	1.44
100	Isoindole-1,3-dione	33.188	1.42
10	beta-Myrcene 1,6-octadiene	6.444	1.34
95	Iron, monocarbonyl-(1,3-butadien)	32.810	1.33
124	6-Phenyl-3,5-dithioxo-2,3,4,5	36.261	1.31
37	alpha-Terpineol	11.101	1.27
108	Anhydro 5-hydroxy-3-piperonyl	34.023	1.25
97	2-Butanone, 4-(dimethyl phenylsil)	32.993	1.18
105	*n*-1-Adamantyl-*p*-nitrobenzalimine	33.812	1.17
138	Bicyclo [4.3.0] nona-3,7-diene	37.457	1.12
123	Acetamide, 2-(adamantan-1-yl)	36.163	1.01
	Total volume		67.04

Rosemary is a valuable medicinal plant that has been studied in different aspects. I reviewed some studies on the essential oil of its samples collected from different regions worldwide. The reasons for the differences or similarities have also been explained. Soliman et al. (1994) analyzed the essential oil of this medicinal plant from Egypt with GC-MS and stated that 43 components were available in sample 1, which represented 82% of total volume, while verbenone (12.3%), camphor (11.3%), bornyl acetate (7.6%), and limonene (7.1%) were significant. Moreover, in their sample II, thirty-seven components occupied 86% of the total volume, and camphor (14.9%), alpha-pinene (9.3%), and 1,8-cineole (9.0%) were dominant. The results in each sample differed from another, and this subject indicated the effects of maintenance and sampling conditions on the changes of secondary metabolites in plants. Touafek et al. (2004) investigated the chemical properties of the essential oil of *R. officinalis* from Algerian Sahara and concluded that thirty compounds represented 98.2% of the essential oil volume while 1,8-cineole (29.5%), 2-ethyl-4,5-dimethylphenol (12.0%), and camphor (11.5%) were significant. Their results, to some extent, were similar to the previous study in Egypt, indicating the impact of similar environmental conditions (two countries from Africa) on the composition of the essential oil. Santoyo et al. (2005) studied the chemical composition of *R. officinalis* essential oil by supercritical fluid extraction and stated that in comparison with chromatography-mass spectroscopy analysis, fractions resulted in

Table 3. Fifty-six constituents in *R. officinalis* essential oil had the lowest percentage (≤ 0.1 in total)

Peak	Compound	Run time (min)	Percentage
3	Thujene	5.288	0.10
88	p-Mentha-1(7), 8(10)-dien-9-ol	32.026	0.10
112	3-Methyl-1-phenyltriazene	34.847	0.10
85	1-Epoxy-2-methyl-3-isobutenyl-1	31.792	0.09
36	*p*-Cymen-8-ol	10.935	0.08
67	10,10-Dimethyl-2,6-dimethylenebi	25.452	0.08
72	1,2-Diphenyl tetramethyl disilane	30.899	0.08
83	Thymyl acetate 2-isopropyl-5	31.632	0.08
109	1,4-Cyclohexadiene, 1,3,6-tris	34.601	0.08
118	beta-Phellandrene cyclohexene	35.626	0.08
24	D-Fenchyl alcohol bicyclo	9.030	0.07
30	*exo*-Methyl camphenilol	9.917	0.07
39	Bicyclo [3.1.1] hept-2-ene-2-ethan	11.582	0.06
41	Naphthalene, decahydro-2-methyl	12.658	0.06
43	1,4-Cyclooctadiene	13.047	0.06
47	alpha-Terpinolene cyclohexe	16.394	0.06
57	Cyclohexene, 1-methyl-4-(5-methyl)	22.042	0.06
63	2-(4a, 8-Dimethyl-2,3,4,4a,5,6)	24.176	0.06
71	Farnesyl acetone	30.728	0.06
73	Isoterpinolene cyclohexene	31.065	0.06
114	*trans*-Decalin, 2-methyl	35.002	0.06
19	Copaene tricyclo	7.943	0.05
27	*trans*-Pinocarveol bicyclo	9.676	0.05
46	Phenol, 2-methyl-5-(1-methylethyl)	15.067	0.05
51	Copaene	17.882	0.05
60	1 Tricyclo [6.4.0.0 (3,7)] dodecane	23.243	0.05
135	Linalool 1,6-octadien-3-ol	37.113	0.05
23	1,3-Cyclopentadiene	8.910	0.04
25	1-(1'-Ethoxyethyl) bicyclo [2.1.1]	9.190	0.04
28	Bicyclo [3.1.1] hept-3-en-2-ol	9.728	0.04
59	Naphthalene, 1,2,3,5,6,8a	22.465	0.04
61	Bicyclo [3.2.0] hept-2-ene-6-one	24.021	0.04
66	*trans*-*Z*-alpha-Bisabolene epoxide	25.355	0.04
78	1-Dodecanone, 2-(imidazol-1-yl)	31.328	0.04
79	*trans*-Sabinene hydrate	31.391	0.04
82	1-Cyclopropene-1-pentanol	31.506	0.04
110	*cis*-Ocimene	34.647	0.04
44	2-Cyclohexen-1-one, 3-methyl-6	13.894	0.03
53	Benzene, 1,2-dimethoxy-4	18.883	0.03
56	alpha-Selinene	21.658	0.03
58	Benzene, isocyanato	22.236	0.03
64	2-Oxabicyclo [9.1.0] dodeca-3,7-D	24.760	0.03
69	di-*epi*-alpha-Cedrene-(I)	26.556	0.03
74	4-[2-(Adamantan-1-yloxy)]-ethylamide	31.105	0.03
76	1,3-Dioxan-2,2-dimethyl-4	31.225	0.03
80	(6e, 9z)-6-(Dimethyl phenylsilyl)	31.414	0.03
81	1,2-bis (Dimethyl phenylsilyl) ethane	31.449	0.03
115	Methyl isopropyl disulphide	35.076	0.03
116	*trans*-Verbenol bicyclo [3.1.1]	35.099	0.03
65	beta-Myrcene	24.857	0.02
70	Pyrimidine, 5-methyl-2	30.356	0.02
75	Cyclopentanone, 2-(1-adamantyl)	31.191	0.02
77	Tricyclo [3.3.1.1(3,7)] decane	31.271	0.02
111	3-(4-Morpholino)bicyclo	34.681	0.02
113	beta-Selinene naphthalene	34.899	0.02
1	1,3,6-Octatriene, 3,7-dimethyl	5.150	0.01

the detection of 33 compounds in the essential oil while the main components were alpha-pinene, 1,8-cineole, camphor, verbenone, and borneol, which occupied 80% of the total volume. Martínez et al. (2009) performed GC-MS analysis of rosemary essential oil from its aerial parts and reported that the major compounds were alpha-pinene (14.10%), camphene (11.47%), beta-pinene (12.02%), myrcene (3.31%), alpha-phellandrene (7.87%), eucalyptol (8.58%), 2-bornanone (3.42%), camphor (8.75%), isoborneol (3.48%), borneol (4.85%), and borneol acetate (6.49%). Hussain et al. (2010) studied *R. officinalis* essential oil in Pakistan and stated that GC-MS analysis revealed major components as 1,8-cineol (38.5%), camphor (17.1%), alpha-pinene (12.3%), limonene (6.23%), camphene (6.00%), and linalool (5.70%). Their results were somewhat in agreement with my results. One of the main reasons for this position is the geographical similarities between the two countries (Iran and Pakistan); of course, the studied plant parts were effective

in this regard. Boutekedjiret et al. (2011) studied the essential oil of *R. officinalis* in Algeria by GC-MS and concluded that more than 90% of its volume was occupied by 1,8-cineole (52.4%) and camphor (12.6%) which were significant. In another study, Rašković et al. (2014) studied the chemical composition of the isolated rosemary essential oil by gas chromatography-mass spectrometry in Tunisia and concluded that 29 chemical compounds were available while the main constituents were 1,8-cineole (43.77%), camphor (12.53%), and alpha-pinene (11.51%). The results for alpha-pinene were somewhat similar to my research; but, in general diversity of compounds in their study was very low compared to mine, which was responsible for differences. Jafari-Sales and Pashazadeh (2020) studied the chemical composition of rosemary essential oil collected from Tabriz in northwest of Iran and concluded that 19 compounds were identified where 1,8-cineole and alpha-pinene had the highest volume in the essential oil. One of the main reasons for these differences is the geographical differences between the two regions in Iran (Tabriz and Kermanshah). Also, the variety of compounds in my study was remarkable, indicating the suitability of Kermanshah province in the west of Iran for the growth of this medicinal plant.

4. Conclusions

The medicinal plants are god-given natural resources in each region; therefore, essential issues in plant science are their identification, taxonomy, and study of chemical features. According to this research, rosemary has valuable secondary compounds that could seriously be focused on them in medical, pharmacology, and toxicology. The author of the article hopes that next researchers study the chemical content of the other medicinal plants belonging to different species and families for their practical application in the future.

Acknowledgments

None.

Conflict of interest

The author confirms that there is no known conflict of interest.

CRediT authorship contribution statement

Mohammad Asadi: M. Asadi performed all parts of the article alone.

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Supplementary File

None.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 37-41

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

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SHORT REPORT

OPEN ACCESS

Phenolic compounds of natural knotweed (*Polygonum cognatum* Meissn.) populations from Turkey

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ARTICLE INFO

Article History:

Received: 25 October 2021
Revised: 20 December 2021
Accepted: 20 December 2021
Available online: 21 December 2021

Edited by: B. Tepe

Keywords:
Knotweed
Phenolic compounds
Polygonum cognatum
Turkey

ABSTRACT

The phenolic compositions of two different *Polygonum cognatum* samples collected from the Cumra and Manisa regions of Turkey were investigated for the first time. Both tested samples were rich in different phenolic compounds, mostly rutin, isorhamnetin, and catechin. The rutin content of Cumra's sample was relatively higher than that of Manisa's sample. *P. cognatum* has potential regarding rutin content as a functional dietary food or may be used as an ingredient to enrich functional foods. Our study will contribute to the previous works performed by different researchers on *P. cognatum*, commonly consumed in Turkey, to reveal its beneficial properties.

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1. Introduction

Polygonum genus contains over 300 species spread out in Europe, North and South America, Asia and North Africa (Jansone, 2015). Also, the *Polygonum* genus has 27 species that are spread in Turkey. Knotweed (*Polygonum cognatum* Meissn.) is one of these 27 species, a perennial plant with a slender woody stock. Its stems are prostrate and green like the leaves. The ochreae on the stems are hyaline, 3-nerved, conspicuous, longer than or as long as the internodes (rarely shorter in very lax). Its leaves are oblong-elliptic in shape and petiolate, often slightly mucronate. There are many flowers in fascicles in the leaf axils. The perianth is pinkish, 4-5 mm in length, hardening, and accrescent in fruit. It grows widespread in the roadsides, slopes, cliffs, cultivated lands, and 720-3000 m altitudes (Davis, 1967).

P. cognatum belongs to the Polygonaceae family (Yıldırım et al., 2003), and it is locally named "madimak" in Turkey (Macar and Kalefetoglu, 2018; Dereli et al., 2019). The knotweed young shoots and leaves are used to treat several diseases such as diabetes, stomachache, abdominal pain, and anemia in central parts of Turkey (Önen et al., 2014; Sargin et al., 2015, Polat, 2019). The plant is commonly consumed as salad and dishes by local people in many provinces. It was also reported to possess antioxidant, antimicrobial, diuretic, antifungal, insecticidal, and antidiabetic activities (Yıldırım et al., 2003; Baytop, 1999; Dereli et al., 2019). These mentioned properties are attributed to tannins, flavonoid glycosides sterols, triterpenes, polyuronides, and saponins of *P. cognatum* (Dereli et al., 2019). The knotweed was previously reported to contain phenolic compounds, vitamin C, and carotenoids (Yıldırım et al., 2003; Önen et al., 2009). *P. cognatum* samples collected from the Sivas region in Turkey were recorded to be rich in vitamin E and Zn,

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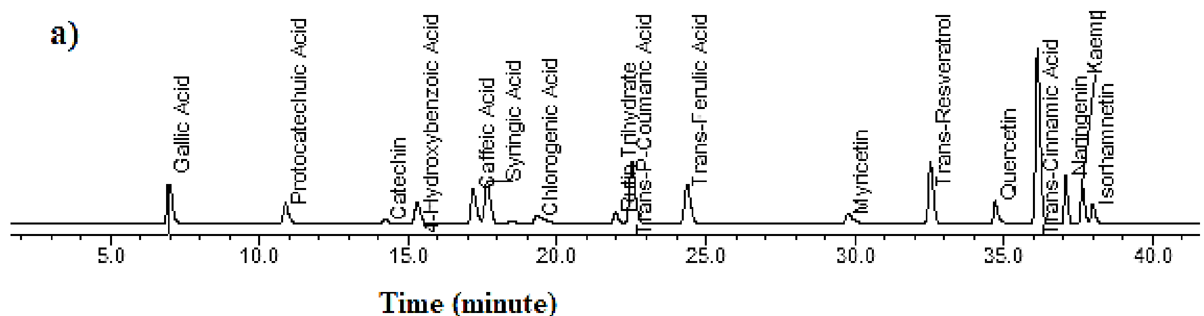
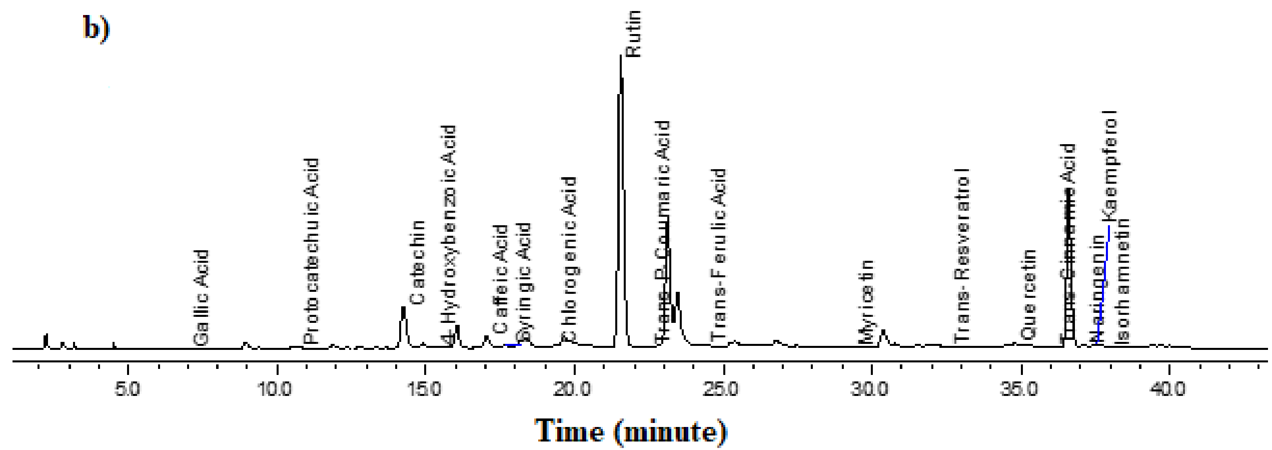
e-ISSN: 2791-7509
doi:
© 2022 IJPBP. Published by Kilis 7 Aralik University (No: 029). All rights reserved.

Please cite this article as: Gümüşçü, A., Dinç, S., Kara, M., Akkuş, M., Gümüşçü, G., 2022. Phenolic compounds of natural knotweed (Polygonum cognatum Meissn.) populations from Turkey. International Journal of Plant Based Pharmaceuticals, 2(1), 37-41.

Fe, and Mn elements (Ulusoy et al., 2017). Similarly, folic acid levels of *P. cognatum* samples of the Sivas district were found to be 176, 25, 41, 28, and 10 times higher than those of fruit juices, spinach, broccoli, green beans, brussels sprouts, and soya bean tissue, respectively (Ulusoy et al., 2018). Bioactive components of "madimak" consumed in high amounts and is very famous locally still need to be clarified because of attracting the attention of consumers as potential functional food. Although there have been some studies on bioactive properties and components of natural knotweed (*P. cognatum*) populations from Turkey, to the best of our knowledge, the phenolic composition of *P. cognatum* has not been studied so far. This research was designed to determine the phenolic components of *P. cognatum* collected from two different regions, Cumra and Manisa, Turkey.





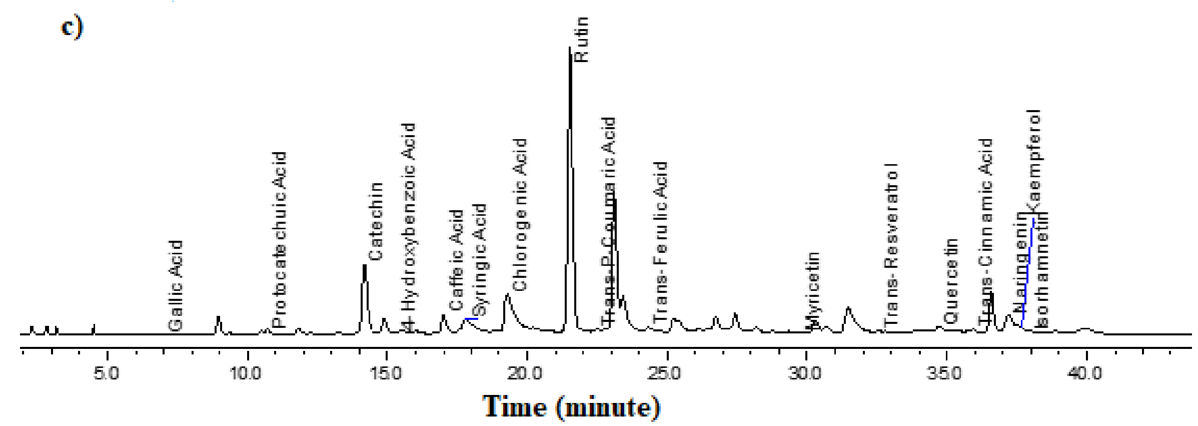


Figure 1. HPLC chromatogram of phenolic compounds of a) standard solutions, b) *P. cognatum* from Manisa, c) *P. cognatum* from Cumra

2. Materials and methods

2.1. Plant materials

This study was performed in Advanced Technology, Research and Application Center laboratories, Selçuk University, in 2016. Knotweed samples were collected from wild flora in Manisa and Çumra (Konya) localities. The sample from Manisa was collected from Salihli-Ödemiş road, near Allahdiyen village, roadside, and altitude was 781 m, on 4th June. The sample from Cumra was collected from the near cemetery, roadside, and altitude was 1016

m, on 26th May. Plant samples were comprised of aerial parts at the flowering stage, and each of them was 100 grams.

2.2. Chemicals

Standards of caffeic acid, catechin, gallic acid, kaempferol, myricetin, naringenin, *p*-hydroxybenzoic acid, rutin trihydrate, *trans-p*-coumaric acid, *trans*-ferulic acid, *trans*-resveratrol, *trans*-cinnamic acid were bought from Ehrenstorfer GmbH, protocatechuic acid was from HWI Analytik, rutin trihydrate and syringic acid were from Alfa Aesar. Methanol, glacial acetic acid, and acetonitrile were obtained from Merck (Germany).

2.3. Extraction of phenolic compounds

Extraction of phenolic compounds in dried *P. cognatum* was carried out using methanol as solvent at room temperature for 24 h in a reflux condenser. After the evaporation of methanol in a rotary evaporator (IKA RV 05 Staufen, Germany), liquid-liquid extraction was performed with diethyl ether and ethyl acetate, respectively. After the evaporation of the solvent, the dried extract was dissolved in methanol for further HPLC analysis (Kara et al., 2015).

Table 1. Phenolic compounds of *P. cognatum* from Cumra and Manisa (mg/100 g sample)

Parameter	Retention time (min)	Cumra	Manisa
Gallic acid	6.973	0.016 ± 0.001	-
Protocatechuic acid	10.880	0.152 ± 0.002	0.037 ± 0.006
Catechin	14.242	8.492 ± 0.113	2.635 ± 0.032
4-Hydroxybenzoic acid	15.304	0.097 ± 0.002	0.021 ± 0.003
Caffeic acid	17.178	0.289 ± 0.004	0.110 ± 0.006
Syringic acid	17.651	0.064 ± 0.008	0.043 ± 0.003
Chlorogenic acid	19.326	0.432 ± 0.078	0.043 ± 0.005
Rutin trihydrate	21.961	20.353 ± 0.151	11.404 ± 0.401
trans-p-Coumaric acid	22.513	0.019 ± 0.001	0.391 ± 0.005
trans-Ferulic acid	24.364	0.231 ± 0.012	0.088 ± 0.001
Myricetin	29.776	0.074 ± 0.002	0.133 ± 0.018
trans-Resveratrol	32.543	0.43 ± 0.004	0.046 ± 0.004
Quercetin	34.712	0.036 ± 0.001	0.167 ± 0.021
trans-Cinnamic acid	36.129	0.006 ± 0.001	0.038 ± 0.001
Naringenin	37.081	0.034 ± 0.002	0.024 ± 0.001
Kaempferol	37.643	0.234 ± 0.021	0.212 ± 0.046
Isorhamnetin	37.991	0.453 ± 0.022	7.086 ± 0.433
Total		31.407 ± 0.149	22.480 ± 0.806

2.4. Phenolic compounds of *P. cognatum*

Separation of phenolic compounds of *P. cognatum* was conducted on HPLC system (Shimadzu, Japan) equipped with UV-Vis detector along with ODS 3 column (250 mm × 4.6 mm id, 5 μm particle; Inertsil) using the gradient HPLC method (Kara et al., 2015; Dinc et al., 2018). 0.05% glacial acetic acid in water (A) and acetonitrile (B) mixtures were used as mobile phase at 1.0 ml/min with the following gradient: 0-2 min 8-10% B; 2-27 min 10-30% B; 27-37 min 30-56 %B; 37 min 8% B, before it returned to the initial conditions. The temperature of the column was kept constant at 30 °C. Each 20 μl of the sample was injected, and detection was performed at 280 nm. Seven different concentrations of calibration solutions were used for the calibration curve. The calibration curve was prepared using concentration ratio versus peak area. The calibration equation and R^2 value were calculated using linear regression analysis. R^2 values were greater than 0.99. The solutions of standards and samples were injected three times. Limit of detection (LOD; S/N=3) and limit of quantification (LOQ; S/N=10) were calculated using a certain ratio of signal to noise (S/N). LOQ of standard solutions were between 0,000000156375 ppm (*trans*-resveratrol) and 0,000166248 ppm (catechin).

Quantification of phenolic compounds was performed with different concentrations of phenolic standards (1-55 mg/l-8 point) based on the peak areas of samples (Figure 1a).

3. Results and discussion

In this study, we first investigated the phenolic composition of the whole part of *P. cognatum* collected from two different districts of Turkey in 2016. Plant phenolics, known for their health benefits, exist omnipresent in plant foods and are composed of phenolic acids and polyphenols. Flavonoids constitute the largest subclass (Bondonno et al., 2020). Phenolic acids possess free radical scavenging capacity and hinder oxidative deterioration in emulsion model systems. In recent years, the food industry has taken advantage of their utilization as natural antioxidants to retard the oxidative deterioration of foods (Kiokias et al., 2020). 17 phenolic compounds were investigated in this study, and their chromatograms are presented in Figures 1b and 1c, also and the quantitative results are also given in Table 1. Both samples collected from Cumra and Manisa regions are found to contain almost all phenolic compounds tested. The total quantity of phenolic compounds of the Cumra sample was higher than that of Manisa. Rutin, a flavonol glycoside, was found at the highest level in both samples. The ratio of rutin in the Cumra sample was considerably higher than that of Manisa. Due to the lack of published data on phenolic components of *P. cognatum,* we compared our data with those of buckwheat which also belongs to Polygonaceae (knotweed family). Rutin quantity of sample from Cumra was considerably higher than those of different buckwheat cultivars previously reported (Kreft et al., 2006). On the other hand, the rutin content of buckwheat species *F. tataricum* was higher than Cumra's sample. Rutin, present in substantial quantity in plants, has a broad range of physiological activities. It can diminish the cytotoxicity of oxidized LDL cholesterol and the risk of heart diseases (Atanassova and Bagdassarian, 2009). Medicinally utilization of rutin has the advantage of reducing capillary fragility associated with some hemorrhagic diseases or hypertension in humans (Jiang et al., 2007). The other phenolic compound at the highest ratio in Cumra's sample was catechin. Although Manisa's sample was also high in catechin, its level was lower than Cumra's sample. Catechins have many health benefits in cardiovascular diseases and cancer (Lorenzo and Munekata, 2016). Isorhamnetin content of Manisa's sample was relatively higher than Cumra's sample. Isorhamnetin has many pharmacological characteristics such as antimicrobial, antioxidant, anticancer, neurological, cardiovascular, etc. (Kandakumar and

Manju, 2017). Resveratrol is another phenolic compound previously investigated in *Polygonum* species. In our study, the resveratrol quantity of Cumra's sample was higher than that of Manisa's sample. Resveratrol has prevalent utilization in medicine, health products, and cosmetic industries considering pharmaceutical characteristics such as anti-inflammatory, anticancer, and cardioprotective activities (Wang et al., 2013). Resveratrol levels in our samples were relatively lower than those of *P. cuspidatum* species studied by other researchers (Wang et al., 2013; Chu et al., 2005; Kuo et al., 2014).

4. Conclusions

In this study, the phenolic composition of *P. cognatum*, commonly named "madimak" in Turkey, was analyzed for the first time using the samples from Cumra and Manisa. *P. cognatum* is found to be one of the plants which are rich in the phenolic compound in Turkey. The total quantity of phenolic compounds of *P. cognatum* collected from Cumra was much higher than that collected from Manisa. The highest rutin content of *P. cognatum* may be considered an important dietary source of rutin.

Acknowledgments

None.

Conflict of interest

The authors confirm that there are no known conflicts of interest.

CRediT authorship contribution statement

Ahmet Gümüşçü: Resources, Investigation, Data curation, Supervision.
Saliha Dinç: Conceptualization, Visualization, Investigation, Methodology.
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Supplementary File

None.

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Yıldırım, A., Mavi, A., Kara, A.A., 2003. Antioxidant and antimicrobial activities of *Polygonum cognatum* Meissn extracts. *Journal of the Science of Food and Agriculture*, 83(1), 64-69.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 42-50

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RESEARCH ARTICLE OPEN ACCESS

Effects of systemic hydroxytyrosol application in experimental periodontitis of rats

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ARTICLE INFO

Article History:

Received: 09 December 2021
Revised: 30 December 2021
Accepted: 30 December 2021
Available online: 31 December 2021

Edited by: B. Tepe

Keywords:
Alveolar bone loss
Antioxidant
Experimental periodontitis
Hydroxytyrosol
Oxidative stress

ABSTRACT

This study aimed to determine the effects of systemically administered hydroxytyrosol (HT) on alveolar bone resorption and oxidative stress parameters in experimental periodontitis (EP). Thirty-two rats were divided randomly into four groups; 1) periodontally healthy + serum physiologic (PH-SP), 2) PH + hydroxytyrosol (PH-HT), 3) experimental periodontitis + SP (EP-SP), and 4) EP-HT. Following induction of EP, 10 mg/kg of systemic HT (test)/SP (control) was administered and continued for 14 days. The animals were euthanized on the 15th day, and the jaws were removed for histopathologic, histomorphometric, and immunohistochemical analyses. Enzyme-linked immunosorbent assay (ELISA) was used to analyze serum and gingival tissue malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) levels. The receptor activator of NF-kB ligand (RANKL), osteoprotegerin (OPG) levels, and RANKL/OPG ratio were analyzed via immunolabeling. Serum and tissue MDA, SOD, and GSH-Px levels did not differ between the groups. The immunohistochemical evaluation showed that RANKL levels and RANKL/OPG ratio in HT applied groups were significantly lower than SP applied groups. Within the limits of this study, daily administration of hydroxytyrosol at a dose of 10 mg/kg for 14 days could prevent alveolar bone destruction in experimental periodontitis. Besides, the antioxidant effect of HT could not be revealed.

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1. Introduction

Periodontal disease, an inflammatory disease of the periodontium, is one of the most important causes of tooth loss. It has been characterized by the imbalance between the biofilm attached to the tooth surface and the host defense mechanism (Berezow and Darveau, 2011). With the development of periodontal disease, periodontium loses its health and bleeding on probing, periodontal pocket formation, the recession of the gingiva, loss of alveolar bone, and periodontal attachment occurs (Hornef et al., 2002; Kassebaum et al., 2014; Kinane, 2001; Schroeder and Listgarten, 1997).

The interaction between the receptor activator of nuclear factor-kappa B (RANK)-the RANK ligand (RANKL)-osteoprotegerin (OPG) plays a critical role in the alveolar bone loss associated with periodontal disease. RANKL provides activation and maturation of osteoclasts. Besides, OPG hampers RANKL to bind to the RANK receptor and inhibits osteoclastogenesis by blocking the differentiation and activation of osteoclasts. While RANKL is a potent stimulator of bone resorption, OPG acts as a bone protective molecule (Bartold et al., 2010).

Free radicals are atoms or molecules with one or more unpaired electrons in their orbits. They are readily degradable, have a short turnover time, and are quite reactive. There is a protective antioxidant system against those free radicals and their harmful effects on the human body. This antioxidant system prevents radical formation, repairs oxidative damage, cleans the damaged molecules, and prevents the organism from mutations (Young and Woodside, 2001). Under normal physiological conditions, there is a balance between the antioxidant system and free radical formation. If this equilibrium is disturbed towards the oxidative side, it causes oxidative stress and leads to tissue damage (Chapple and Matthews, 2007). Conversely, the shift to the antioxidant side will reduce the soft and hard tissue injuries, and thereby the oxidative damage can be able to reduce.

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e-ISSN: 2791-7509
doi:
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Please cite this article as: Sengun, M.C., Gunpinar, S., 2022. Effects of systemic hydroxytyrosol application in experimental periodontitis of rats. International Journal of Plant Based Pharmaceuticals, 2(1), 42-50.

Several animal and clinical trials have been focused on the preventive effects of antioxidant chemicals such as resveratrol (Tamaki et al., 2014), flavonoids (Formica and Regelson, 1995), and melatonin (Kose et al., 2016) to reduce the tissue damage of periodontal diseases. Additionally, it has been reported that the administration of antioxidants in the treatment of periodontal disease regulates proinflammatory cytokines and can improve tissue repair by reducing soft and hard tissue loss (Chapple and Matthews, 2007).

Hydroxytyrosol (HT) is an antioxidant found abundantly in olive and olive oil, which are the main ingredients of the Mediterranean diet. It is a phenolic compound that neutralizes reactive oxygen species via chain breaking. Phenol compounds can prevent or delay the oxidation of organic molecules. They reflect their effect via transferring a proton of their Hydrogen atom to the ROO- chain of the radical (Foti, 2007). It shows the anti-inflammatory effects by inhibiting the cyclooxygenase and lipoxygenase enzymes and inhibiting the enzyme activities of immune cells such as neutrophils, mast cells, and macrophages (Brezani et al., 2017). It has been shown that HT can be beneficial and protective against many acute and chronic diseases with its anti-inflammatory and antioxidant effects. It enhances cardiovascular health (Khurana et al., 2013), reduces the risk of endothelial dysfunction (Fabiani et al., 2008), inhibits tumor cell proliferation and DNA damage, decreases edema and tissue destruction in rheumatoid arthritis (Silva et al., 2015), and has a neuroprotective role in diabetic rats (Reyes et al., 2017).

The soft and hard tissue destruction due to oxidative stress is balanced with the antioxidant system in periodontal tissues. For this reason, the homeostatic equilibrium between reactive oxygen species and the antioxidant system is critical in periodontal destruction. HT, which is the main phenolic component of olive oil and an effective antioxidant, has been reported to have a protective role in the development of chronic inflammatory diseases. However, Zhang et al. (2021) revealed that HT suppressed osteoclast differentiation and mitochondrial stress in *in vitro* model, the role of HT in the development of periodontal disease, which is also a chronic inflammatory disease, has not been elucidated yet. In this study, we hypothesized that HT treatment could regulate oxidative stress and decrease alveolar bone resorption and inflammation during the development of periodontal disease. In light of all this information, this study aimed to investigate the histopathological, histomorphometric, and immunohistochemical effects of systemic HT administration on alveolar bone resorption. The second objective was to analyze the gingival and serum levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) enzymes in experimental periodontitis (EP) model in rats.

2. Materials and methods

2.1. Animals and experimental model

This study protocol was approved by the Bolu Abant Izzet Baysal University Faculty of Medicine Ethics Committee (Decision no. 2016/41). The rats used in this study were obtained from the Bolu Abant Izzet Baysal University Experimental Animal Laboratories. 32 male Albino Wistar rats weighing 200-300 grams and 10-12 weeks of age were used. All animals were housed at Bolu Abant Izzet Baysal University Experimental Animal Application and Research Center, kept 12 hours light/dark cycle and appropriate environment temperature (20 °C ± 4 °C) with 60 to 70% humidity. They were hosted in the plastic-based wire cages and could reach food and water ad libutum.

Thirty-two rats were divided randomly into four groups:
- periodontally healthy + serum physiologic (PH-SP)
- periodontally healthy + hydroxytyrosol (PH-HT)
- experimental periodontitis + serum physiologic (EP-SP)
- experimental periodontitis + hydroxytyrosol (EP-HT)

2.2. Induction of experimental periodontitis

To induce the experimental periodontitis model, rats were anesthetized with 90mg/kg ketamine hydrochloride and 10mg/kg xylazine hydrochloride intramuscularly. The EP model was induced for each rat by placing 3/0 silk sutures around the cervix of the right and left upper second molars with some subgingival location. Sutures were kept in the area for 14 days and checked without anesthesia application.

Oral gavage applications were initiated at the next day following placing the ligatures (Silva et al., 2015). Test solution was prepared as follows: HT (purity > 85%) solution [LGC, Cas No:10597-60-1, hydroxytyrosol (purity > 85%], Toronto Ontario, Canada) was dissolved in 2% methanol and was diluted with serum physiologic (SP). Rats in the test groups received 10 mg/kg HT via gavage for 14 days, once a day in the morning. For the control groups, to contain similar methanol levels, 70 ml of SP was mixed with 1.4 ml of 2% methanol and applied with the same protocol as the test group. Rats were sacrificed on the 15th day of suturing via taking whole cardiac blood.

2.3. Histopathologic and histometric analyses

Following the sacrifice, upper jaws were collected and divided into two parts with carbon disk, and half-jaws were randomly selected for histological evaluation. Those samples were fixed in 10% neutral formaldehyde and decalcified with 10% EDTA (pH 7.4) solution. After decalcification, the tissues were washed with distilled water, passed through the increasing alcohol solutions and xylene, and embedded in paraffin. Sections with a thickness of 5 µm were taken with a microtome. Histopathologic examination was performed by staining the sections with hematoxylin & eosin.

Alveolar bone loss (ABL); was determined histomorphometrically by measuring the distance between the cement-enamel junction (CEJ) and the alveolar bone crest (AC) with a light microscope with 40X magnification. Measurements were also performed separately on mesial, furcation, and distal areas in the buccal region of the second molar by the researcher who was blinded for the study groups. The hematoxylin & eosin-stained sections were evaluated under a light microscope (Nikon Eclipse i5, Nikon Instruments Inc, Tokyo, Japan). The measurements were captured and evaluated with 'Image J' (Image J v.1.5, National Institutes of Health, Maryland, USA) program.

2.4. Immunohistochemical analyses

After deparaffinization and rehydration, tissue sections were incubated with 3% hydrogen peroxide to block endogenous enzyme activity. After washing with tap water and distilled water, antigens were recovered via incubating the section in a microwave at 200 W for 20 min with citrate buffer. After washing the samples with PBS, the samples were blocked with 2% bovine serum albumin for 20 min. After blocking, the samples were incubated overnight at + 4 °C with the RANKL (RANKL/TNFSF11 Antibody, NB100-56512, Novus Biological, Littleton, USA) and OPG (Osteoprotegerin/TNFRSF11B Antibody, NB100-56505, Novus Biological, Littleton, USA) primary antibodies. After that, the secondary antibody was ligated using a

detection kit (Osteoprotegerin/TNFRSF11B Antibody, NB100-56505, Novus Biological, Littleton, USA). After washing with PBS, samples were incubated with streptavidin-peroxidase for 10 minutes and stained with 3,3'-diaminobenzidine (DAB) chromogen. Thus, the primary antibody became visible. After immunohistochemical staining, sections were examined with a camera attached (Nikon DS-Fi1c, Nikon Instruments Inc, Tokyo, Japan), light microscope (Nikon Eclipse i5, Nikon Instruments Inc, Tokyo, Japan), and evaluated with an image analysis system (NIS Elements v. 4.0, Nikon Instruments Inc, Tokyo, Japan). Intensities of RANKL and OPG staining and staining areas were measured in all sections. The staining intensity was determined as the intensity of the pixel in the computer image taken from the sections. The staining area was calculated with the ratio of the stained cell area (µm) to the entire stained area [range of interest (µm)]. Measurements were made at three regions randomly selected at 200X magnification in the whole section.

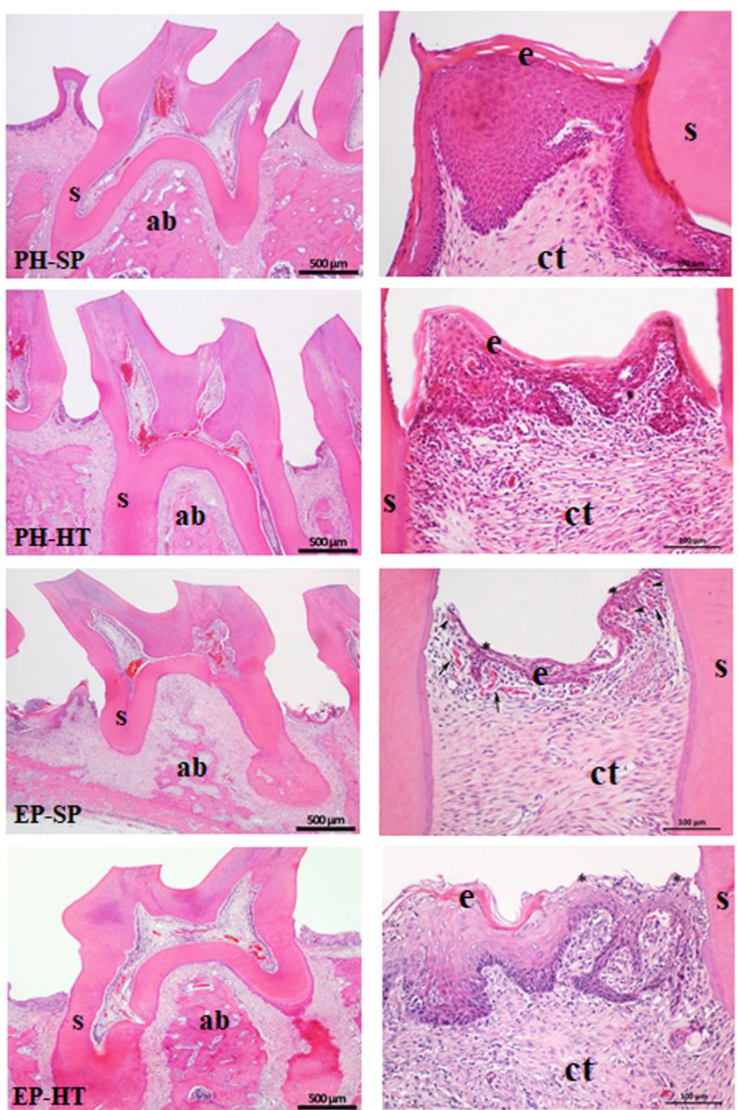

Figure 1. Alveolar bone loss and histopathological images of the study groups

Normal morphology epithelium and connective tissue in PH-SP and PH-HT groups. Spillage (*), vascularization (→), and mild, severe inflammation (›) in the keratin layer and surface epithelial cells of the EP-SP group. Spillage (*) in the keratin layer and surface epithelium in the EP-HT group. PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol, e: epithelium, ct: connective tissue, s: cementum. Hematoxylin & eosin staining, 40X magnification, bar: 500 µm (left images) 200X magnification, bar: 100 µm (right images).

2.5. Biochemical analyses

The gingival tissue samples with a total size of 3 mm were obtained around the sutures and immediately stored at -80 °C in sterile tubes containing 400 µl phosphate-buffered saline (PBS) (Panbiotech, P04-31500, 500ml, Germany) and 0.05% Tween-20 until analysis. On the day of analysis, samples were dissolved in PBS with the ratio of 100mg tissue/100µl PBS, transferred to tubes, and each sample was disintegrated entirely with tissue homogenizer (IKA WERKE, Wilmington, USA) at least 10 minutes. Freeze-thawing was applied to homogenates which were homogenized in 1 ml PBS, twice at -20 °C, and following the final thawing, samples were centrifuged, and the supernatants were separated for analysis.

To separate the serum component of cardiac blood, samples were centrifuged (1200 g, 10 minutes) at +4 °C, and aliquoted serum samples were stored at -80 °C until assayed.

2.6. Serum and gingival tissue levels of MDA, SOD, and GSH-Px

Malondialdehyde (MDA) (Cayman Rat MDA, Cat. No: 706002, Cayman Chemical Inc. Michigan, USA), glutathione peroxidase (GSH-

Px) (Cayman Rat Gsh-Px, Cat. No: 10009055, Cayman Chemical Inc. Michigan, USA) and superoxide dismutase (SOD) (Cayman Rat SOD, Cat. No: 703102, Cayman Chemical Inc. Michigan, USA) levels in the serum and gingival tissue samples were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The linear measurement range was 0-50 nmol/ml, 50-344 nmol/min/ml, and 0.005-0.05 units/ml for MDA, GSH-PX and SOD, respectively. The samples out of the linear range were reevaluated with appropriate dilutions.

In order to assess toxicity, gamma-glutamyl transferase (GGT) (U/L), aspartate transaminase (AST) (U/L), alanine aminotransferase (ALT) (U/L), alkaline phosphatase (ALP) (U/L), blood urea nitrogen (UREA) (mg/dL) and creatinine (CREA) (mg/dl) levels were evaluated. Measurements were done spectrophotometrically using enzymatic and colorimetric methods using the manufacturer's instructions (Architect c 8000, Abbott Laboratuvari, Illinois, USA).

2.7. Statistical analyses

For all evaluated parameters, the normality was tested by the Shapiro-Wilk test. If the data distribution was normal, a parametric test, one-way ANOVA-Tukey, otherwise a nonparametric test, Kruskal Wallis-Mann-Whitney U test was employed to determine the differences between groups. All analyses were performed using the SPSS 19 (Statistical Package for Social Sciences) package program, and values of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Histopathologic and histomorphometric findings

Images of histological sections are given in Figure 1. Levels of bone loss in the study group were summarized in Table 1. Measurements of alveolar bone loss in EP groups were statistically higher than those of PH groups ($p < 0.05$). On the other hand, there was no statistically significant difference between experimental periodontitis groups (EP-SP and EP-HT) ($p > 0.05$).

Table 1. Alveolar bone loss measurement values in the study groups (µm)

	PH-SP (n=8)	PH-HT (n=8)	EP-SP (n=8)	EP-HT (n=8)	$p^{\alpha,\beta}$
Mesial					
Mean ± sd	195.63 ± 50.40	154.16 ± 21.34	595.23 ± 193.66^{a,b}	437.68 ± 203.49^{a,b}	0.000$^{\alpha}$*
Median	191.79	160.37	563.22	371.19	
Min-Max	122.52-302.92	120.02-181.24	358.97-894.36	249.89-767.41	
Furcation area					
Mean ± sd	117.78 ± 23.37	98.35 ± 12.30	406.99 ± 183.97^{a,b}	318.66 ± 211.54^{a,b}	0.000$^{\beta}$*
Median	113.45	96.98	394.05	228.83	
Min-Max	91.73-152.06	80.86-118.75	181.14-639.24	151.19-775.31	
Distal					
Mean ± sd	516.01 ± 132.74	519.48 ± 154.27	1124.76 ± 352.79^{a,b}	856.59 ± 293.52^{a}	0.000$^{\alpha}$*
Median	486.00	491.71	1036.42	854.84	
Min-Max	358.62-762.59	288.30-754.36	755.02-1858.73	524.98-1312.39	
Total					
Mean ± sd	276.47 ± 65.05	257.33 ± 55.45	708.99 ± 194.52^{a,b}	537.64 ± 198.86^{a,b}	0.000$^{\alpha}$*
Median	270.57	242.70	678.96	497.53	
Min-Max	193-48-405.86	183.08-351.45	464.10-1086.02	324.87-868.62	

$^{\alpha}$One Way ANOVA, $^{\beta}$Kruskal Wallis. *$p < 0.05$. n: number of samples.
a: Difference from PH-SP, b: Difference from PH-HT.
PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol, µm: Micrometer, sd: Standart deviation, Min: Minimum, Max: Maximum.

3.2. RANKL, OPG, and RANKL/OPG immunohistochemical findings

RANKL staining of 2 of the 32 hard tissue samples could not be evaluated because of the pouring and folding of the sections. Both the staining area and intensity levels of RANKL and OPG were summarized in Table 2, and the images of the sections were depicted in Figure 2.

The RANKL staining area in the EP-HT group was statistically lower than that of the EP-SP group ($p < 0.05$). When the levels of OPG staining area were compared between groups, it was found that these levels were low in the EP-SP group than PH-SP group as expected, but the results did not reach significance ($p > 0.05$). The RANKL/OPG staining area ratio was significantly higher in the PH-SP group than in the PH-HT and EP-HT groups ($p < 0.05$).

The RANKL staining intensity of the EP-HT group was significantly lower than the PH-SP group ($p < 0.05$). It was determined that EP-SP group RANKL staining intensity was significantly higher than that of PH-HT ($p < 0.05$); on the other hand, these measurements were lower in the EP-HT than EP-SP group ($p < 0.05$). When the OPG staining intensity was compared, then the levels of PH-SP and PH-HT groups were significantly higher than those of EP-SP and EP-HT groups ($p < 0.05$). The RANKL/OPG staining intensity of the EP-SP group was found to be higher than the other groups ($p < 0.05$).

3.3. Biochemical analyses

3.3.1. Serum MDA, SOD and GSH-Px levels

Serum MDA, SOD, and GSH-Px levels in both groups were summarized in Table 3. Measurements of the level of these enzymes did not differ between the groups ($p > 0.05$).

3.3.2. Gingival tissue MDA, SOD, and GSH-Px levels

The tissue MDA levels of the EP groups were higher than those of PH control, but the results were not statistically significant ($p > 0.05$). When the tissue levels of SOD were compared between groups, it was determined that the levels in the EP-induced groups were lower than the PH-HT group ($p < 0.05$). On the other hand, the levels of SOD were found to be increased in the EP group with HT treatment, but the results did not reach significance ($p > 0.05$) (Table 3).

3.3.3. Serum GGT, AST, ALT, ALP, UREA, and CREA

It was determined that the enzyme mentioned above levels were below the toxic level, and these levels were not statistically significant between the groups ($p > 0.05$) (Table 4). Serum GGT levels, a marker of liver toxicity, were under the toxic levels in all groups (< 4 U/L); therefore, these results were not given in the tables.

This study evaluated the effects of 10 mg/kg systemic HT administration on alveolar bone destruction and oxidative stress parameters in rats with EP. We revealed that systemic HT administration decreased the level of RANKL staining. On the other hand, although the decreased alveolar bone loss was determined in HT groups via morphometric analysis, the results did not reach significance. Besides, tissue and serum MDA, SOD, and GSH-Px levels did not differ between those treated with HT or SP in periodontitis groups. Therefore, the results of this study showed that 10 mg/kg systemic HT administration could not reduce the reactive oxygen species and could not present antioxidant properties.

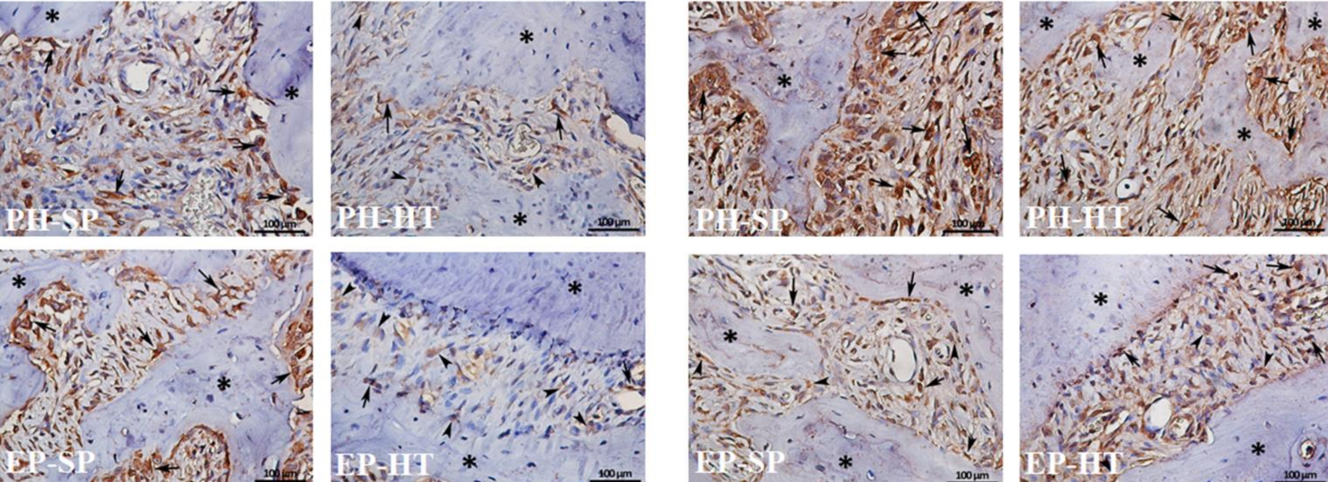

Figure 2. Histological images of RANKL and OPG immunostaining in the experimental groups

(→): Severe immune-positive stained cells, (›): Weakly immune-positive stained cells., (*): Alveolar bone. PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol. (200X magnification, bar: 100 µm.)

Table 2. RANKL and OPG immunohistochemical results

	PH-SP (n=7)	PH-HT (n=7)	EP-SP (n=8)	EP-HT (n=8)	$p^{\alpha,\beta}$
Staining area					
RANKL (+) cell number (%)					
Mean ± sd	5.79 ± 1.15	4.24 ± 1.02	7.76 ± 2.94 ^{b,d}	2.65 ± 1.11	0.000$^{\alpha}$ *
Median	6.11	3.95	6.82	2.95	
Min-Max	4,11-7,65	3.19-6.38	3.68-13.15	0.93-4.04	
OPG (+) cell number (%)					
Mean ± sd	24.99 ± 7.92	34.61 ± 9.72	25.19 ± 5.93	22.67 ± 5.97	0.084$^{\beta}$
Median	25.05	33.48	22.94	24.41	
Min-Max	10.24-34.02	25.89-54.92	18.05-34.70	19.17-33.66	
RANKL/OPG					
Mean ± sd	0.26 ± 0.16^{b,d}	0.12 ± 0.03	0.32 ± 0.14^{b,d}	0.11 ± 0.06	0.010$^{\beta}$ *
Median	0.24	0.12	0.28	0.10	
Min-Max	0.12-0.62	0.06-0.18	0.15-0.58	0.03-0.20	
Staining intensity					
RANKL					
Mean ± sd	66.80 ± 9.96^{d}	54.52 ± 9.02	77.75 ± 8.84^{b,d}	52.41 ± 12.34	0.010$^{\beta}$ *
Median	63.66	56.33	82.5	54.5	
Min-Max	54.66-82.00	36.00-62.33	60.66-85.00	32.00-68.33	
OPG					
Mean ± sd	93.04 ± 9.59^{c,d}	92.76 ± 8.68^{c,d}	65.37 ± 8.03	75.19 ± 8.48	0.000$^{\alpha}$ *
Median	92.00	91.66	66.83	73.00	
Min-Max	79.33-109.66	82.00-104.33	49.33-73.66	64.66-91.66	
RANKL/OPG					
Mean ± sd	0.73 ± 0.16	0.57 ± 0.09	1.20 ± 0.17^{a,b,d}	0.69 ± 0.13	0.000$^{\beta}$ *
Median	0.75	0.58	1.16	0.76	
Min-Max	0.54-1.03	0.42-0.71	0.96-1.48	0.45-0.82	

$^{\alpha}$One Way ANOVA, $^{\beta}$Kruskal Wallis. *$p < 0.05$. n: number of samples.
a: Difference from PH-SP, b: Difference from PH-HT, c: Difference from EP-SP, d: Difference from EP-HT.
PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol, RANKL: The receptor activator of NF-κB ligand, OPG: Osteoprotegerin, sd: Standart deviation, Min: Minimum, Max: Maximum.

In our study, the amount of alveolar bone loss was calculated by measuring the distance between CEJ and AC in the second molars of rats. The amount of alveolar bone loss was higher in the experimental periodontitis groups compared to the PH groups. Although HT treatment in the EP group showed a decreased bone loss, the results were not statistically significant. On the other hand,

Zhang et al. (2021) determined decreased alveolar bone loss in mice periodontitis model treated with HT via Micro-CT analysis. The researchers applied 20 mg/kg HT one week before the induction of periodontitis and continued for two weeks. Conversely, in our study, 10 mg/kg HT was administered concurrently with the periodontitis induction. Therefore, our results might be due to the fact that the relatively low doses of antioxidants would not be able to prevent bone resorption. Furthermore, only two-dimensional measurement was utilized rather than calculating the bone loss volume.

Table 3. Serum and gingival tissue MDA (nmol/ml), SOD (U/ml) and GSH-Px (nmol/min/ml) levels in the study groups

	PH-SP (n=8)	PH-HT (n=8)	EP-SP (n=7)	EP-HT (n=8)	p^{Ω}
Serum levels					
MDA (nmol/ml)					
Mean ± sd	4.01 ± 0.70	4.42 ± 1.08	4.37 ± 0.69	4.54 ± 1.26	0.728
Median	4.00	4.44	4.22	4.63	
Min-Max	3.04-5.10	2.18-5.53	3.55-5.41	1.84-5.78	
SOD (U/ml)					
Mean ± sd	0.74 ± 0.13	0.55 ± 0.11	0.65 ± 0.11	0.60 ± 0.22	0.117
Median	0.76	0.58	0.66	0.61	
Min-Max	0-54-0.98	0.41-0.71	0.47-0.81	0.15-0.86	
GSH-PX (nmol/min/ml)					
Mean ± sd	147.25 ± 46.19	114.12 ± 64.22	143.50 ± 44.25	160.37 ± 60.23	0.401
Median	166.00	101.00	128.50	151.00	
Min-Max	63.50-198.50	33.50-243.50	93.50-203.50	93.50-293.50	
Gingival tissue levels					
MDA (nmol/ml)					
Mean ± sd	0.86 ± 0.45	0.99 ± 0.70	0.94 ± 0.12	0.79 ± 0.43	0.848
Median	0.70	0.80	0.93	0.79	
Min-Max	0.45-1.75	0.40-2.41	0.79-1.16	0.25-1.46	
SOD (U/ml)					
Mean ± sd	8.23 ± 2.26	10.12 ± 1.87[a,b]	7.21 ± 1.23	7.39 ± 1.89	0.015*
Median	7.23	9.46	6.94	7.41	
Min-Max	5.81-12.29	8.19-13.83	6.24-9.35	5.20-10.93	
GSH-PX (nmol/min/ml)					
Mean ± sd	1.29 ± 0.58	1.86 ± 0.51	1.20 ± 0.44	1.55 ± 0.86	0.170
Median	1.39	1.79	1.19	1.38	
Min-Max	0.35-2.19	1.23-2.22	0.80-1.96	0.42-3.07	

$^{\Omega}$One Way ANOVA, *p < 0.05. n: number of samples.
a: Difference from EP-SP, b: Difference from EP-HT.
PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol, MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, nmol/ml: nanomole/milliliter, U/ml: Units per millilitre, sd: Standart deviation, Min: Minimum, Max: Maximum.

Table 4. Serum AST (U/L), ALT (U/L), ALP (U/L), UREA (mg/dl) and CREA (mg/dl) levels in the study groups

	PS-SF (n=8)	PS-HT (n=8)	DP-SF (n=7)	DP-HT (n=8)	p^{Ω}
AST (U/L)					
Mean ± sd	129.50 ± 41.57	97.50 ± 10.90	104 ± 14.92	95.87 ± 12.19	0.610
Median	109.00	96.00	103.00	93.00	
Min-Max	98.0-219.0	83.0-114.0	81.0-128.0	84.0-120.0	
ALT (U/L)					
Mean ± sd	62.37 ± 10.08	51.12 ± 6.53	57.71 ± 7.11	55.12 ± 11.95	0.445
Median	61,50	52,50	60.00	58.00	
Min-Max	48.0-78.0	41.0-59.0	48.0-67.0	29.0-67.0	
ALP (U/L)					
Mean ± sd	240.62 ± 26.04	208.75 ± 51.28	217 ± 39.77	242.75 ± 71.39	0.128
Median	238.50	206.00	201.00	258.00	
Min-Max	206.0-288.0	110.0-282.0	173.0-283.0	116.0-332.0	
UREA (mg/dl)					
Mean ± sd	50.87 ± 5.43	50.75 ± 4.30	48.42 ± 2.50	53.75 ± 5.99	0.329
Median	(43-58)	(43-58)	(45-51)	(45-60)	
Min-Max	43.0-58.0	43.0-58.0	45.0-51.0	45.0-60.0	
CREA (mg/dl)					
Mean ± sd	0.54 ± 0.02	0.51 ± 0.02	0.52 ± 0.03	0.52 ± 0.02	0.294
Median	0.54	0.51	0.52	0.52	
Min-Max	0.51-0.59	0.49-0.55	0.50-0.60	0.48-0.56	

$^{\Omega}$One Way ANOVA, *p < 0.05. n: number of samples.
PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol, AST: Aspartate transaminase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, UREA: Blood urea nitrogen, CREA: Creatine, U/L: Units per liter, mg/dl: Milligrams per deciliter, sd: Standart deviation, Min: Minimum, Max: Maximum.

The interaction of RANK-RANKL-OPG plays a critical role in alveolar bone destruction associated with periodontal disease as well as systemic conditions such as osteoporosis. Although the alteration in RANKL and OPG levels are utilized alone, it is more valid to determine the RANKL/OPG ratio to reveal the alveolar bone loss. In the literature, immunohistochemical evaluation of RANKL and OPG was presented by staining area and/or staining intensity. The 'intensity' of the staining indicates the degree of expression of the cells in the histological sections, while the staining area refers to the number of expressing cells within a specific area in the sections (Fedchenko and Reifenrath, 2014). The staining intensity is assessed by computer software which gives precise values, while the staining area was determined via counting the stained cells in the section separately by the researchers to record numerical values. Assessment of the staining area is a more practical technique commonly used in the literature; on the other hand, the staining

intensity offers more detailed information but requires more time (Fedchenko and Reifenrath, 2014). This study evaluated both staining area and staining intensity of RANKL and OPG levels and RANKL/OPG ratio. In line with the literature, RANKL levels and RANKL/OPG ratio were increased, and OPG levels were decreased in periodontal disease (Arabacı et al., 2015; Saglam et al., 2015). Moreover, considering the results of this study, it can be concluded that the assessment of staining intensity rather than staining area reveals more sensitive results in immunohistochemical analysis.

Previous studies have shown that alveolar bone loss can be prevented by systemic administration of various antioxidant agents as a result of decreased RANKL and increased OPG levels (Arabacı et al., 2015; Saglam et al., 2015). In this study, the effect of HT administration on RANKL and OPG levels were evaluated, and both staining area and staining intensity values of RANKL were decreased, but OPG levels did not change in EP by HT administration. This can be explained by the fact that the need for OPG, a competing inhibitor of RANKL, may be reduced as a result of decreased RANKL levels (Zhou et al., 2013). When RANKL/OPG ratio was compared, systemic HT application decreased both the staining area and the intensity levels in EP. These results were supported with the previous studies showing decreased RANKL level, RANKL/OPG ratio, and increased OPG levels after the administration of various antioxidants such as sumac (Saglam et al., 2015), resveratrol (Tamaki et al., 2014), melatonin (Arabacı et al., 2015) and curcumin (Zhou et al., 2013) in periodontal disease.

In the literature, it has been reported that HT could be applied in different amounts such as 2 mg/kg (Mnafgui et al., 2016), 5 mg/kg (Silva et al., 2015), and 10 mg/kg (Pirozzi et al., 2016); the physiological lower limit has been reported as 5 mg/kg by the European Food Safety Authority (Cristina Vilaplana-Perez et al., 2014). Pirozzi et al. (2016) reported that HT administration at a dose of 10 mg/kg per day reduces liver damage and regulates oxidative stress by demonstrating anti-inflammatory and antioxidant characteristics without showing any toxic reaction (Pirozzi et al., 2016). Furthermore, pretreatment with 20 mg/kg HT partially decreased alveolar bone loss in a mice periodontitis model (Zhang et al., 2021). In the light of this information, systemic HT administration at a dose of 10 mg/kg per day was utilized concurrently with the induction of periodontitis in this study.

Lipid peroxidation levels in biological fluids of subjects with periodontal disease are controversial. It has been reported that periodontitis patients had higher serum and tissue MDA levels than healthy controls. Besides, decreased MDA levels in GCF and saliva have reduced inflammation. On the contrary, periodontitis subjects had lower serum MDA levels than healthy individuals (Akalın et al., 2007; Baltacioglu et al., 2014; Saglam et al., 2018; Tsai et al., 2005). In our study, serum MDA levels were lower in EP than healthy controls, and tissue MDA levels were similar among groups. Furthermore, HT administration did not affect either serum or tissue MDA levels in EP. These results were compatible with the literature, which reports that administration of antioxidants did not alter the serum MDA levels (Tas et al., 2015; Wang et al., 2017).

Superoxide dismutase and GSH-Px enzyme levels in biological fluids and local tissues have been studied to clarify the antioxidant mechanism in periodontal disease. Sobaniec and Sobaniec-Lotowska (2000) reported that serum SOD, GSH-Px, and glutathione reductase enzymes were lower, and MDA levels were higher in EP-induced rats compared to the periodontally healthy group (Sobaniec and Sobaniec-Lotowska, 2000). In parallel, it was determined that the MDA levels in gingival tissue could be significantly reduced by administering the pharmacological imitation of SOD (Di Paola et al., 2005). In a clinical study, Ellis et al. (1998) found that the catalase and SOD levels in gingival tissues obtained from the areas with a periodontal pocket depth of more than 6 mm were lower than those in healthy tissues with a pocket depth of less than 3 mm (Ellis et al., 1998). In contrast, Akalin et al. (2005) analyzed SOD enzyme levels in gingival samples of 26 cases with periodontitis and 16 periodontally healthy individuals and reported that SOD levels were significantly higher in periodontitis patients than in healthy individuals (Akalin et al., 2005). Huang et al. (2000) reported that GSH-Px levels in GCF were negatively correlated with pocket depth and attachment loss in patients with chronic periodontitis, and the GCF levels were increased after periodontal treatment (Huang et al., 2000). In our study, although serum and tissue SOD and GSH-Px levels were lower in EP groups, the results did not reach significance. Additionally, HT administration did not significantly affect those except SOD levels in the periodontally healthy group. These results could be explained by the increased oxidative stress and decreased antioxidant mechanism in periodontal disease. Furthermore, antioxidant mechanisms might be activated in the later stages of chronic inflammation. These results also support the hypothesis that serum enzyme levels may not reflect the local tissue response in periodontitis (Karakan et al., 2017).

In this study, serum levels of ALT, AST, GGT, ALP, urea, and creatinine, indicative of hepatic and renal damage, were also investigated. All enzyme levels were found to be below the toxic threshold, and they were consistent with the previous studies in the literature (Dalcico et al., 2013; Franca et al., 2017; Wang et al., 2013).

The results of our study should be discussed precisely due to not having enough published papers that investigated the possible effect of HT administration on EP. In a very recently published literature, the researchers demonstrated that HT application suppressed bone destruction via the prevention of osteoclast activation *in vitro* and pretreatment with daily administration of 20 mg/kg HT partially decreased alveolar bone resorption and oxidative stress *in vivo* mice periodontitis model (Zhang et al., 2021). These researchers used the prophylactic application of HT. That might increase the tissue antioxidant levels of HT, which might prevent periodontitis-induced alveolar bone loss. Conversely, in our study, the administration of HT was started simultaneously with the induction of periodontitis. Therefore, discussing the results of that research with the current study may not be convenient.

Furthermore, the limitation of this study is that the histomorphometric measurements of alveolar bone loss were carried out in two dimensions only. The fact that a different antioxidant substance was not compared with HT and the total oxidant/antioxidant levels have not been evaluated can be considered limitations.

4. Conclusions

The hypothesis that systemically administrated HT at a dose of 10 mg/kg could reduce alveolar bone destruction was supported in immunohistochemical evaluation but not by the results of histomorphometric measurements. Therefore, it can be suggested that the effect of HT on alveolar bone loss should be further analyzed with a three-dimensional volume assessment via micro-computed tomography. Moreover, different application doses and frequencies of HT compared with different antioxidants should be investigated to clarify HT's antioxidant characteristics in EP.

Acknowledgments

This study was supported by Bolu Abant Izzet Baysal University Scientific Research Center (Project number 2017.06.05.1144). The authors would like to thank Dr. Buket Tekçe for her kind assistance in biochemical analysis.

Conflict of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Mehmet Cihan Sengun: He performed all the experimental procedures and drafted the manuscript.
Sadiye Gunpinar: She supervised all the experimental study and edited the original manuscript.

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Supplementary File

None.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 51-63

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

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RESEARCH ARTICLE

OPEN ACCESS

Comparative toxicity of cinnamon oil, cinnamaldehyde and their nano-emulsions against *Culex pipiens* (L.) larvae with biochemical and docking studies

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ARTICLE INFO

Article History:

Received: 02 December 2021
Revised: 15 January 2022
Accepted: 15 January 2022
Available online: 19 January 2022

Edited by: B. Tepe

Keywords:
Culex pipiens
Cinnamon oil
Cinnamaldehyde
Nano-emulsions
Larvicidal activity
Biochemical studies
Molecular docking

ABSTRACT

The larvicidal activity of cinnamon oil and its main component, cinnamaldehyde, was compared with their nano-emulsions (NEs) against *Culex pipiens* mosquito larvae. Oil-in-water (O/W) NEs preparation was based on the coarse emulsion followed by high-energy ultra-sonication. The droplet size, polydispersity index (PDI), viscosity, zeta potential, and pH of NEs were investigated. The droplet sizes of the NEs were 95.67 nm for cinnamon oil and 174.59 nm for cinnamaldehyde. The NEs recorded high negative zeta potentials (-30.0 and -21.20 for cinnamon oil and cinnamaldehyde, respectively). The larvicidal activity results showed that the cinnamaldehyde (LC_{50} = 94.46 and 72.91 mg/l for T and NE, respectively) had higher activities than cinnamon oil (LC_{50} = 154.08 and 123.13 mg/l for T and NE, respectively) after 24 h of exposure against *C. pipiens* larvae. These results proved that NE formulation enhanced the activity of tested compounds against larvae. The *in vitro* effect on the acetylcholinesterase (AChE), adenosine triphosphatase (ATPase), and gamma-aminobutyric acid transaminase (GABA-T) were demonstrated, and the data proved that the NEs formulations were higher than their pure compounds. Non-formulated cinnamon oil and cinnamaldehyde caused 17.26% and 30.83% of AChE, respectively, while their NEs caused 46.40% and 60.59% inhibition. Furthermore, the molecular docking studies indicated that the affinity binding of cinnamaldehyde on AChE and GABA-T was higher than ATPase. This work describes bio-products with potential use against *C. pipiens* larvae as eco-friendly products.

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1. Introduction

The mosquitoes are at the center of worldwide entomological research due to their importance as vectors of several viral and parasitic diseases affecting about 700 million people worldwide (Li et al., 2022; Wilder-Smith et al., 2017). According to the World Health Organization (WHO), more than 80 % of the world's population lives in areas at risk of at least one vector-borne disease that causes more than 700.000 deaths annually (WHO 2017). *Culex* is a predominant mosquito that mainly thrives in tropical and sub-tropical areas, breeds in dirty waters like congested drains and impaired septic tanks near human dwellings. In Egypt, *Culex* species can transmit a nematode worm (*Wuchereria bancrofti*), responsible for the filarial disease (Holder et al., 1999). Filariasis is the fastest spreading insect-borne disease to humans worldwide; 146 million cases are reported worldwide every year. About 3492 species of mosquito are recorded; 100 species of them are vectors and can transmit many diseases to humans and mammals (Ghosh et al., 2012). Once limited to tropical and subtropical zones, numerous vector-borne diseases have emerged in temperate areas because of climate change. Hence, temperate countries may be the most threatened by the emergence and re-emergence of vector-borne diseases (Karuppusamy et al., 2021; Rocklöv and Dubrow, 2020).

Vector control is the most critical step due to the absence of a drug or vaccine to prevent disease outbreaks. Most mosquito control

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e-ISSN: 2791-7509
doi:
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Please cite this article as: Taktak, N.E.M., Badawy, M.E.I., Awad, O.M., El-Ela, N.E.A., 2022. Comparative toxicity of cinnamon oil, cinnamaldehyde and their nanoemulsions against Culex pipiens (L.) larvae with biochemical and docking studies. International Journal of Plant Based Pharmaceuticals, 2(1), 51-63.

programs target the larval stage in their breeding sites with larvicides because they are most concentrated, immobile, sensitive, and accessible at this stage. At the same time, the use of adulticides only reduces the adult population temporarily (Rehman et al., 2014). Moreover, the insecticide application is in a defined area, decreasing environmental contamination. Pyrethroids and organophosphates are the main groups used to control mosquitos in Egypt and worldwide. Organophosphate insecticides were used outdoor as larvicides, while pyrethroids were used indoors as adulticides (Zahran et al., 2017). However, synthetic insecticides to control mosquitoes have created resistance among target species, adverse effects on non-target organisms, and a secondary impact on the environment and public health.

These drawbacks have prompted researchers to explore new insect-selective products with low toxicological risks, environmentally safer, target-specific, and cost-effective insecticides as an alternative vector control tool (Alsaraf et al., 2021; Zeghib et al., 2020). Therefore, enormous efforts have been made to explore the efficiency of plant-based products against mosquitoes (Wangai et al., 2020; Zahran et al., 2017; Zeghib et al., 2020). Plant-based insecticides have bioactivity, biodegradability, and human and environmental safety compared to traditional insecticides. They also have various novel modes of action, which is crucial in combating insect resistance (Norris et al., 2018). Insecticidal properties of natural bioactive compounds derived from plants, especially essential oils, are suitable against mosquito larvae (El Gohary et al., 2021). These essential oils are complex natural mixtures of volatile, semi-volatile organic compounds, odorous, and secondary metabolites, principally mono- and sesquiterpenes, which are considered among the best alternatives for the control of disease vectors (Alsaraf et al., 2021; Taktak and Badawy, 2019). Monoterpenes are secondary plant metabolites with insecticidal, nematicidal, acaricidal, and larvicidal properties (El-Sabrout et al., 2020; Kweka et al., 2016). The physical and chemical properties of monoterpenes, such as chemical instability, volatility, and low water solubility, rendered them unsuitable for widespread use against Culex larvae (Moretti et al., 2002). As a result, incorporating essential oils (EOs) and monoterpenes into NEs could improve the system to be more effective than bulk substances. In addition, NEs provide several benefits, such as a substantial increase in water solubility, dissolution rate, dispersion uniformity, and significant bioavailability after application (El Gohary et al., 2021).

It is generally understood that insects exposed to these products may encounter toxic compounds that have relatively nonspecific effects on a wide range of molecular targets. Among the targets are proteins (enzymes, receptors, signaling molecules, ion-channels, structural proteins), nucleic acids, biomembranes, and other cell components (Rattan, 2010). As a result, insect physiology is impacted in several ways, including the nervous system malfunctioning, which is the most important one. Rattan (2010) reviewed several physiological effects of plant secondary metabolites on insects, including inhibition of acetylcholinesterase (AChE), blockage of the gamma-aminobutyric acid (GABA)-gated chloride channel, disruption of sodium and potassium ion exchange, and inhibition of cellular respiration (Rattan, 2010). In particular, inhibition of AChE activity is significant since it is responsible for terminating nerve impulse transmission through the synaptic pathway (Badawy et al., 2018; Rattan, 2010).

Therefore, the main objective of this study was to prepare NEs of cinnamon EO and cinnamaldehyde as larvicidal agents against *C. pipiens*. Droplet size distribution, polydispersity index (PDI), viscosity, pH, stability, and surface morphology of the produced NEs were examined. The larvicidal effect against *C. pipiens* of the technical grades (T) was compared to the NEs. The *in vitro* biochemical studies were also investigated on AChE, adenosine triphosphatase (ATPase), and GABA transaminase (GABA-T). In addition, molecular docking of cinnamaldehyde and a reference larvicide temephos was examined with some enzymes.

2. Materials and methods

2.1. Chemicals and reagents

Cinnamon (*Cinnamomum verum*) EO was purchased from LUNA Co. for the Perfumes and Cosmetics Industry (6 October City, Giza, Egypt). A trace gas chromatography Ultra/Mass Spectrophotometer ISQ (GC/MS Thermo Scientific) instrument was used to analyze the components of this oil. Cinnamaldehyde (≥ 95%) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). A reference larvicide temephos (90%) was purchased from Kalyani Industries Pvt. Ltd. Acetylthiocholine iodide (ATChI), adenosine triphosphate (ATP), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), Folin-Ciocalteu phenol reagent, gamma-aminobutyric acid (GABA), α-ketoglutarate, 2-mercaptoethanol, β-nicotinamide adenine dinucleotide (β-NAD), *p*-nitrophenyl phosphate, trichloroacetic acid (TCA), Tris-HCl [tris (hydroxymethyl) aminomethane hydrochloride], triton X-100 and Tween 80 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Other commercially available solvents and chemicals were purchased from El-Gomhouria for Trading Chemicals And Medical Appliances Co., (Adeb Ishak St, Manisha, Alexandria, Egypt) and used without further purification. Cinnamon oil was stored in dark vials at 4 °C until tested.

2.2. C. pipiens mosquito rearing

A colony of a susceptible strain of *C. pipiens* culture was reared in the High Institute of Public Health insectary, Alexandria University, Alexandria, Egypt. The larvae were feeding on biscuits until pupated in shallow trays with 2-3 liters of dechlorinated water. In adult cages (30 x 30 x 30 cm), male adults were fed on 30% sucrose solution, while females were fed on pigeon blood four times a week. The use of a live pigeon in our research was approved by the High Institute of Public Health's Ethics committee and confirmed by Alexandria University under reference number 481. The egg rafts were moved from adult cages to white trays containing dechlorinated water for egg hatching.

2.3. NEs preparation

The cinnamon oil NE was prepared from 10% active ingredient as an organic phase with a polar phase mixture of Tween 80 (10%) and water (80%). While NE of cinnamaldehyde was prepared as follows: active ingredient was dissolved in DMSO to prepare the organic phase, Tween 80 dissolved in distilled water at 50 °C to prepare the polar phase. Considering the concentrations of the active ingredient, DMSO and Tween 80 were 2.5%, 5%, and 2.5%, respectively, in the prepared total volume (Abdelrasoul et al., 2020). The organic phase was dropped into the polar phase by stirring at room temperature for 30 min at 4000 rpm to form the coarse emulsion. Using a high-energy ultrasonic procedure, the ultrasonic probe was used to transform the coarse emulsion into a NE (Figure 1). The ultrasonication process was carried out for 5 min at a rate of 9 cycles per second and a power of 15 kHz for cinnamon oil and 5 kHz for cinnamaldehyde (Munawiroh et al., 2017).

2.4. Characterizations of the NEs

2.4.1. Droplet size and polydispersity index (PDI)

At room temperature, droplet size and PDI of the NE were determined by Dynamic Light Scattering (DLS) technique with a Zetasizer NanoZSlaser diffractometer (Malvern Instruments Ltd, Worcestershire, UK) working at 633 nm and equipped with a backscatter detector (173˚). At a fixed 90° angle, the droplet size (nm) was characterized by distribution curves in intensity (%). The NEs were 500-fold diluted in ultrapure water and sonicated for 5 min at 9 cycles/sec and 5 kHz power before being measured to prevent multiple scattering effects. The droplet size was expressed as a mean diameter in nanometer (Mibielli et al., 2021; Silva et al., 2012).

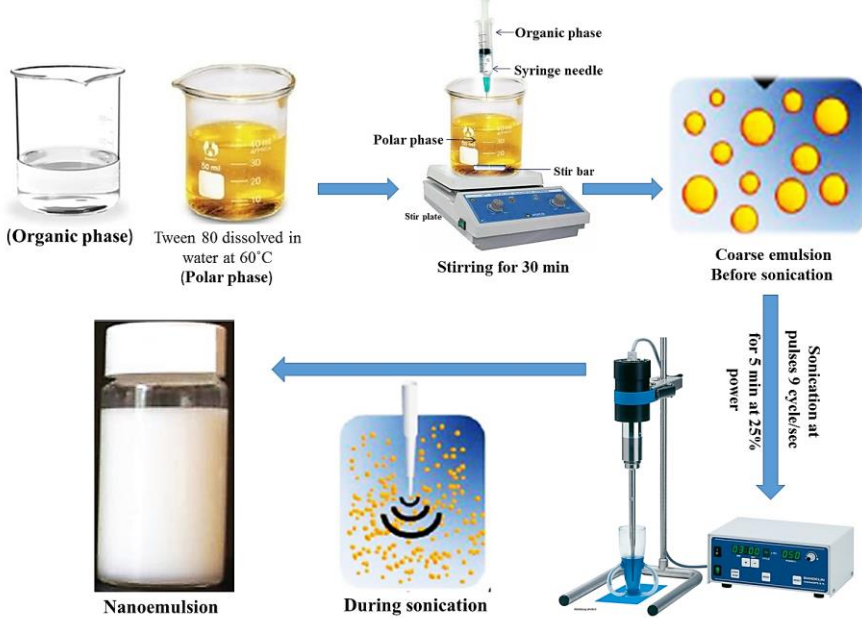

Figure 1. Schematic illustration of NEs preparation

2.4.2. Viscosity and pH measurements

The dynamic viscosity was determined without further dilution using a digital viscometer (a Rotary Myr VR 3000) with an L2 spindle spinning at 200 rpm at 25 °C. The viscosity was measured three times, and the results were expressed in mPa.s. The pH values of the prepared NE were determined using a digital pH meter (Crison pH Meter Basic 20, EU) (Badawy et al., 2017; Drais and Hussein, 2015).

2.4.3. Stability tests

Three samples from each formulation were centrifuged for 30 min at 5000 rpm, and phase separation, creaming, and cracking were observed. The NE should have enough stability without phase separation. Other thermodynamic stability tests were performed on stable formulations. Three samples of each NE were held at 25 and 40 °C then subjected to a heating-cooling test. The thermodynamic stability examinations were done by storing the NE at -21 °C for 24 h and then at 21 °C until melting for 24 h. A transparent tube was filled with 25 ml of each freshly prepared NE for three months at 25 °C as a storage period, the transition from steady-state to creaming and coalescence was investigated (Badawy et al., 2017; Drais and Hussein, 2015).

2.4.4. Zeta potential (ζ)

Using the Zetasizer NanoZSlaser (Malvern Instruments, Worcestershire, UK) at 25 °C, the zeta potential of NEs was investigated (Silva et al., 2012). Zeta potential was measured by transferring the samples to electrophoretic cells, then applying an electrical potential ± 150 mV. The formulations were 200-fold diluted in ultrapure water and sonicated for 5 min at 9 cycles/sec and 5kHz power before being analyzed to avoid multiple scattering effects (Smoluchowski, 1916).

2.4.5. TEM

TEM (JEOL JEM-1400 Plus transmission electron microscope, USA, Inc.) equipped with 20 mM aperture at 20 Kv was used to examine the surface morphology and topology of prepared NEs. Bright-field imaging with increasing magnification and diffraction modes was chosen. The sample from each NE was diluted with distilled water (1:100) and added to 200-mesh form war-coated copper TEM sample holders (EM Sciences, Hatfi eld, PA, Japan).

2.5. Toxicity assay against C. pipiens larvae

The larval bioassay was performed on *C. pipiens* larvae to compare the effect of cinnamon oil and cinnamaldehyde and their NEs by dipping method (WHO, 2005). Tested compounds and their NEs were tested against third instar larvae of *C. pipiens* to obtain the LC_{50} values. The technical cinnamaldehyde and pure cinnamon oil were dissolved in Tween 80/DMSO (0.05%) then dissolved in de-chlorinated water. In comparison, their NEs were dissolved directly in de-chlorinated water. After preliminary screening for all products, different stock solutions for each compound were prepared to determine the toxicity. The tested concentrations of cinnamaldehyde were between 50 to 600 mg/l. In contrast, the tested concentrations of cinnamon oil were between 20 to 300 mg/l. The acute toxicity of technical temephos (90%) was also evaluated by mixing different concentrations (2, 4, 6, 8, 10, 20, 30, and 40 µg/l) prepared in DMSO with dechlorinated tap water. In a control experiment, DMSO and Tween 80 were mixed with de-chlorinated water. Twenty *C. pipiens* larvae were put into plastic

cups containing 100 ml of de-chlorinated water. Mortality caused by the compounds was recorded as larval mortalities percentage of dead versus live larvae numbers after 24 and 48 h. The 50% lethal concentration (LC_{50}) values of the compounds were calculated using probit analysis (Finney, 1971).

2.6. Biochemical studies

2.6.1. Preparation of enzyme homogenates

After 24 h of exposure to LC_{50} values of tested compounds, surviving larvae were homogenized in 10 mM NaCl (1%, w/v) Triton X-100 and 40 mM sodium phosphate buffer (pH 7.4) at 4 °C to assess ATPase, AChE, and GABA enzyme activities. The homogenate was centrifuged at 4 °C for 20 min at 5000 rpm. The supernatant was either used right away for an enzymatic assay or processed at -20 °C for later use (Taktak and Badawy, 2019).

2.6.2. Total protein assay

According to Lowry et al. (1951) method, total protein in crude enzyme was determined using BSA for the standard curve. Protein extract (200 µl) was added to 1900 µl alkaline copper reagent [48 ml of sodium carbonate 2% (w/v) in 0.1 N sodium hydroxide + 1ml sodium-potassium tartrate 1% (w/v) + 1 ml copper sulfate 0.5% (w/v)] and immediately mixed with 200 µl Folin-Ciocalteu phenol reagent. After 30 min of incubation at 25 °C, the absorbance was recorded at 600 nm. By comparing the protein content of the measured samples to the BSA standard curve, the protein content of the samples was calculated.

2.6.3. Acetylcholinesterase (AChE) assay

AChE activity was determined by Ellman colorimetric method (Ellman et al., 1961). The reaction mixture contained 10 µl ATChI (0.075 M), 1340 µl phosphate buffer (pH 8), 100 µl of the crude enzyme, and 50 µl DTNB (0.01 M). Reaction mixture free of crude enzyme sample was checked as a blank, while a mixture containing non-treated larval extract was also studied as a control. Using a Unico 1200 Spectrophotometer (Laxco Inc, USA), the absorbance was measured at 412 nm after 10 minutes of incubation at 37 °C. The enzyme activity was expressed as OD_{412}.mg protein^{-1}.min^{-1}.

2.6.4. Adenosine triphosphatase (ATPase) assay

The Koch method was used to measure the activity of ATPase (Koch, 1969). The reaction mixture was prepared with 400 mM Na^+, 20 mM K^+, 5 mM Mg^+, and 5 mM ATP, and 200 l of the crude enzyme was transferred. After that, the volume was completed to 950 µl with tris-HCl buffer (pH 7.4). The reaction was stopped with 200 µl of TCA after 10 min of incubation at 37 °C. 10 ml of a fresh color reagent containing 5 g of ferrous sulfate dissolved in a solution of ammonium molybdate and sulfuric acid (10 N) was added to the reaction mixture. A reaction mixture free of crude enzyme sample was examined as a blank. As a control, a reaction mixture containing untreated larval extract was tested. The enzyme activity was measured according to the absorbance of the developed color at 740 nm and expressed as OD_{740}.mg protein^{-1}.min^{-1} (Kessler et al., 2014).

Table 1. The chemical constituents of the cinnamon EO isolated from *C. verum* by GC/MS

Rt (min)	Compound	Molecular formula	Molecular weight	Area (%)	RI
6.60	α-Pinene	$C_{10}H_{16}$	136	1.03	919
8.49	β-Pinene	$C_{10}H_{16}$	136	0.73	980
10.65	β-Cymene	$C_{10}H_{14}$	144	1.01	1050
13.10	1,8-Cineole	$C_{10}H_{18}O$	154	6.12	1129
18.99	Linalool	$C_{11}H_{18}O_2$	182	2.02	1319
24.40	*(-)*-Camphor	$C_{10}H_{16}O$	152	0.54	1494
25.43	*(E)*-Cinnamaldehyde	C_9H_8O	132	80.09	1527
26.01	Eugenol	$C_{10}H_{12}O_2$	164	1.06	1545
26.89	Methyl linoleate	$C_{19}H_{36}O_2$	296	3.18	1574
29.55	*(E)*-Cinnamyl acetate	$C_{11}H_{12}O_2$	176	2.56	1660
32.09	β-Terpinyl acetate	$C_{12}H_{20}O_2$	196	0.43	1742
36.89	Caryophyllene	$C_{15}H_{24}$	204	0.63	1896
	Total			99.40	

Rt: Retention time. RI: Retention index.

2.6.5. Gamma aminobutyric acid transaminase (GABA-T) assay

The activity of GABA-T was assayed according to Pandey and Singh (1985) with minor modifications. Enzyme extract (100 µl) was added to 1450 µl of 50 mM Tris-HCl (pH 8.5), 100 µl of 2 mM α-ketoglutarate, 100 µl of 2-mercaptoethanol (20 mM), and 20 µl of β-NAD (1.1 mM). The reaction was started by adding 200 µl of GABA (3 mM) to the mixture. Finally, 30 µl of 1% triton-x 100 were added to the previous mixture. Samples including the blank and control samples were incubated at 25 to 30 °C for 30 min. The absorbance was measured at 340 nm, and the enzyme activity was presented as OD_{340}.mg protein^{-1}.min^{-1}.

2.7. Molecular docking

Using molecular docking studies, digital values were used to determine the effectiveness of cinnamaldehyde and temephos against various enzyme targets. The crystal structures of AChE (PDB: 5X61), ATPase (PDB: 4BYG), and GABA-T (PDB: 1SF2) were obtained from protein data bank (PDB) (http://www.rcsb.org/pdb/home/home.do). The selection of AChE PDB was based on the model for *C. pipiens* AChE1 that was built using the template protein of malaria mosquito vector *Anopheles gambiae* (PDB: 5X61, Chain B), with 99% query coverage and 92.36% similarity (Rao et al., 2021). 3D structure of the tested enzymes was visualized using Molecular Operating Environment (MOE) 2014.13 software (Chemical Computing Group Inc, Montreal, Quebec, Canada) and examined for missing atoms, bonds, and contacts (Chemical Computing Group, 2008). The tested compounds were converted to 3D, and the energy was minimized by the MMFF94 function (Halgren, 1999). A triangle-matching algorithm was chosen to dock the compounds into the active sites of the enzymes. The contributions of hydrophobic, ionic, hydrogenated, and Van der Waals interactions were used to quantify the free energy of binding. A ligand was considered adequate for a minimum docking score value (or interaction energy calculation) of an enzyme-ligand complex.

2.8. Statistical analysis

Statistical analysis was performed using the IBM SPSS software version 25.0 (SPSS, Chicago, IL, USA) (IBM, 2017). Mortality percentages were calculated for each treatment and corrected using Abbott's equation (Abbott, 1925). Means and standard error (SE) were obtained from three independent replications for each treatment. According to the probit analysis, the log dose-response lines (LdP line) were used to determine the LC_{50} values (Finney, 1971). The least-square regression analysis was used to determine the 95% confidence limits. Analysis of variance (ANOVA) of the biochemical data was conducted and means property values were separated ($p \leq 0.05$) with Student-Newman-Keuls (SNK).

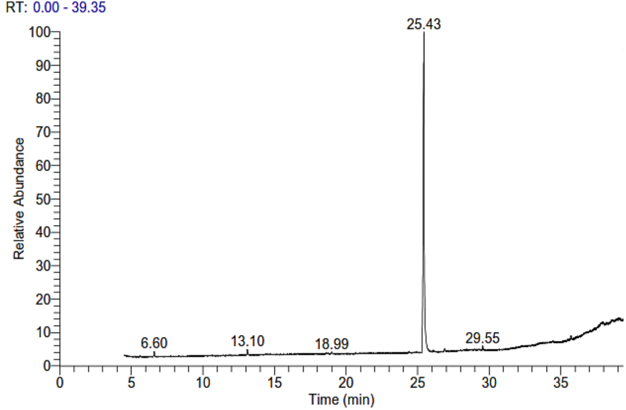

Figure 2. GC/MS chromatogram of cinnamon essential oil

3. Results and discussion

3.1. GC/MS analysis of cinnamon oil

The GC/MS chromatogram of the oil is shown in Figure 2. The chemical composition of cinnamon oil was done using GC/MS analysis. A chromatogram of *C. verum* EO constituents showed that the oil is a mixture of 12 different components, representing 99.40% of the total oil (Table 1). The identified chemical compounds belong to olefinic hydrocarbons, monoterpenes, sesquiterpenes, and other essential phytochemicals. The major components detected in the oil, according to retention time and their relative abundances, were α-pinene (1.03%), β-cymene (1.01%), 1,8-cineole (6.12%), linalool (2.02%), *(E)*-cinnamaldehyde (80.09%), eugenol (3.18%), methyl linoleate (1.06%) and *(E)*-cinnamyl acetate (2.56%). However, other constituents, including β-pinene, *(-)*-camphor, β-terpinyl acetate, and caryophyllene, were less abundant with a percentage of lower than 1%. The GC/MS analysis results of the cinnamon EO showed that cinnamaldehyde was the major component of this essential oil. According to studies carried out by several investigators, the major component of cinnamon EO was cinnamaldehyde in the range of 44-97% (Adinew, 2014; Wang et al., 2009; Yu et al., 2020). These findings agree with the current study since the concentration of cinnamaldehyde was 80.09%. On the contrary, the results obtained by Ghosh et al. (2013) proved that eugenol was found to be the main component of cinnamon oil (*C. zeylanicum*) with 59.918% of the total peak area. In conclusion, most *C. verum* oil components were similar to those reported in this oil chemistry.

Table 2. The visual appearance, droplet size, PDI, zeta potential, dynamic viscosity, and pH of the prepared NEs

Nanoemulsion	Visual appearance	Droplet size (nm) ± SE	PDI ± SE	Zeta potential (mV)	Viscosity (mPa.s) ± SE	pH	Stability after centrifugation at 5000 rpm
Cinnamon oil NE	Milky	95.67±0.04	0.33±0.05	-30.3	7.03±0.02	6.11	√
Cinnamaldehyde NE	Milky	174.59±0.08	0.58±0.13	-21.20	6.93±0.07	6.08	√

(√) refer to the stable state, (×) refer to the non-stable state. PDI: Polydispersity index.

3.2. Characterizations of the prepared NEs

3.2.1. Droplet size and polydispersity index (PDI)

Table 2 presented the droplet size and PDI data of produced NEs. There is a significant difference between the droplet size values for cinnamon oil and cinnamaldehyde, which were 95.67 and 174.59 nm, respectively. In general, the scale of 10-500 nm corresponding to the average droplet size of O/W NEs (Izquierdo et al., 2002; Kabri et al., 2011). This finding showed that all compounds were successfully prepared at the nanometric scale. In agreement with our results, the findings obtained by Abdelrasoul et al. (2018) reported that the mean particle size of nano-sized cinnamaldehyde was 128.07 nm. While the particle size detected by Mibielli et al. (2021) in the carvacrol, NE was 354.20 nm. Under room temperature and accelerated stability assessment, no phase separation, creaming, or sedimentation were observed (Balaji et al., 2017; Qin et al., 2017). The tiny droplets in a NE contribute to its long-term physical stability, which is why this sort of formulation is often known as "approaching thermodynamic stability". (Izquierdo et al., 2002; Solans et al., 2005). NEs have several advantages over traditional emulsions due to the small size of the droplets: higher optical visibility, more excellent stability against droplet aggregation and gravitational separation, and increased bioactivity of encapsulated components (McClements, 2012). For these reasons, NEs have emerged as an alternative drug, pesticide carriers, and other vital applications (Feng et al., 2018; Tang et al., 2013).

The PDI of the prepared NEs were 0.33 and 0.58 for cinnamon and cinnamaldehyde, respectively (Table 2). The PDI reflects the distribution, homogeny, and stability of particle size in the emulsion. These findings indicated that the size distribution of all NEs was

relatively narrow. In agreement with our results, the PDI value of cinnamaldehyde NE prepared by Abdelrasoul et al. (2018) was 0.322. While the PDI value of carvacrol NE formulated by Mibielli et al. (2021) was 0.104. The PDI, which ranges from zero to one, is a dimensionless estimate of the width of the size distribution measured from the cumulate analysis (Kentish et al., 2008). A lower PDI value indicates the presence of a uniform droplet size distribution (homogeneous system), while a PDI value closer to one indicates the presence of a large variety of droplet sizes (heterogeneity of the system) (Tyagi et al., 2012).

3.2.2. Zeta potential

Table 2 and Figure 3 show that the NEs revealed negative zeta potential (-30.30 and -21.20 for cinnamon and cinnamaldehyde, respectively). This finding implies a sufficiently high negative surface charge for droplet-droplet repulsion and, as a result, improved NE stability (Bruxel et al., 2012). The zeta potential is a better way to optimize sample stability and save time consumed in shelf-life tests. It is considered a potent indicator of NE stability, resist flocculation and aggregation for more extended periods, and is associated with to surface potential of the droplets (Benita and Levy, 1993). The charge acquired by a particle or molecule in a given medium is its zeta potential. It arises from the surface charge, concentration, and types of ions in the solution. Since particles of similar charge will repel each other, those with high charges will resist flocculation and aggregation for more prolonged periods making such samples more stable. This finding means that the stability can be modified by altering the pH, the ionic concentration, the type of ions, and additives such as surfactants and polyelectrolytes (Mahdi et al., 2011).

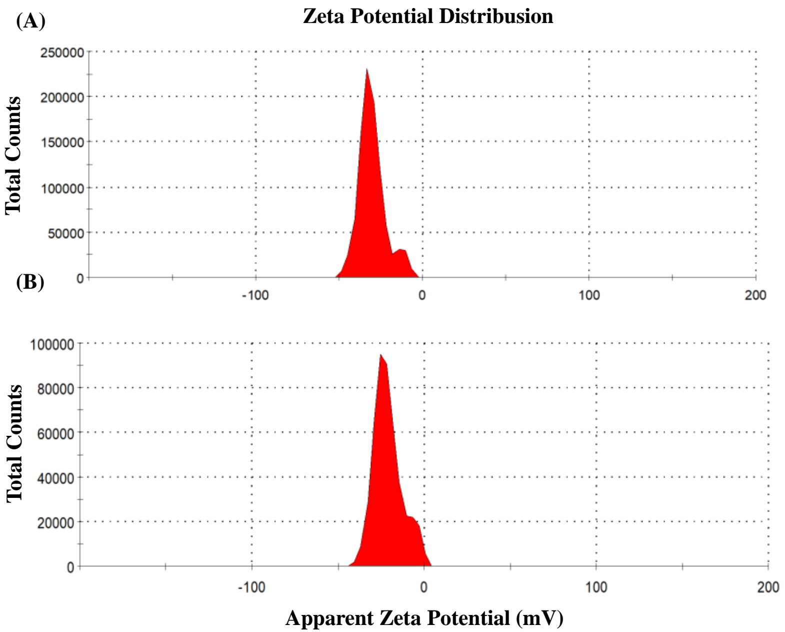

Figure 3. Zeta potential distribution graph of NEs of cinnamon oil (A) and cinnamaldehyde (B)

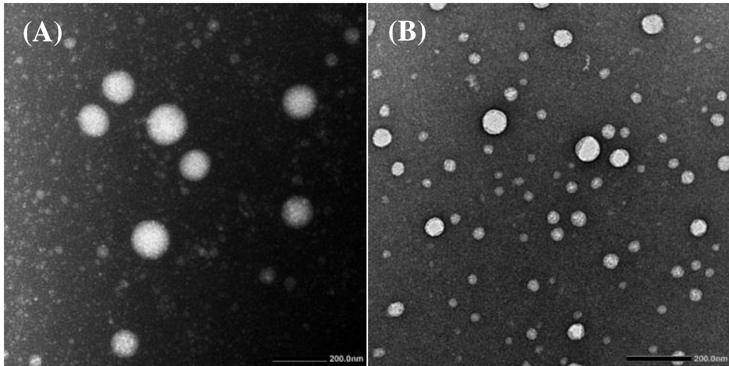

Figure 4. Transmission electron micrograph of cinnamon NE (A) and cinnamaldehyde NE (B). The TEM was performed on a JEOL JEM-1400 Plus transmission electron microscope operating at an acceleration voltage of 80.0 kV with a 20 mm aperture. Print magnification 30000x.

3.2.3. Viscosity and pH measurements

The viscosity values of the NEs were 7.03 and 6.93 for cinnamon and cinnamaldehyde, respectively (Table 2). The pH values were 6.11 and 6.08 for cinnamon and cinnamaldehyde, respectively (Table 2). As mentioned previously, the pH range of a NE has a significant impact on its stability. The surface charge of the globule changes as the pH value changes, affecting their stability during storage. A rise in globule surface charge facilitates electrostatic repulsion and decreases NE flocculation and dissolution (Pengon et al., 2018).

3.2.4. Thermodynamic stability studies

An accelerated storage testing was carried out to predict the long-term physical stability of the NEs (Badawy et al., 2017). The recorded data of visual stability, centrifugation, and heating-cooling

stability of NEs showed that all prepared NEs were stable at 5000 rpm of centrifugation, at 25 °C for up to 3 months, and under heating-cooling cycle test.

3.2.5. TEM

The morphological study of the structure of NE droplets is carried out using TEM. TEM micrographs of NEs were presented in Figure 4, showing their spherical form and typical NE appearance at magnification 30.000x.

3.3. Larvicidal efficacy of tested compounds and their NEs

After 24 and 48 h of exposure, the larvicidal activity of cinnamon oil and technical (T) cinnamaldehyde was compared with their NEs and temephos against *C. pipiens*. Table 3 presented the LC_{50} values with 95% confidence limits and other statistical parameters. Cinnamaldehyde T and NE were more effective (LC_{50} = 70.44-94.46 mg/l) than the EO products (LC_{50} = 112.34-154.08 mg/l). Cinnamaldehyde NE gave the LC_{50} value of 72.90 mg/l more than the T form (94.46 mg/l) after 24 h of the experiment. At the same time, this compound gave LC_{50} values 70.44 and 84.91 mg/l after 48 h of exposure for NE and T, respectively. NE form of EO gave an LC_{50} value of 123.13 mg/l, which was more effective than non-formulated EO (LC_{50} = 154.08 mg/l) after 24 h of exposure. It can be observed that the larvicidal behavior of the NE was the best. Temephos as a reference insecticide against mosquito larvae exhibited the LC_{50} of 0.012 and 0.009 mg/l after 24 and 48 h, respectively.

Table 3. Larvicidal activity of cinnamon EO, its main component cinnamaldehyde, and their NEs against *C. pipiens*

EOs	Time of exposure (h)	Formulation	LC_{50}[a] (mg/L)	95% confidence limits (mg/L)		Slope[b] ± SE	Intercept[c] ± SE	(χ^2)[d]
				Lower	Upper			
Cinnamon oil	24	EO	154.08	144.56	163.65	7.28±0.46	-15.94±1.02	12.81
		NE	123.13	117.53	128.15	6.96±0.52	-14.55±1.12	4.18
	48	EO	145.75	136.52	154.65	7.48±0.48	-16.18±1.06	12.73
		NE	112.34	105.47	118.18	6.19±0.52	-12.69±1.12	9.13
Cinnamaldehyde	24	T	94.46	87.52	103.56	10.23±0.61	-20.25±1.21	56.15
		NE	72.90	69.05	76.23	7.01±0.60	-13.05±1.16	5.88
	48	T	84.91	77.09	90.17	9.95±0.92	-19.20±1.82	10.79
		NE	70.44	60.61	77.33	7.02±0.62	-12.97±1.19	9.18
Temephos	24	T	0.012	0.008	0.017	3.33±0.19	-3.54±0.20	90.08
	48	T	0.009	0.007	0.011	4.26±0.28	-3.99±0.26	57.96

EO: Essential oil, T: Technical, NE: Nanoemulsion, [a] The concentration causing 50% mortality, [b] Slope of the concentration-mortality regression line, [c] Intercept of the regression line, [d] Chi-squared value, SE: Standard error

Table 4. The activity of cinnamon EO, its main component cinnamaldehyde, and their NEs on AChE, ATPase, and GABA-T activity in *C. pipiens* larvae after 24 h of the treatment with LC_{50} of each treatment

Treatment	Type of formulation	Enzyme activity (OD.mg protein^{-1}.min) ± SE			Change in activity (%) ± SE		
		AChE	ATPase	GABA-T	Inhibition of AChE	Activation of ATPase	Activation of GABA-T
Untreated larvae	-	0.93[a]±0.00	1.07[e]±0.01	0.110[e]±0.00	0.00[e]±0.00	0.00[e]±0.00	0.00[e]±0.00
Cinnamon oil	EO	0.77[b]±0.00	1.38[d]±0.02	0.161[c]±0.00	17.26[d]±0.08	28.56[d]±2.80	46.13[c]±5.58
	NE	0.50[d]±0.00	1.88[c]±0.02	0.213[b]±0.00	46.40[b]±0.57	75.42[c]±2.51	93.14[b]±6.62
Cinnamaldehyde	T	0.64[c]±0.00	2.37[b]±0.01	0.146[d]±0.01	30.83[c]±0.49	120.53[b]±2.06	32.21[d]±4.36
	NE	0.37[e]±0.01	2.93[a]±0.05	0.409[a]±0.00	60.59[a]±1.59	172.59[a]±3.07	271.23[a]±14.05
Temephos	T	0.18[f]±0.01	-	-	80.65[a]±1.01	-	-

EO: Essential oil, T: Technical, NE: Nanoemulsion, OD: Optical density, SE: Standard error, AChE: Acetylcholinesterase, ATPase: Adenosine triphosphatase, GABA-T: Gamma-aminobutyric acid transaminase. Values in the column with different letters are significantly different at $p \leq 0.05$ using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test.

Based on the literature review, a natural product with LC_{50} < 100 mg/l could be a promising candidate for mosquito control (Dias and Moraes, 2014; Kharoubi et al., 2021; Ríos et al., 2017). In our earlier study, twenty-one monoterpenes from different chemical groups were evaluated against *C. pipiens* larvae, and the results indicated that (±)-β-citronellol and cinnamyl acetate had the highest mortality (LC_{50} = 5.520 and 5.603 mg/l, respectively) (Taktak and Badawy, 2019). In addition, our results are approximately similar to the data obtained by Pavela (2015), who proved that the LC_{50} values of *p*-cymene and linalool against *C. quinquefasciatus* larvae were 21 and 247 mg/l, respectively (Pavela, 2015). Moreover, Michaelakis et al. (2014) proved that the LC_{50} value of linalool was more than 200 mg/l against *C. pipiens* larvae (Michaelakis et al., 2014). In addition, the data observed for cinnamon and eucalyptus oils showed a 100% larvicidal effect at 1000 mg/l and a 100% knockdown effect at 10% against *C. quinquefasciatus* (Manimaran et al., 2012).

In recent years, nanotechnology in pest management has been increasing, and one of the most important goals is to prepare nano pesticides (Jesser et al., 2020). Essential oils or monoterpenes in nanometric emulsions enhanced mosquitocidal activity (Nenaah et al., 2021). For example, the NE of neem oil with the hydrodynamic size of 30.12 nm and PDI of 0.262 exhibited noteworthy larvicidal activity against *C. quinquefasciatus* (LC_{50} = 11.75 mg/l after 24 h) (Mishra et al., 2018). Currently, extensive efforts are being made to study the potential toxic behavior of prevalent nano pesticides in mosquitoes and other pest ecosystems (Kumar et al., 2020; Mishra et al., 2018). However, limited experimental studies have been carried out to shed light on the toxicity profile of these nanometric pesticides towards the non-target species prevalent in the target ecosystem (Côa et al., 2020; Taktak et al., 2021).

3.4. Biochemical studies

To explain some biochemical actions of the tested compounds on *C. pipiens* larvae, the effect of the LC_{50} values of these compounds on the activity of AChE, ATPase, and GABA-T extracted from larvae after 24 h of the treatment was studied. Generally, the activities of AChE and GABA-T were inhibited after treatment, while ATPase activity was increased (Table 4). The activity of enzymes in untreated larvae was 0.93, 1.07, and 0.11 for AChE, ATPase, and GABA-T, respectively. Cinnamaldehyde NE inhibited the AChE enzyme by 60.59%, while the unformulated form decreased the activity by 30.83% compared to the untreated larvae. At the same time, the

effectiveness of cinnamon NE (46.40% inhibition) was more than the pure oil (17.26% inhibition) on AChE activity. NE of cinnamon oil caused 75.42% activation of ATPase enzyme compared to its pure oil (28.56% activation). In contrast, cinnamaldehyde NE caused more effect (172.56% activation) than the T form (120.53%). All tested compounds, at the level of the LC_{50}, proved an activation of GABA-T activity. Cinnamaldehyde NE was the most active product, which caused 271.23% activation for GABA-T compared to 32.21, 93.14, and 46.16% activation for technical cinnamaldehyde, cinnamon NE, and cinnamon EO, respectively.

Table 5. Molecular docking, binding scores, and binding interactions of cinnamaldehyde and temephos within the active sites of AChE (PDB ID: 5X61), ATPase (PDB ID: 4BYG), and GABA-T (PDB ID: 1SF2)

Enzyme	Compound	Docking score (ΔG, kcal/mol)	Van der Waals	H-Bond			Hydrophobic Interactions (π-interactions)			RMSD
				(Amino acid-ligand atom)	Interaction	Distance (Å)	(Amino acid-ligand atom)	Interaction	Distance (Å)	
AChE	Cinnamaldehyde	-4.32	Gly A445, Gly B445, Ile A231, Ile A446, Ile B231, Ile B446, Phe A490, Phe B490, Trp A441, Trp B441, Tyr A282, Tyr A493, Tyr A494, Tyr B282, Tyr B493, Tyr B494	Cys A447-O1 Cys B447-O1	HBA HBA	3.04 3.04	-	-	-	1.274
	Temephos	-7.41	Asn B438, Asp A233, Asp B233, Gly A445, Gly B445, Ile A231, Ile B231, Leu B444, Phe B449, Trp A441, Trp B441, Tyr A282, Tyr A493, Tyr A494, Tyr B282, Tyr B493, Tyr B494, Val B232, Val B235	Cys A447-S1 Cys A447-S1 Ile A446-S1 Ile A446-S1	HBA HBA HBA HBA	3.78 3.75 4.45 4.45	-	-	-	1.301
ATPase	Cinnamaldehyde	-2.62	Ala B486, Glu A488, Glu B488, Gly A497, Gly B497, His B503, His B515, Val B496	-	-	-	-	-	-	2.353
	Temephos	-4.53	Ala B489, Arg B268, Glu A488, Glu B488, Thr B299, Pro B490	Gly B300-S1 Ser B301-S1	HBA HBA	4.14 4.05	-	-	-	1.835
GABA-T	Cinnamaldehyde	-4.06	Arg A192, Arg C192, Glu A270, Glu B270, His A206, His C206, Phe A189, Phe C189, Phe B351, Phe D351, Lys A329, Tyr B348, Tyr D348, Thr B353, Thr D353, Ile A72, Ile C72	Lys C329-O1	HBA	3.19	-	-	-	0.982
	Temephos	-3.06	Glu A229, Glu B229, Gln A395, Gln B395, Ile A402, Ile B402, Phe A230, Phe B230, Pro A226, Pro B226, Ser A403, Ser B403, Tyr A225, Tyr B225	Arg A222-S1 Arg B222-S1 Pro A399-S12 Pro B399-S12	HBD HBD HBA HBA	3.39 3.46 3.47 3.47	-	-	-	2.007

RMSD: The root means square deviation of the pose, in Å, from the original ligand. This field is present if the site definition is identical to the ligand definition.

According to some authors, the inhibition of acetylcholinesterase by terpenes has no relationship with their larvicidal properties (Cai et al., 2018; Seo et al., 2015). Miyazawa and Yamafuji (2005) proved that bicyclic hydrocarbons with allylic methyl group uncompetitive inhibitors for AChE. In contrast, bicyclic alcohols and ketones exhibited weak inhibition. Inhibition of AChE by α-pinene, terpineol, linalool, β-myrcene, nerol, and geraniol was found at high concentrations (Zarrad et al., 2017). In another study, fenchone, S-carvone, and linalool, followed by estragole, produced a higher inhibition on AChE, but *(E)*-anethole was not active (López and Pascual-Villalobos, 2010). The effects of twenty-one monoterpenes on AChE, acid and alkaline phosphatases, ATPase, and GABA-T were investigated (Taktak and Badawy, 2019). The authors proved that all compounds proved a significant inhibition of all tested enzymes except ATPase. Monoterpenes such as thymol, linalool, menthol, camphor, carvone, and borneol have been discovered to bind to ionotropic GABA receptors in insects (Tong, 2010). Glycolysis produces ATP in the insect body, both in the cell wall and cytoplasm. Consequently, essential oils and their constituents affect the intracellular and extracellular ATP balance (Faleiro, 2011). *In vivo* activation of ATPase at these points may be due to changes in ATP balance as a result of the influence of essential oils and their constituents on cell membranes.

3.5. Molecular docking

Table 5 and Figure 5 show the docking results of cinnamaldehyde with AChE (PDB: 5X61), ATPase (PDB: 4BYG), and GABA-T (PDB: 1SF2), respectively. The data presented as docking scores and different interactions include H-bonds, Van der Waals, and hydrophobic. The binding affinity values of cinnamaldehyde were -4.32, -2.62, and -4.06 kcal/mol on the active sites of AChE, ATPase, and GABA-T, respectively. It can be noted that the highest binding affinity was found with AChE and GABA-T. However, temephos as a reference insecticide exhibited binding affinity values -7.41, -4.53, and -3.06 kcal/mol on the active sites of AChE, ATPase, and GABA-T, respectively (Table 5).

Figure 5A shows the recognized binding modes and molecular orientations (2D and 3D) of cinnamaldehyde with AChE. Seventeen amino acids surround the compound through Van der Waals interactions. The oxygen atom formed two HBA interactions with the amino acids Cys A447 and Cys B447. No hydrophobic interactions were observed. Cinnamaldehyde exhibited low binding affinity with high docking energy toward the active sites of the ATPase (ΔG = -2.62 kcal/mol). It was interacted with ATPase by Van der Waals through ten amino acids (Ala B486, Glu A488, Glu B488,

Gly A497, Gly B497, His B503, His B515, and Val B496). Cinnamaldehyde did not show hydrogen bonds to amino acids or hydrophobic interactions with ATPase in the active pockets. The binding confirmation of the cinnamaldehyde with ATPase is shown in Figure 5B. This compound is surrounded by fourteen amino acids through Van der Waals interactions in GABA-T (Figure 5C). The O1 atom formed one HBA with the amino acid Lys C329 via hydrogen bonding interaction (3.19 Å).

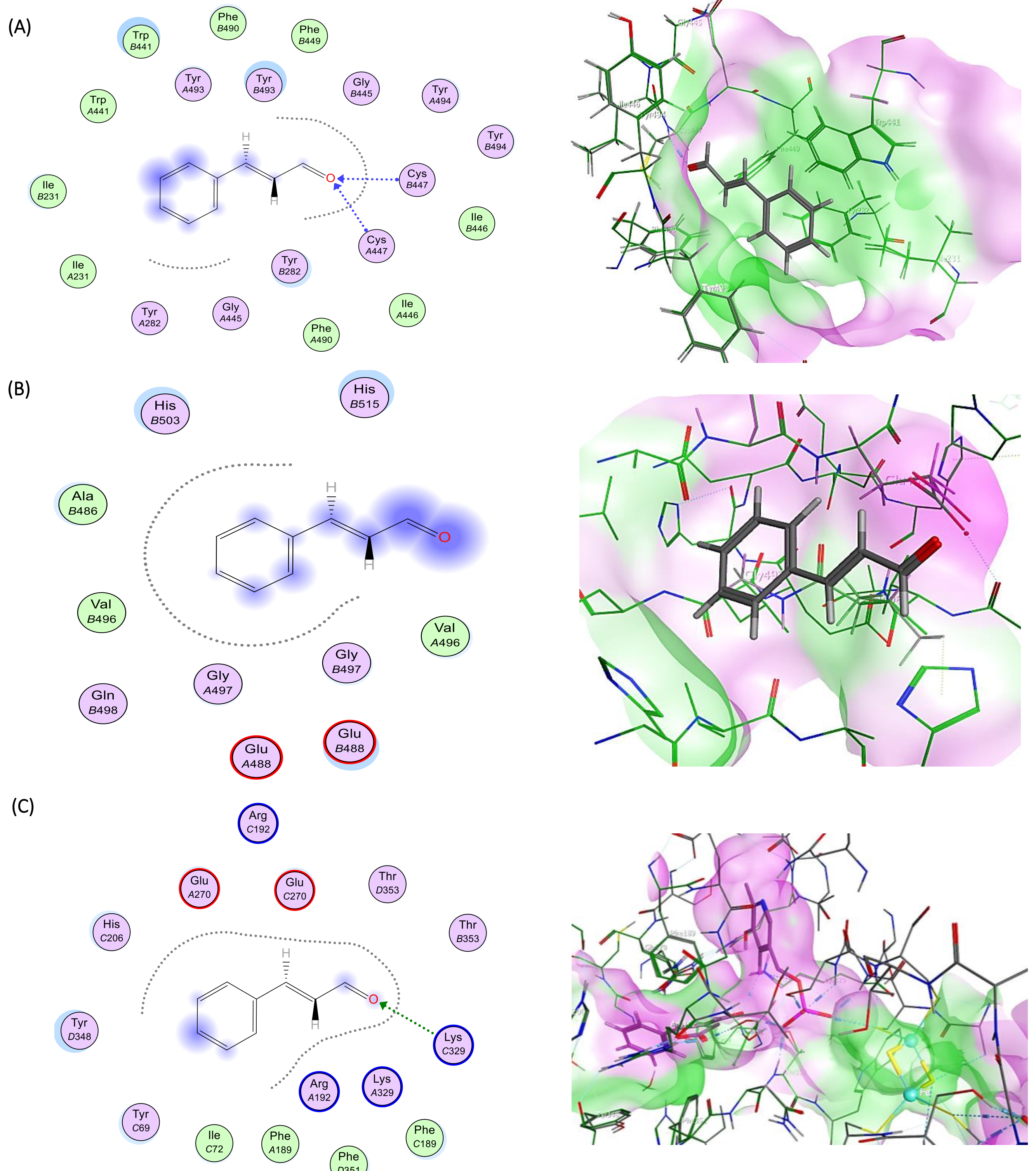

Figure 5. Docking view of cinnamaldehyde on the binding sites of AChE (PDB: 5X61) (A), ATPase (PDB: 4BYG) (B), and GABA-T (PDB: 1SF2) (C). Left are the 2D interaction diagrams, and right are the complex structures in 3D.

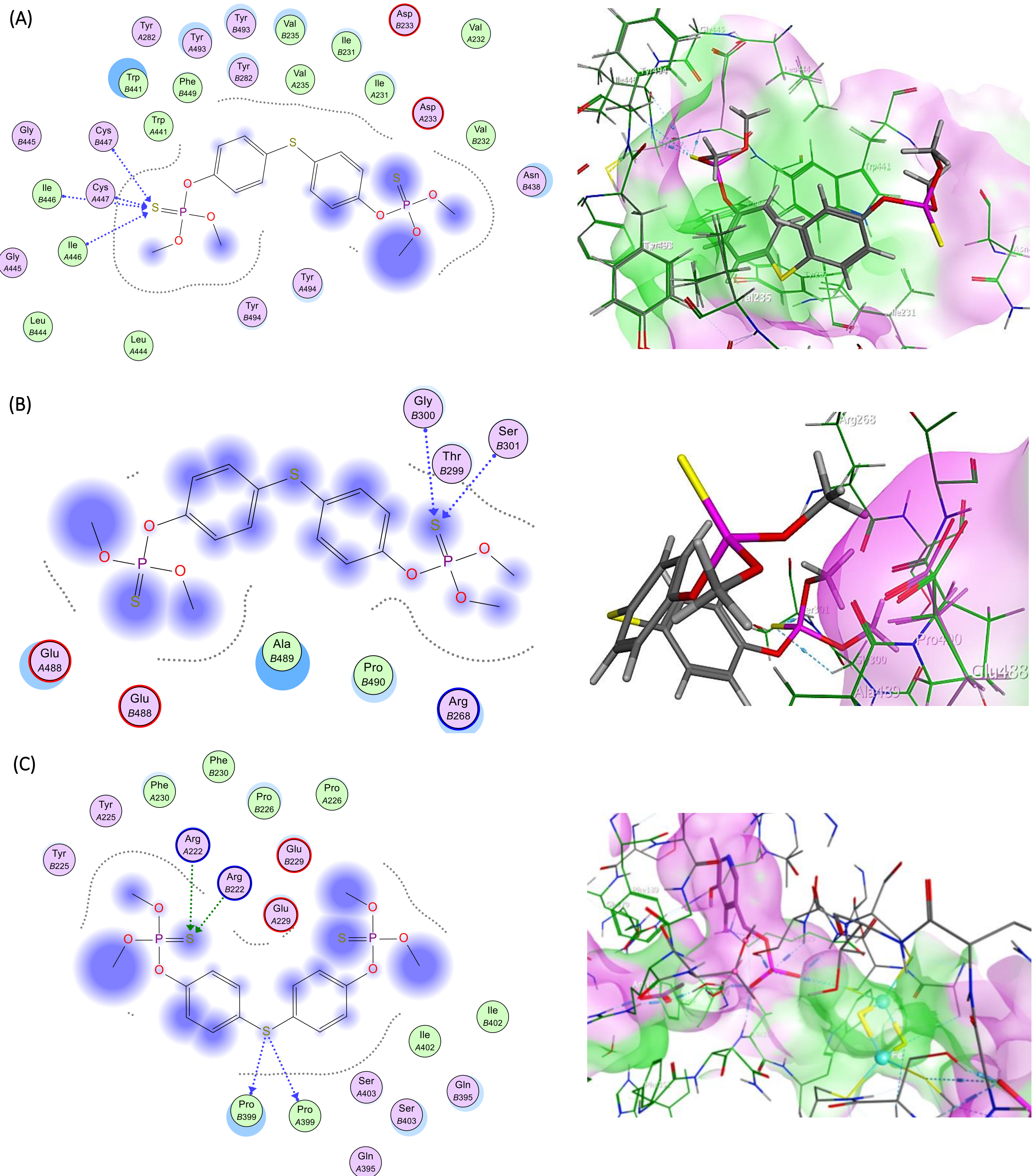

Figure 6. Docking view of temephos on the binding sites of AChE (PDB: 5X61) (A), ATPase (PDB: 4BYG) (B), and GABA-T (PDB: 1SF2) (C). Left are the 2D interaction diagrams, and right are the complex structures in 3D.

Figure 6 shows the recognized binding modes and molecular orientations (2D and 3D) of temephos with the target enzymes. The insecticide bonded with AChE Van der Waals, hydrogen bonding, and hydrophobic interactions. Sulfur atom in the thiophosphate group interacted with four amino acids, Cys A447-S1, Cys A447-S1, Ile A446-S1, and Ile A446-S1 through HBA (Figure 6A). No hydrophobic interactions were found. The binding confirmation with ATPase is shown in Figure 6B. The sulfur atom in the thiophosphate group formed two HBAs with the amino acids Gly B300 and Ser B301 with 3.39 and 3.46 Å, respectively. The insecticide is surrounded by fourteen amino acids through Van der Waals interaction in GABA-T (Figure 6C). The sulfur atom in the thiophosphate group formed two HBDs with the amino acids Arg A222 and Arg B222 with 3.39 and 3.46 Å, respectively. Moreover, the sulfur atom of sulfanylphenoxy moiety formed two HBAs with the amino acids Pro A399 and Pro B399 with 3.39 and 3.46 Å, respectively. No hydrophobic interactions were found.

Because the mechanism of larvicidal activity of monoterpenes is not wholly understood, molecular docking is a method to predict and understand molecular recognition. It detects the predominant binding mode and binding affinity between the protein and ligand and gives a 3D structural explanation of the interaction in several biological processes (Gumede et al., 2012; Lie et al., 2011). This tool was used to elucidate the biological activities of some larvicides with their pharmacokinetic properties (Kumar et al., 2018; Zeng et al., 2016). The docking study revealed that cinnamaldehyde has low binding energy and a high affinity for the functional pocket of the enzyme targets and can bind to the active sites of those enzymes.

4. Conclusions

Natural pesticides derived from natural resources are urgently needed to control mosquitoes without causing harm to beneficial organisms or the environment. Therefore, NEs of cinnamon oil and cinnamaldehyde were prepared and characterized as alternatives to synthetic mosquitocides. The NEs enhanced the mosquitocidal activity against *C. pipiens* larvae and displayed a remarkable effect. As a result, cinnamaldehyde had more larvicidal activity than cinnamon oil 24 h after exposure. Furthermore, the action of the NEs was higher than the unformulated products on the target enzymes (AChE, ATPase, and GABA-T). Additionally, the molecular docking study demonstrated that cinnamaldehyde had a greater affinity for AChE and GABA-T than ATPase. These results indicate that cinnamon oil and cinnamaldehyde NEs have an overall significant effect on *C. pipiens* larvae and might prove helpful as mosquitocide agents.

Acknowledgments

None.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

CRediT authorship contribution statement

All authors contributed to the study's conception and design. They performed material preparation, data collection, and analysis. Nehad E.M. Taktak performed the preparation, characterization of the NEs, the bioassay techniques, and molecular docking studies. In addition, she also wrote the draft of the manuscript. Osama M. Awad, Nadia E. Abou El-Ela, and Mohamed E.I. Badawy designed the experiments, assisted in the characterization of the NEs, analyzed and interpreted the data, and wrote the manuscript. All authors participated in article proofreading, sentence correction and approved the final manuscript.

Ethics approval and consent to participate

Approval of the Ethics Committee of the High Institute of Public Health, Alexandria University, Egypt, was obtained on 10th July 2018 with a reference number of 481. The study was carried out in compliance with the International Guidelines for Research Ethics. Consent to Participate is not applicable.

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Supplementary File

None.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 64-81

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS
https://ijpbp.com

REVIEW

OPEN ACCESS

Edible seeds with potential anti-obesity impact: A Review

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ARTICLE INFO

Article History:

Received: 17 December 2021
Revised: 16 January 2022
Accepted: 16 January 2022
Available online: 21 January 2022

Edited by: B. Tepe

Keywords:
Alternative medicine
Dietary supplements
Functional food
Seeds
Obesity
Weight loss

ABSTRACT

Obesity and related metabolic diseases prevalence rates have risen dramatically in the recent decades, leading to severe health issues and increased mortality rates. A pressing need is evolved for a potential solution addressing obesity undermining. Bariatric dietary supplements and alternative medicine are recently gaining growing attention as a panacea for obesity owing to their rich nutritional profile and bioactive compounds. This systematic review was conducted to evaluate the current knowledge of some purported dietary seeds commonly used as functional food; quinoa (*Chenopodium quinoa*, L.), chia (*Salvia hispanica* L.), Hab El-Rashad (*Lepidium sativum*, L.), pumpkin (*Cucurbita pepo* L.) and fenugreek (*Trigonella foenum-graecum*). Due to the limited scientific evidence for their efficacy, future studies should empirically investigate dietary intervention structure to evaluate the impact on body mass status.

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1. Introduction

Obesity is a major worldwide health problem that has increased widely in the past years. Recently, obesity rates or overweight people have continued to rise. The World Health Organization (WHO) defined obesity as an excessive accumulation of fats that lead to high health risk (Chooi et al., 2019). Obesity is illustrated by body mass index (BMI), which is the individual's weight in kilogram divided by the square of height in meters. WHO and CDC's Division of Nutrition, Physical Activity, and Obesity (DNPAO) defined the normal range of BMI as18.5 to 24.9 kg/m^2, overweight range as 25 to 29 kg/m^2, obese range as 30 to 39 kg/m^2, while severe obesity range is considered to be ≥ 40 kg/m^2 (Chooi et al., 2019; Petersen et al. 2019).

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e-ISSN: 2791-7509
doi:
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Nearly one-third of the global population suffers from obesity and its cardiometabolic complications, leading to premature mortality (Chooi et al., 2019). Obesity incidence is a multifactorial problem (Figure 1) that plays a significant role in the pathogenesis of different diseases. Obesity increases the implications of many chronic diseases, including type 2 diabetes, hypertension, cardiovascular diseases, heart attacks, strokes, fatty liver, and several types of cancer (Blüher, 2019).

Consequently, the Food and Drug Administration (FDA) has been approving several dietary supplements as a potential means to control obesity and its related risk factors. Nowadays, edible fruits seeds use is very common and trendy as food supplements with anti-obesity effects. The use of these seeds could be due to the interplay between the secondary metabolites content and their potential effect to modulate the oxidative stress and inflammation that accompany the obesity besides their hypoglycemic effect, decreasing lipogenesis and enhancing lipolysis (Rodríguez-Pérez et al., 2019).

Please cite this article as: Hosny, H., Omran, N., Handoussa, H., 2022. Edible seeds with potential anti-obesity impact: A Review. International Journal of Plant Based Pharmaceuticals, 2(1), 64-81.

In 1985, WHO estimated that about 65% of the world population predominately depended on plant-based traditional medicines for their primary health care. According to a survey done by WHO, 80% of 122 compounds identified from 94 plant species were used for the similar ethnomedical purpose. About 80% of the world's population currently relies on traditional medicines for their primary health needs, mainly by using plant extracts (Cragg and Newman, 2013).

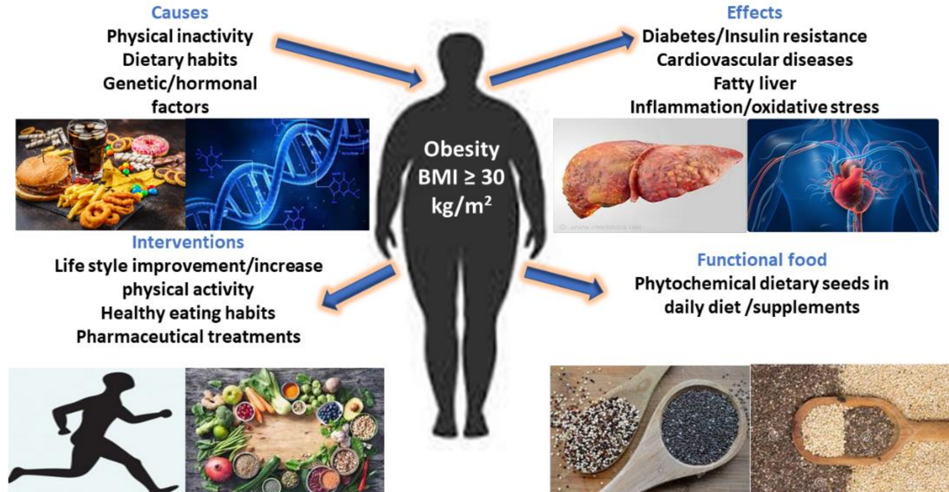

Figure 1. Schematic overview of the vicious cycle of obesity showing possible effects, cause, medical and functional food interventions

In the past years, seeds have been used widely against the battle of many chronic diseases, including obesity and related diseases. Every day, there is growing evidence about the use of dietary seeds and natural products either in food industries or in drugs and supplementary products (Moreno-Valdespino et al., 2020). Ancient people used medicinal plants to treat many illnesses, including obesity, and recently the use of medicinal plants has been widely increasing for their benefits and fewer side effects compared to allopathic medicines (Hussain et al., 2019). The nutritional supplements industry worldwide is now moving towards more beneficial attractive options by choosing particular types of food with known mechanisms to help decrease the worldwide tragedy of obesity (Monteiro and Cannon, 2019).

This review differs from previous societal ones by focusing specifically on seeds recommended by many health care professionals who provide treatments for obesity. The purpose of this review is to provide background information on their role in food intake and body weight in humans and their mechanism of action. These seeds are quinoa, chia, Hab El-Rashad, pumpkin, and fenugreek seeds.

2. Materials and methods

In the present review, several scientific databases, including PubMed, Web of Science, Wiley, Science Direct, Google Scholar, ACS Publications, Taylor & Francis, Springer, and Europe PMC, were searched for relevant literature using the keywords: obesity, natural products, dietary seeds, phenolic compounds, quinoa, chia, Hab El-Rashad, pumpkin and fenugreek seeds. All citations were combined using EndNote X8 (Thomson Reuters, Toronto, Canada). A total of 10.404 citations were recognized, and duplicates were removed (*n* = 2.176).

3. Inclusion/exclusion criteria

Data included in this review was based on the information derived from studies demonstrating the nutritional content of the seeds' nutritional content and their anti-obesity, antioxidant, anti-inflammatory effects in vivo and in vitro reports describing seeds' pharmacological and biological effects.

In the past years, edible seeds commonly used in the food industry have gained lots of importance due to many pharmacological activities that increased their application in the field of nutrition and as potential food supplements, as shown in Table 1.

4. Edible dietary seeds

4.1. Quinoa seeds

Quinoa (*Chenopodium quinoa*) seeds, as shown in Figure 2A, are widely cultivated in South America. They have a rich nutritional profile, as shown in Table 2, and a climate-resistant ability, making them an important seed that will be used widely in the future food industry (Bakhtavar and Afzal, 2020). Quinoa belongs to the family Amaranthaceae, order Caryophyllales which belongs to the core eudicots. It was known as 'suba' by the Bogota culture and 'jupha' or 'quinia' by the Bolivia culture. The species were domesticated within the South American range region, around the Titicaca Lake, approximately 5000 years ago. Presently it extends to the north and the south of the continent on the range of mountains, from Colombia to the south of Chile, where it is often found growing wild or cultivated (Burrieza et al., 2019). Quinoa is an herbaceous plant that grows annually, and its seeds are used as an alternative to cereals with high nutritional content.

For many years, it has been an indigenous grain in the Andes and has recently gained growing attention due to its higher protein, fat, and fiber content. Generally, fermented products have a strong

antioxidant activity and are regarded as excellent antioxidant foods (Hirich et al., 2020).

Quinoa is an essential crop of food that can meet the demands of basic human nutrition stated by the United Nations of Food and Agriculture Organization. It is helpful for human health because of its nutrient abundance, including amino acids, minerals, dietary, and active polysaccharides, as demonstrated in Table 3 (Teng et al., 2020). Previous research has shown that quinoa has a strong anti-obesity effect. Yao et al. (2015) stated that quinoa seed consumption that lasted for 30 days resulted in an extensive decrease in body weight, levels of triglycerides (TGs), and low-density of lipoprotein (LDL) among 22 students aged 18-45 years (Yao, et al., 2015).

Table 1. Phyto-therapeutic seeds pharmacological activity

Seed	Pharmacological activity	Type of study	Dose/extract form	Result	Reference
Quinoa (*C. quinoa*)	Anti-obesity	3T3-L1 cell culture	12.5 and 25 mg/ml total extract	Renders triglycerides accumulation, down regulation of PPAR- γ and C/EBPα, controlling adipogenesis	(Yao et al., 2015)
		Human (22 patients age range 188-45 years)	Seeds incorporated in cereal bar for 30 days	Improved lipid profile (decreased serum cholesterol and triglycerides)	(Graf et al., 2015)
		Human (age range 18-45 years)	2.05 g/kg/day methanolic extract	Decreased serum triglycerides, total cholesterol, and LDL	(Ng and Wang, 2021)
	Antioxidant	Randomized clinical trial	Seed powder	Prevents lipid peroxidation, radical scavenging	(Fernández-López et al., 2020)
		Animal model (rats)	0.88 gm/kg/day methanolic extract	Increased liver glutathione and superoxide dismutase expression	(Ng and Wang, 2021)
		Animal model (mice)	20 mg/kg seed powder	Decreased lipid peroxidation, increased levels of glutathione and superoxide dismutase	(Saxena et al., 2017)
	Anti-diabetic	Animal model (rats)	310 gm/kg total extract	Decreased serum lipid profile, serum glucose	(Paśko et al., 2010)
		Human (age range 18-45)	20 gm/kg/day alcoholic extract	Improved glycemic profile, decreased serum glucose levels	(Ng and Wang, 2021)
	Anti-inflammatory	Animal model (rats)	100 gm/kg seeds	Decreased levels of protein carbonyls and interleukin (IL)-6	(Noratto et al., 2019)
		Human (age range 18-45)	25 gm/kg/day methanolic extract	Decreased levels of IL-6, no significant effect on TNF-α level	(Ng and Wang, 2021)
	Antiviral Antimicrobial	*Pomacea canaliculata*	100 and 500 µg/ml total extract	The predominance of saponins resulted in decreased permeability into bacterial/viral membranes leading to the lysis effect	(El Hazzam et al., 2020)
	Analgesic Immunostimulant	Animal model (mice)	70-90 mg/kg seeds	Increased anti-body responses (IgG/IgA)	(El Hazzam et al., 2020)
	Cosmetic agent (Emulsifying agent)	The cell-free broth at room temperature	Ethanolic extract	Emulsification potential in a different range of pH and temperature, to be incorporated in skin and hair products	(Bezerraa et al., 2020)
	Hepatoprotective	Human (age range 18-45)	4.8 gm/kg/day ethanolic extract	Improved liver functions, including ALT and AST levels, improved liver histopathological analysis	(Ng and Wang, 2021)
		Animal model (mice)	20 mg/kg seed powder	Improvement in histopathology of the liver after administration of quinoa seed powder	(Saxena et al., 2017)
Chia (*S. hispanica*)	Anti-obesity	Animal model (rats)	50 mg/kg seeds	Decreased serum lipid profile and rat's body weight	(Panchal, 2012)
		Human (77 participants)	Chia seed powder	Increased adiponectin levels, decreased C-reactive protein, and induced weight loss	(Felemban et al., 2020)
		Animal model (rats)	Chia seed oil	Improved lipid and glycemic profiles, decreased body weight	(Melo et al., 2019)
	Antioxidant	Dynamic gastrointestinal model simgi®	0.75 and 0.95% w/w seeds	Decreased serum glucose level and improved glycemic profile	(Tamargo et al., 2020)
		A gastrointestinal tract simulation	Ethanolic extract	Induced antioxidant activity through radical scavenging and *in vitro* ferrous ion chelation	(Melo et al., 2019)
	Anti-diabetic	Animal model (rats)	20% seeds	Normalizing fasting blood sugar levels, accompanied by decreased body weight	(Alamri, 2019)
		Randomized clinical trial (15 human participants)	25 gm/day chia seeds	Decreased serum glucose levels approaching normal levels	(Felemban et al., 2020)
		Randomized clinical trial	-	Decreased insulin resistance, improved glycemic profile, and decreased BMI	(Felemban et al., 2020)
	Anti-inflammatory	Animal model (rats)	133 gm/kg seeds	Decreased lipid peroxidation and oxidative stress	(Marcinek and Krejpcio, 2017)
		Animal model (rats)	Seed powder	Improvement in inflammation status through decreasing inflammatory cytokines	(Melo et al., 2019)
		Animal model (Wistar rats)	Seed powder	Decrease levels of thiobarbituric acid reactive substances (TBARS)	(Melo et al., 2019)
	Anti-hyperlipidemic Anti-cancer	-	-	Enrichment of α-linolenic acid induces apoptosis in tumor cells, normalizing lipid profile to normal levels	(Gazem and Chandrashekariah, 2016)

Seed	Pharmacological activity	Type of study	Dose/extract form	Result	Reference
		Animal model (mice)	Chia seed oil	Remarkable decreases in the tumor weight, activating caspase activity and promoting apoptosis	(Melo et al., 2019)
	Antimicrobial and antifungal	Human (20 patients with the duodenal ulcer)	1.4 gm total extract	Decreasing the symptoms and inhibition of *Helicobacter pylori* growth	(Pachi et al., 2020)
	Anti-hypertensive	Human (males and females suffering from hypertension)	Seed powder	Significant decrease in blood pressure with no renal or hepatic alteration	(Felemban et al., 2020)
		Randomized single-blind trial (11 men and 9 women, age range 18-75)	Chia seed oil	Control and decrease blood pressure and induce vasodilatation	(Melo et al., 2019)
Hab El-Rashad (*L. sativum*)	Anti-obesity	Animal model (rats)	100 mg/kg methanolic extract	Improvement of lipid and glycemic profiles along with decreased insulin resistance	(L'hadj et al., 2019)
		Animal (rats)	Seed powder	Improved lipid profile, decreased total cholesterol, and triglycerides, decreased body weight	(Shah et al., 2021)
	Antioxidant	*In vitro* analysis on human blood	40 µg/ml oil extract	Free radical scavenging	(Alqahtani et al., 2019)
		In vitro DPPH assay	Ethanolic extract	Significant antioxidant activity was determined	(Baregama and Goyal, 2019)
		Animal model (rats)	Ethanolic extract	The radical scavenging effect, decreased lipid peroxidation	(Shah et al., 2021)
	Anti-diabetic	Animal model (mice)	200 mg/kg ethanolic extract	Decreased fasting blood glucose levels along with decreased body weight	(Desai et al., 2017)
		Animal model (mouse)	Crude extract	Decreased serum glucose level and improved lipogenesis in diabetic mouse	(Shah et al., 2021)
		Animal model (rats)	Ethanolic extract	Improve insulin resistance and pancreatic beta cells integrity	(Shah et al., 2021)
	Anti-inflammatory	*In vitro* analysis on human blood	100, 200, 300 µg/ml oil extract	The anti-inflammatory effect was manifested by the protection of cell membrane	(Alqahtani et al., 2019)
		Animal model (rats)	Seed oil	Modulating inflammatory mediator leukotriene B4	(Shah et al., 2021)
		Animal model (rats)	Methanolic extract (50, 100, 200 mg/kg/day)	Decreased circulating inflammatory cytokines, decreased liver inflammation	(Shah et al., 2021)
	Antimicrobial	*Salmonella typhi* and *Streptococcus pneumonia*	Methanolic extract	Inhibition of bacterial growth	(Al-Snafi, 2019)
		Staphylococcus aureus, *Bacillus cereus* and *Escherichia coli*	Methanolic extract	Inhibition of bacterial growth manifested in zone inhibition	(Baregama and Goyal, 2019)
		Streptococcus equine and *Corynebacterium pseudotuberculosis*	Ethanolic extract (200 mg/ml)	Inhibition of growth	(Shah et al., 2021)
	Anti-fungal	*Aspergillus flavus*	Methanolic extract (30 mg/ml)	Complete growth inhibition	(Baregama and Goyal, 2019)
	Cytotoxic activity	Hep2 cells	Ethyl acetate extract	A potent cytotoxic effect was detected owing to the rich flavonoid content in the seed extract	(Baregama and Goyal, 2019)
	Diuretic activity	Animal model (rats)	Alcoholic extract (100 mg/kg)	Increased water excretion along with sodium and potassium excretion	(Patel et al., 2009)
	Anti-hypertensive	Animal model (rats)	Aqueous extract	Decreased blood pressure without affecting the heart rate	(Shah et al., 2021)
		Animal model (mice)	Aqueous extract (1000 mg/kg)	Decreased blood pressure	(Shah et al., 2021)
Pumpkin (*C. pepo*)	Anti-obesity	Animal model (rats)	100 mg/kg total extract	Decreased body weight and enhanced lipid profile	(Kalaivani et al., 2018)
		Animal model (rats)	Hydro-alcoholic extract (400 mg/kg/day)	Improved weight loss and modulates lipid profile	(Ghahremanloo et al., 2018)
	Antioxidant	*In vitro* analysis	50 mg/kg seeds	Radical scavenging activity, decreased lipid peroxidation	(Sharma et al., 2020)
		Animal model (rats)	Alcoholic extract	Decreased lipid peroxidation due to abundant polyphenols	(Adnan et al., 2017)
	Anti-diabetic	Animal model (rats)	200 mg/kg total extract	Decreased blood glucose levels and fasting blood glucose approaching normal levels	(Kushawaha et al., 2017)
		Animal model (diabetic rats)	Seed powder	Inhibit bile acid absorption, modulate glycemic profile	(Adnan et al., 2017)
		Animal model (rats)	Aqueous extract	Control diabetes through inhibition of α-amylase	(Sharma et al., 2020)
	Anti-inflammatory	Animal model (mice)	40-50 mg/kg seeds	Decreased the expression of inflammatory cytokines including IL-12, IL-1β, and TNF-α	(Dong et al., 2021)
	Hypotensive	Animal model (rats)	2 gm/day seed oil	Decreased blood pressure due to relaxation of vessels	(Kaur et al., 2019)
	Hepato-protective	Animal model (rats)	Seed powder	Decreased liver damage through histopathological examination	(Sharma et al., 2020)
	Anti-cancer activity	Animal model (rats)	Methanolic extract	Inhibit tumor growth	(Sharma et al., 2020)
		In vitro cell lines: breast carcinoma (MCF7) and liver carcinoma (HEPG2)	Seed oil	Significant cytotoxic effect on (MCF7) and (HEPG2) cell lines	(Adnan et al., 2017)

Seed	Pharmacological activity	Type of study	Dose/extract form	Result	Reference
	Antimicrobial activity	*Staphylococcus aureus* and *Escherichia coli*	Seed oil	Inhibition zone of growth of 60%	(Adnan et al., 2017)
Fenugreek (*T. foenum-graecum*)	Anti-obesity	Animal model (rats)	300 and 500 mg/kg alcoholic extract	Decreased lipid profile and body weight	(Gurunath, 2019)
		Animal model (rats)	Aqueous extract	Decreased fat accumulation and improved lipid profile through improving lipid metabolism and decreasing lipase enzyme	(Yao et al., 2020)
		Randomized clinical trial (39 overweigh males)	Aqueous extract	Decreased the fat absorption and prevented lipid accumulation	(Yao et al., 2020)
	Antioxidant	Animal (rats)	Daily intake of seeds	Decreases oxidative stress and decreased LDL oxidation	(Srinivasan, 2019)
		Animal (rats)	Aqueous extract	Decreased lipid peroxidation and increased glutathione and beta-carotene	(Almatroodi et al., 2021)
		In vitro analysis (DPPH assay)	Seed oil	Hight antioxidant percent showing radical scavenging activity	(Almatroodi et al., 2021)
	Anti-diabetic	Animal model (rats)	200 mg/kg aqueous extract	Decreases serum glucose levels	(Alsieni et al. 2021)
		Human (diabetic patients)	Fenugreek gum	Decreased serum glucose and improved insulin resistance	(Yao et al., 2020)
	Anti-inflammatory	Animal model (mice)	0.2 mL/g/day ethanolic extract	Decreased expression of inflammatory cytokines including TNF-α, IL-6, IL-1β, and MCP-1 and decreased infiltration of macrophages in adipose tissues	(Zhou et al., 2020)
		Animal model (induced arthritic rats)	Petroleum ether extract (0.5 ml/kg)	Decreased edema and decreased inflammatory cytokines, also modulating cyclooxygenase enzyme (COX)	(Almatroodi et al., 2021)
	Galactagogue	Human (women)	580 and 610 mg in capsules (3 times per day)	Increased secretion of breast milk in 24-72 hours	(Yao et al., 2020)
	Antimicrobial and antiviral	Animal model (rats)	0.05-1.6 µg/mL	Decreased microbial growth	(Mohamadi et al., 2018)
	Anti-carcinogenic	Animal model (rats) induced breast cancer	200 mg/kg ethanolic extract	Inhibited hyperplasia of mammary glands	(Jhajhria and Kumar, 2016)
		A-549 male lung carcinoma and MCF-7 female breast cancer cell lines	Ethanolic extract	Induced apoptosis and decreased tumor activity	(Jhajhria and Kumar, 2016)
		Animal model (mice)	Seeds powder	Protects against cyclophosphamide-induced apoptosis and lipid peroxidation in the urinary bladder of experimental mice	(Jhajhria and Kumar, 2016)
	Hepatoprotective	Thiamethoxam induced liver injury cell line	Polysaccharide fenugreek extract	Reduced liver toxicity and induced healing	(Yao et al., 2020)
		Animal model (carbon tetrachloride-induced liver injury in rats)	Ethanolic extract	Decreased liver injury through radical scavenging activity	(Almatroodi et al., 2021)
		Animal model (high-fat diet-induced mice)	Seed powder	Decreased liver enzymes (ALT and AST) and improved histopathological analysis	(Almatroodi et al., 2021)

Saponin, 20-hydroxyecoyson, and dietary fiber found in quinoa, as depicted in Table 3, were reported to have an anti-obesity effect. It was reported that saponins in quinoa seeds inhibit triglyceride (TG) accumulation in mature adipocytes (Ellulu et al., 2017). It was also stated that mice treated with quinoa seed extract showed lower levels of inflammation manifested in less inflammatory markers as well as decreased insulin resistance. Quinoa administration may prevent diet-induced obesity and control adipocyte-specific obesity expression of a gene in mice (Foucault et al., 2012).

It is reported that high-risk patient groups will benefit from quinoa, for example, people with diabetes, dyslipidemia, and obesity, due to its high nutritional value characteristics, therapeutic characteristics, and being a material free of gluten. Such characteristics are directly linked to the quinoa seed levels of minerals, fibers, vitamins, fatty acids, antioxidants, and phenolics that significantly impact human nutrition and well-being maintenance conditions (Navruz-Varli and Sanlier, 2016).

According to recent studies, it was found that quinoa seeds have a significant ability to enhance the health conditions related to obesity as well as reduce body weight. A study performed on induced obesity in Wistar rats showed that the test group subjected to quinoa seeds had lower glucose levels and an enhanced lipid profile manifested in decreased levels of LDL, total cholesterol, and triglycerides proving the significant impact of quinoa seeds on obesity (Paśko et al., 2010).

4.2. Chia seeds

Chia (*Salvia hispanica* L.) seeds, as depicted in Figure 2B, belong to the family Lamiaceae. It is an annual herbaceous plant native to Northern Guatemala and Southern Mexico. Its name comes from the word "salvere" in Latin related to the healing properties of the well-known *Salvia officinalis* herb for culinary and medicinal purposes, also called Mexican chia or salba chia. (de Falco et al., 2017).

Chia seeds play a significant role as a functional food and nutritional supplement shown in Table 2. The composition and concentration of their bioactive compounds, as shown in Table 3, depending on several factors: climatic conditions, geographical origin, and extraction methods. Moreover, chia seeds do not produce toxic compounds and are gluten-free (de Falco et al., 2017).

Chia seeds are of high interest nowadays as anti-obesity nutraceuticals. It was reported that chia seed extracts directly relate

to the downregulation of the expression of genes related to lipid synthesis (Chooi et al., 2019).

Chia seeds are rich in dietary fibers, which decrease the sense of hunger and omega-3 necessary for the emulsification and absorption of many essential nutrients, including liposoluble vitamins A, D, E, and K. Moreover, it was reported that chia seeds also improve the metabolism of glucose that could be an excellent alternative strategy to develop successful, safe anti-obesity nutraceuticals for the future (Rubavathi et al., 2019).

According to recent studies, it was stated that chia seeds are rich in α-linolenic acid (ALA), which was believed to be related to decreasing body weight. Test subjects treated with chia seeds showed a significant decrease in body weight compared to those fed with a high-fat diet that suggested that α-linolenic acid decreased lipoid accumulation and improved lipid profile of these subjects. The exact mechanism behind the ability of chia seeds to reduce body weight is still under investigation, but it is considered a promising weight loss herbal nutraceutical (Han et al., 2020).

Table 2. The nutritional profile of phytotherapeutic seeds

	Fibers		Ash		Protein		Fat		Moisture		Reference
Quinoa	14.40	Avg% 17.02	1.58	Avg% 1.56	16.71	Avg% 16.78	5.5	Avg% 5.9	8.35	Avg% 8.225	(Nowak et al., 2016)
	19.651		1.53		16.85		6.3		8.10		
Chia	29.80	Avg% 30.3	4.32	Avg% 4.33	17.51	Avg% 17.405	16.05	Avg% 16.125	4.5	Avg% 4.425	(Kulczyński et al., 2019)
	30.83		4.34		17.3		16.2		4.35		
Fenugreek	10.36	Avg% 10.615	3.43	Avg% 3.45	24.5	Avg% 25.05	6.5	Avg% 6.85	2.77	Avg% 2.89	(Afzal et al., 2016)
	10.87		3.47		25.6		7.2		3.01		
Pumpkin	14.80	Avg% 15.16	4.15	Avg% 4.10	18.5	Avg% 18.195	17.85	Avg% 17.675	5.97	Avg% 6.12	(Syed et al., 2019)
	15.52		4.04		17.89		17.5		6.27		
Hab El-Rashad	29.85	Avg% 30.035	4.31	Avg% 4.34	20.65	Avg% 20.88	17.67	Avg% 18.35	7.86	Avg% 7.91	(Gokavi et al., 2004)
	30.22		4.36		21.10		19.03		7.95		

Chia seeds are a healthy source of fibers, proteins, antioxidants, essential fatty acids, and minerals. These seeds are considered by many to be a "superfood" that contributes to human nutrition and fights obesity by helping to raise the satiety index. Furthermore, chia seeds are considered gluten-free, making them a safe alternative for many food products that increase the obesity risk (Caruso et al., 2018).

Figure 2. Dietary seeds (A); quinoa (*C. quinoa*), (B); chia (*S. hispanica*), (C); Hab El-Rashad (*L. sativum*), (D); pumpkin (*C. pepo*), (E); fenugreek (*T. foenum-graecum*)

4.3. Hab El-Rashad seeds

Hab El-Rashad seeds, as demonstrated in Figure 2C, is originally called *Lepidium sativum* L. and is considered to be edible plant that belongs to cruciferous plants. This species was grown in ancient Greece as a grain. *L. sativum* was a nutritional source for ancient Egyptians who used it as an essential food source long before the invention of bread (Al-Fuhaid, 2018). Hab El-Rashad is also well known as garden cress which is widely used in food industry and in dietetic products (Rafińska et al., 2019).

In Egypt and Saudi Arabia *L. sativum* is widely known with its Arabic name Hab El-Rashad. It is a widely spread medicinal plant cultivated in different temperate climates around the world. It has different names according to the place of origin, such as Garten-Kresse in Germany, Shahi in Iran and Agretto in Italy (Al-Fuhaid, 2018).

Hab El-Rashad seeds are mainly used in traditional medicine to treat asthma, hypertension, hepatotoxicity, and hyperglycemia. It is also known as Haliv in India and has been historically used in the lactating women's diet as well as treating diarrhea and dysentery (Rafińska et al., 2019).

L. sativum seeds has drawn great attention in the recent years for many research aspects, especially its high nutritional content as shown in Table 2 and its biological effect in reducing the risk of obesity and its related health problems. It was reported that the Hab El-Rashad seeds had hypercholesterolemic and hypoglycemic effect which help decreasing the onset of obesity. The exact mechanisms are still under investigation; however, these seeds are promising remedies for treatment of obesity (Abdulmalek et al., 2021).

A recent study revealed that dietary supplementation with Hab El-Rashad seeds powder (*L. sativum*) had a tremendous positive effect on metabolic rate, oxidative stress, and related histopathological injuries manifested in obesity. It was also reported that Hab El-Rashad seeds induced weight loss in test subjects along with the enhancement of the lipid profile through lowering the levels of total cholesterol, triglycerides and LDL in Sprague Dawley rats that were exposed to high-fat ratios nutrition (L'hadj et al., 2019). Several authors attributed this beneficial effect to the phytochemical molecules, especially the flavonoid group which has the mimetic insulin property of improving sensitivity to insulin playing a significant role in enhancing the condition of obesity (Alharbi and Hanan, 2017).

Table 3. Anti-obesity main bioactive compounds in phyto-therapeutic seeds

Seed	Bioactive compound	Structure	Reference
Quinoa (*C. quinoa*)	20-Hydroxyecoyson		(Ellulu et al., 2017)
	Oleanolic acid		(Ellulu et al., 2017)
	Phytolaccagenic acid		(Tang and Tsao, 2017)
	Kaempferol		(Tang and Tsao, 2017)
	Quercetin		(Tang and Tsao, 2017)
Chia (*S. hispanica*)	α-Linolenic acid (ALA)		(Han et al., 2020)

Seed	Bioactive compound	Structure	Reference
	Oleic acid		(Han et al., 2020)
	Chlorogenic acid		(Marcinek and Krejpcio, 2017)
	Caffeic acid		(Marcinek and Krejpcio, 2017)
Hab El-Rashad (*L. sativum*)	Benzyl isothiocyanate (BITC)		(Yokoyama et al., 2020)
	Resveratrol		(Marrelli et al., 2020)
	Apigenin		(L'hadj et al., 2019)
	Quercetin		(L'hadj et al., 2019)
	Naringenin		(L'hadj et al., 2019)

Seed	Bioactive compound	Structure	Reference
Pumpkin (*C. pepo*)	*p*-Hydroxybenzoic acid		(Dotto and Chacha, 2020)
	Coumaric acid		(Dotto and Chacha, 2020)
	Linoleic acid		(Hernández-Pérez et al., 2021)
	Oleic acid		(Hernández-Pérez et al., 2021)
	Trigonelline		(Hernández-Pérez et al., 2021)
Fenugreek (*T. foenum-graecum*)	4-Hydroxyisoleucine		(Zhou et al., 2020)
	Gentianine		(Kandhare et al., 2018)
	Carpaine		(Kandhare et al., 2018)

Seed	Bioactive compound	Structure	Reference
	Diosgenin		(Kandhare et al., 2018)
	Gitogenin		(Kandhare et al., 2018)
	Galactomannan		(Kandhare et al., 2018)

Insulin resistance, type 2 diabetes, dyslipidemia, and cardiovascular disorders are associated with obesity. It is understood that adipokines, adipose tissue markers, and other adipose-derived peptides influence the intake of foods where insulin controls their expression (Achari and Jain, 2017). According to recent studies it was reported that Hab El-Rashad seeds are rich in phenolic compounds and flavonoids that may aid to its anti-obesity action as shown in Table 3 (Elshawwa, 2020).

Hab El-Rashad seeds were found to be rich in benzyl isothiocyanate (BITC) which belongs to the glucosinolates. These are sulfur-containing phytochemicals abundant in various cruciferous crops and edible herbal plants. According to recent studies the elevated level of BITC found in Hab El-Rashad seeds plays an important role in increasing the body libido and balancing the thyroid hormone and consequently increasing the metabolic rate which has a positive effect on obesity (Yokoyama et al., 2020).

4.4. Pumpkin seeds

Pumpkin (*Cucurbita pepo* L.) seeds as shown in *Figure 2D*, belongs to family Cucurbitaceae. They are commonly used edible seeds all over the world, as they are rich in phytosterols, proteins, vitamins and minerals (Ademiluyi et al., 2019). *C. pepo* is widely known as pumpkin, pompom in France and pepon or larger melon in Greece. It was originally cultivated by ancient Americans, and it is also cultivated in many other regions including India, Brazil, Argentina and Mexico (Joebstl et al., 2010).

This plant has been employed in the food industry for the production of purees, juices, jams, and alcoholic beverages. Pumpkin seeds as a rich source of bioactive compounds have been used frequently as functional foods or medicines. Moreover, the pumpkin seed has gained attention not only as an edible seed, but also as a potential nutraceutical supplement (Montesano et al., 2018).

The potential of pumpkin seeds to have anti-obesity effect was recently investigated, where a recent study reported that rats fed on high fat diet along with pumpkin seeds showed improvement in body weight and fat accumulation compared to control group which was fed on only high fat diet. The addition of pumpkin seeds showed a more notable decrease in the gaining of body weight, where the control of body weight gain could be due to a variety of mechanisms, including unusual food assimilation or decreased body energy storage. The exact mechanism is still not known but pumpkin seeds are considered to be a promising anti-obesity nutraceutical (Kalaivani et al., 2018).

According to recent studies the pumpkin seeds were found to be of high interest for the food industry serving healthier food products. The seeds were found to have rich nutritional profile as shown Table 2, including proteins, crude fibers, calcium, carotene, and vitamin C which help in weight reduction. Also, it was reported that pumpkin seeds are gluten free which serves as an alternative for many food products, that help in decreasing the burden of obesity and related health problems especially liver diseases resulting from untreated obesity demonstrated in Table 3 (Mala et al., 2018).

It was reported that the ingestion of natural bioactive compounds with a recognized anti-obesity effect, became of special importance in scientific communities as it can help in replacing other medical interventions owing to its ability to control weight gain and help in weight loss (Ghahremanloo et al., 2018). Based on recent studies it was found that pumpkin seeds chloroform extract has a potent effect on adipogenesis, and downregulation of some genes related to obesity including FABP4, PPARGC1A, CEBPB, respectively (Alshammari and Balakrishnan, 2019).

4.5. Fenugreek seeds

Fenugreek (*Trigonella foenum-graecum* L.) seeds as depicted in Figure 2E, is an annual legume crop mainly grown for use as a spice in many parts of the world. The plant is an aromatic herbaceous annual, widely cultivated in Mediterranean countries and Asia (Rafiqi et al., 2019). Fenugreek is one of the oldest and widely spread seeds used by ancient Egyptians and Hippocrates as an herbal medicine decades ago (Yao et al., 2020).

Fenugreek has many names such as Helbeh in Arabic, Methi, Sag methi and Kasuri methi in Hindi, Fenugreek, Bird foot and Greek hayseed in English and Fieno Greco or Trigonella in Italian. Fenugreek has very rich nutritional constituents as shown in Table 2. Also, it is a rich source of calcium, iron, carotene and other vitamins (Kimbonguila et al., 2019).

According to recent pharmacological studies fenugreek has shown that it contains a number of active ingredients that regulate the metabolism of glycolipids and enhance insulin resistance. In addition, it was found that 4-hydroxyisoleucine shown in Table 3 has a strong impact on lowering insulin resistance and therefore helps in decreasing the obesity in test subjects. However, the exact mechanism is still unclear and further research is needed on this seed to investigate its anti-obesity effect (Zhou et al., 2020).

A number of chemical components are present in fenugreek seeds which are responsible for its biological activity including alkaloids (such as trigonelline, gentianine and carpaine), furastanols (such as vicenin-1, vitexin, diosgenin, gitogenin, yamogenin), essential oils, proteins, steroidal saponins, mucilaginous soluble fiber and insoluble fibers. These constituents help in decreasing serum glucose levels, inflammatory mediators causing minor inflammation associated with obesity and insulin resistance in test subjects. All of this makes the fenugreek seed a promising anti-obesity phytomedicine (Kandhare et al., 2018).

It was reported that fenugreek seeds have a potent efficacy in lowering lipids including low-density lipoprotein (LDL) and cholesterol due to the high fiber content when it was compared to other dietary fibers. There has been a suggested mechanism in decreasing blood cholesterol, where fenugreek gum thickens in the stomach in the presence of moisture and forms a gel. This gel appears like fat inside the body where it gives signals to the brain to send a message to the gall bladder to empty its content of bile in the stomach, where the gel traps the bile. This bile should act as a lipid-emulsifying agent, therefore when it is trapped, it would not perform its function, and this prevents the reabsorption of the lipids. Thus, the emulsification and absorption of lipids, including cholesterol, results in the lowering of blood lipid and this, respectively, reduces the risk of many disorders related to obesity, as well as helping in weight loss (Gurunath, 2019).

5. Molecular anti-obesity effect of the seeds

In the shadow of this review, the molecular processes through which bioactive substances found in herbal extracts, functional foods, or dietary supplements exhibit anti-obesity benefits are addressed. Several studies, including clinical trials, animal tests, *in vitro* cell culture, and *in vivo* alternative model systems, have identified specific bioactive compounds that aid in demolishing obesity as shown in Figure 3.

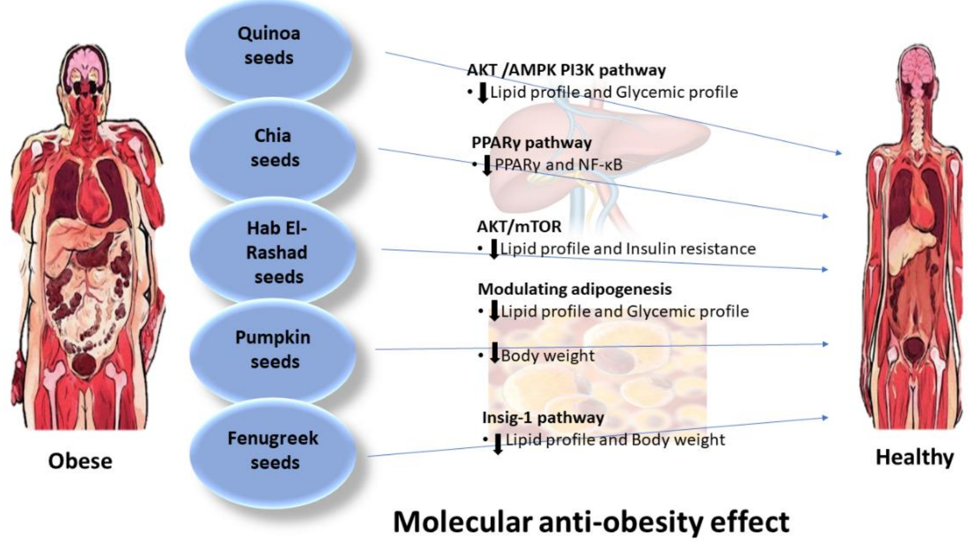

Figure 3. Common pathways involved in the anti-obesity effect of natural products

According to Obaroakpo et al. (2020), quinoa formulated in the form of yogurt showed anti-obesity effect through lowering lipid and glycemic profiles via AKT (protein kinase B)/AMPK (Mitogen-activated protein kinase)/PI3K (phosphatidylinositol 3-kinase) signaling pathway (Obaroakpo et al., 2020). *In vitro* studies on 3T3-L1 adipocytes treated with chia seeds extract showed anti-obesity effect through modulating adipogenesis process via the inhibition of the expression of PPARγ (peroxisome proliferator-activated receptor γ) and NF-κB (nuclear factor kappa light chain enhancer of activated B cells) (Grancieri et al., 2021). Moreover, an *in vivo* study performed on male Wistar rats treated with chia seeds showed decrease in body weight and visceral fat weight through modulating adipogenesis via AMPK (adenosine monophosphate-activated protein kinase) signaling pathway (Oliva et al., 2021). A study performed on obese rats suggested that Hab El-Rashad ethanolic extract showed anti-obesity effect manifested in modulating lipid profile and decreasing insulin resistance along with decreased body weight through regulating AKT (protein kinase B)/mTOR (mammalian target of rapamycin) signaling pathway (Abdulmalek et al., 2021). A study done on Wistar male rats using pumpkin seed oil demonstrated anti-obesity effect through enhanced lipid profile, glucose and insulin levels, and decreased body weight through modulating lipogenesis and adipogenesis process (Kalaivani et al., 2018). Another study was done on Wistar male rats where pumpkin seeds oil was found to decrease total cholesterol, triglycerides and LDL, also decreasing oxidative stress associated with obesity via modulating adipogenesis process (Kalaivani et al., 2020). An *in vivo* study carried out in obesity-induced rats supplemented by fenugreek seeds was found to have anti-obesity effect through modulating lipid profile and decreasing body weight via controlling lipogenesis process through insulin-inducible gene-1 (Insig-1) signaling pathway (Khound et al., 2018).

6. Pharmacological activity of the seeds

6.1. Antioxidant activity

Quinoa seeds are considered to be one of the most important medicinal plants all over the world due to its significant antioxidant activity as it is rich in phenolic compounds including chlorogenic acid and rosmarinic acid, also flavonoids including quercetin and glycosides, which contribute to the antioxidant activity of the seeds (Fernández-López et al., 2020). Phenolic acids and flavonoids are the most abundant polyphenols in quinoa responsible for its antioxidant effect. Several phenolic acids have been reported in quinoa seeds samples including protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, *p*-coumaric, ferulic, sinapic and isoferulic, cholorogenic, rosmarinic and caffeic acid, where ferulic acid and its derivatives were the most abundant phenolic compounds in quinoa seeds (Stikić et al., 2020). Phenolic compounds found in quinoa seeds had a significant ability to scavenge the 2,2-diphenyl-1-picrilhidrazil (DPPH) (Liu et al., 2020). The antioxidant effect of compounds containing phenols is due to the existence of a hydroxyl group connected to an aromatic ring which, through its inhibitory capacity of reactive oxygen species, can provide a defensive barrier against oxidative stress (ROS) which alter many biological actions of the body leading to many diseases including obesity, diabetes, cardiovascular diseases and cancer. Therefore, quinoa seeds were found to have a significant effect as antioxidants to prevent that cascade of events leading to severe illness (Buitrago et al., 2019).

Also, chia seeds were reported to have a potent antioxidant effect. It was shown that ingestion of chia seeds extract decreased oxidative stress that was manifested in reducing MDA (malondialdehyde) levels and increasing GSH (glutathione) levels indicating a potent antioxidant ability (Tamargo et al., 2020). This is linked to the presence of high levels of antioxidant nutrients such as chlorogenic acid, caffeic acid, myricetin, quercetin, kaempferol and omega-3. These nutrients aid in decreasing the oxidative stress and activating hepatic antioxidant enzymes leading to reduction of lipid peroxidation (Mukthamba and Srinivasan, 2016). Chia seeds are considered to be one of the most important antioxidant herbal nutraceuticals, since they represent a very good source of polyphenols and antioxidants such as caffeic acid, rosmarinic acid, myricetin and quercetin. The advantages of chia seeds being a potent antioxidant, have been explored in various fields of study worldwide including the medical, pharmaceutical and food industry. They have shown a great help in decreasing and delaying the onset of many diseases including obesity and related health disorder, aging, cancer and inflammatory diseases (Knez Hrnčič et al., 2020).

It was reported that Hab El-Rashad seeds have potent antioxidant effect where they showed a potential in being a hepatoprotective agent against oxidative stress. Studies revealed that this action might be related to the high content of linolenic acid and phytosterols along with phenolic compounds in the seeds (Alqahtani et al., 2019). A related analysis discovered that providing Wistar rat with Hab El-Rashad seeds for 60 days, increased tocopherol levels and the activity of antioxidant enzymes proving that Hab El-Rashad seeds are a promising herbal antioxidant agent (Umesha and Naidu, 2015). The plant's seeds and leaves have volatile oils and are a good source of amino acids, fatty acids, and minerals. Owing to their high content of phenolic compounds, they have the ability to act *in vitro* as antioxidants, and thus they may have significant preventive effects against chronic disorders (Balgoon, 2019). A recent study showed great potential in Hab El-Rashad seeds being a potent antioxidant, where the levels of glutathione (GSH) and catalase levels were elevated on administration of the seed extract which decreased the lipid peroxidation level and acted as a shield against tissue damage by oxidative stress (Feng et al., 2016).

It was stated that the serviceability of the pumpkin lies in its functionality, containing variant constituents, each of which has an excellent nutritional composition, which can be used in food formulations and is functional in the treatment and prevention of many illnesses related to high oxidative stress, where pumpkin seeds were reported to be a promising anti-oxidant agent due to the presence of triterpenoids, flavonoids, coumarins, cucurbitacins, and carotenoids which were reported to have a direct relation with the antioxidant activity of pumpkin seeds (Sharma et al., 2020). Based on various research works underlying the high levels of polyphenolic compounds in pumpkin ethanolic extract, pumpkin seeds are regarded as a functional food. Species of *Cucurbita* contain numerous nutrients that are important for human wellbeing, including carotenoids and tocopherols present mainly in peels and seed fractions, respectively (Achilonu et al., 2018).

Based on recent studies, it is understood that dietary fenugreek seed counters surplus lipid peroxidation and induces changes in the content of antioxidant molecules in test subjects. It was also reported that an antioxidant property is correlated to the soluble portion of fenugreek seeds. Administration of fenugreek to test subjects reversed the altered levels of antioxidants and antioxidant enzymes behavior indicating that fenugreek seeds have a positive antioxidant activity that can be used to treat many health complications related to the presence of high oxidative stress in body (Srinivasan, 2019). Latest biological studies have shown that fenugreek has antioxidant associated with anti-inflammatory activities which are directly related to the presence of bioactive phytochemicals including flavonoids which were reported to have a potent antioxidant activity through their radical scavenging effect and LDL-antioxidant properties. Moreover, fenugreek seeds rich in glycosides decreased the production of nitric oxide (NO) which increases oxidative stress in cells (Wu et al., 2020).

6.2. Anti-diabetic activity

According to recent studies quinoa, a pseudo cereal free of gluten, high in nutrients, fibers and phytochemicals, including saponins, phytosterols, phytoecdysteroids, phenolics and bioactive peptides, may be a key solution for dietary intervention to combat obesity, metabolic syndrome, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD). This diversity in combination of phytochemicals makes quinoa one of the greatest phenolic compounds associated with anti-diabetic activity (Noratto et al., 2019). It was stated that quinoa intake decreased the glucose levels and enhanced insulin resistance in test subjects which by far had a significant role in diabetic treatment (Paśko et al., 2010). Furthermore, human studies have also shown that the ingestion of quinoa lowered the levels of BMI and glycosylated hemoglobin (HbA1c) and improved satiation and fullness in patients with pre-diabetic condition (Ruiz et al., 2017).

Regarding chia seeds, they are a rich source of α-linolenic acid (ALA) which was found to decrease insulin resistance in type 2 diabetes and the related health conditions. It also decreased the serum glucose level, total cholesterol and triglycerides levels, aiding in the treatment of diabetes, and also delaying the onset of diabetes in borderline test subjects (Akinfenwa et al., 2020). Also, chia seeds have been the subject of recent research due to their promising chemical composition and nutritional value; they contain high amounts of dietary fibers, minerals, proteins, and essential fatty

acids such as linolenic acid. Chia seeds have recently been described as the best source of fatty acids and omega-3. They also have a high content of bioactive ingredients, such as phenolic compounds and tocopherols, which have an anti-diabetic effect (Scapin et al., 2016). Some experiments on rats have shown that six and 12 weeks of consumption of chia seeds can promote health benefits, such as decreased glucose levels, however, the exact mechanism is still unknown (Alamri, 2019).

According to recent studies, in a high fat diet rodent model, Hab El-Rashad seed powder and alcoholic extracts showed a cytoprotective action in pancreatic islets to maintain the integrity of the β cell which control adequate insulin secretion and maintain glucose levels to normal in test subjects (Desai et al., 2017). Furthermore, Hab El-Rashad seeds were reported to contain flavonoids, sulfur, coumarin, triterpenes, sterols, glycosides, and various imidazole alkaloids, where alkaloids exert its action through alpha-glucosidase inhibition and reduce transporting glucose through the intestinal epithelium (Roughani and Miri, 2018). It was reported that Hab El-Rashad seeds ethanolic extract stabilize diabetes through enhancing antioxidant effect, decreasing oxidative stress and improving lipid profile, also regulating insulin pancreatic secretion from the pancreatic islet β cells which is related to decreased levels of glucose, urea, triglycerides, and cholesterol (Jain and Grover, 2018).

Pumpkin seeds were reported to have anti-diabetic effect due to their hypoglycemic and hypolipidemic activity. According to recent studies it was found that in diabetic rats, cellular insulin uptake was increased demonstrating that the capacity of cells to utilize insulin and glucose was increased significantly. It was also reported that pumpkin seeds alcoholic extracts showed a potential in α-amylase inhibition, and aqueous extracts showed significant inhibition of α-amylase and α-glucosidase which are directly linked to the anti-diabetic effect that was recently taken in consideration for pumpkin seeds (Kushawaha et al., 2017). According to recent studies, the use of natural products is increasing in the field of diabetes including the using of pumpkin seeds extracts in the past few years, due to the presence of polysaccharides which were found to be highly active in combating blood sugar content. The polysaccharides were evaluated for their mechanism of action where it was found that pumpkin polysaccharides can stimulate endogenous GLP-1 secretion, decrease oxidative damage, and delay the onset of diabetes. These are considered to be the key steps that explain the anti-diabetic mechanism of pumpkin polysaccharides (Lu et al., 2019).

Fenugreek seeds were also found to have hypoglycemic effect due to a variety of constituents including galactomannan, phenolic compounds and fibers. Clinical studies have shown that 2 to 3 g/day of fenugreek gum is effective in controlling blood sugar, whereas the requirement of other food fibers is much larger (~ 20 g). When ingested, the fenugreek gum thickens and forms gel trapping sugars, fats and starch-hydrolyzing amylase enzymes in the stomach; thus, leading to the slowing down of the sugar absorption and that is of high benefit for diabetic and obese patients (Zameer et al., 2018). According to updated studies it was found that the soluble fiber content including galactomannan was stated to be responsible for the anti-diabetic activity of the fenugreek seeds and clinical data showed that the glycemic control was improved in a small study of mild type-2 diabetes mellitus patients. A reduction in glycosylated hemoglobin (HbA1c) levels and an increased insulin sensitivity was observed in patients receiving fenugreek (Srinivasan, 2019). It was also reported that the hypoglycemic effect was demonstrated in a study that fenugreek inhibited in vitro α-amylase activity in a dose dependent manner and that it was found to be effective and considered of high potential as anti-diabetic agent (Wu et al., 2020).

6.3. Anti-inflammatory activity

Regular consumption of quinoa seeds was reported to have a potent anti-inflammatory effect that takes place by lowering the levels of circulating inflammatory mediators including interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α. This is due to the high fiber content found in quinoa seeds (Noratto et al., 2019). The anti-inflammatory effect of quinoa seeds is directly related to being a potent antioxidant, which is manifested by the high levels of phenolic compounds where, two major groups are classified as phenolic acids and polyphenols (Carciochi et al., 2014). Inflammation is complementary to chronic diseases associated with obesity and dietary changes have shown that inflammation has decreased, with implications for slowing or preventing the onset of certain diseases. The overproduction of ROS in obesity promotes cell injury, secretion of pro-inflammatory cytokines and their development, consequently contributing to inflammation-signaling pathways. Quinoa seeds were reported to decrease chronic inflammation associated with obesity and related health problems through decreasing the oxidative stress and so decreasing the inflammatory mediators onset leading to minor inflammation (Garcia-Mazcorro et al., 2018).

According to recent studies, it was reported that chia seeds exert anti-inflammatory effect that could treat minor inflammation associated with obesity. The seed extracts inhibit the macrophages recruited by adipocytes that aggravate immune response and decrease the expression of MCP-1 (monocyte chemo attractant protein-1), PGE2 (prostaglandins E2), IL-6 (interleukin-6), PAI-1 (plasminogen activator inhibitor-1) and TNF-α (tumor necrosis factor-α) and so it was reported to have a great potential in treating minor inflammation associated with obesity (Marcinek and Krejpcio, 2017). It was also found that chia seeds intake has been able to inhibit NF-κB and TNF-α activation, reducing inflammatory cytokines such as TNF-α, leading to enhanced anti-inflammatory body ability in the normal diet and high-fat diet, resulting in a low-grade inflammatory state. This may be due to the fatty acids, phenolic acids, and other bioactive compounds that are found in chia seeds, including vitamins, minerals, and antioxidant substances. The bioactive compounds in the seeds results in upregulation of PPAR-α expression which control variant gene expression, and cell signaling pathways, reducing inflammation in that way (da Silva et al., 2019).

Hab El-Rashad seeds are used in folk medicine to treat some inflammatory diseases including hepatitis, diabetes mellitus and arthritis (Alqahtani et al., 2019). According to recent studies it was reported that Hab El-Rashad seeds have shown synergistic effects of platelet aggregation inhibition and decrease thromboxane B2 levels in Wistar rats' spleen and lung tissues decreasing the accumulation of inflammatory mediators (Raghavendra and Akhilender Naidu, 2011; Alqahtani et al., 2019). According to recent studies (Akbar, 2020) evaluating the anti-inflammatory effect of Hab-El-Rashad seeds extract on inflammatory shock it was suggested that the presence of lipopolysaccharides (LPS) aggravated an immune response manifested in releasing inflammatory mediators released by macrophages, especially tumor necrosis factor alpha (TNF-α) which cause fever, shock, and could lead to death if not treated. Hab El-Rashad seeds were found to decrease the levels of circulating TNF-α leading to reducing the inflammation effect on the human body. Further research is required to reveal the exact mechanism for more clinical implications (Ahmad et al., 2018).

It was stated that pumpkin seeds extracts possess a potent anti-inflammatory action where 3β-hydroxycholest-7-en-24-one extracted from pumpkin seeds had a direct effect on inhibition of nitric oxide (NO) production and decreased inflammatory macrophages circulation through inhibiting the activation of macrophages, decreasing thereby the onset of inflammation. Accordingly, they can be considered promising anti-inflammatory agents (Ratnam et al., 2017). The anti-inflammatory function of pumpkin seeds was evaluated where the administration of low and high doses of pumpkin seed extracts of various origins showed that different cellular markers such as DNA damage and chromosomal damage as well as inflammation regulation index improved significantly in relation to normal subjects. The lipoxygenase inhibitory activities of pumpkin seed extracts may partly explain this fact (Al-Okbi et al., 2017).

Recent studies showed that fenugreek seeds contained diosgenin which played an important role in inflammation linked to obesity, where it inhibits filtration of macrophages of adipose tissues and decrease inflammatory mediators and cytokines produced in obese test subjects. Further studies are needed to investigate the exact mechanism where diosgenin extracted from fenugreek seeds exerts its anti-inflammatory effect (Zhou et al., 2020). Moreover, fenugreek seeds were reported to exert anti-inflammatory effect through inhibition of the inflammatory biomarkers including TNF-α, IL1β, LT-B4. It was also suggested that it inhibits macrophages and pro-inflammatory cytokines along with decreasing the oxidative stress leading to decreased inflammatory response. The exact mechanism is still under investigation, but fenugreek seeds are considered to be a promising nutraceutical as an anti-inflammatory herbal medicine (Tavakoly et al., 2018).

6.4. Effect on appetite

It was recently reported that quinoa delayed the weight gain in test subjects fed quinoa along with high caloric intake diet through suppressing appetite. The exact mechanism is still not clear, but it was suggested that quinoa boosts satiety and fullness. Studies showed high potential in quinoa seeds as an appetite suppressor (Noratto et al., 2019).

According to recent studies, chia seeds were also found to reduce appetite in a significant way and so it's a promising appetite suppressor that can be used as a safe herbal nutraceutical supplement (Shende and Narvenker, 2020).

Pumpkin seeds were reported to have a potent effect on digestive disorders, stabilizing the appetite and decreasing sugar craving, which also plays a significant role in enhancing glycemic profile and modulating insulin resistance in test subjects (Rajasree et al., 2016).

Recent studies suggested that high content of soluble dietary fibers found in fenugreek seeds lowered the level of blood sugar in diabetic test subjects by postponing carbohydrate gastric emptying leading to appetite suppression for longer duration of time (Yao et al., 2020).

7. Edible seeds in anti-obesity supplements

Nowadays obesity is a high-risk chronic disease, which encouraged the Food and Drug Administration (FDA) to approve many drugs and supplements of natural products to help in decreasing the risk which became a threat to a lot of people. Pharmacotherapy treatments for obesity is of high importance to reduce the risk of many chronic diseases, improve the lifestyle of patients suffering from obesity, and decrease the need for the bariatric surgeries (Gomez, 2017).

The health benefits of human nutrition from quinoa seeds have been extensively documented, such that this crop is now indicated as an important herbal dietetic to produce functional foods and nutraceutical products. It is reported that several food-derived molecules, including proteins and peptides, can show bioactivities and disease prevention in humans and enhancing the quality of life (Capraro et al., 2020).

Chia seeds are a healthy source of fiber, protein, antioxidants, and essential fatty acids, proteins, and minerals. These seeds, considered by many to be a "superfood" that contributes to human nutrition and fights obesity by helping to raise the satiety index. They also have many biological characteristics, and are considered to be gluten free which can be a safe alternative for many food products that increase the obesity risk (Caruso et al., 2018).

A recent study revealed that dietary supplementation with Hab El-Rashad seeds powder (*L. sativum*) had a tremendous positive effect on metabolic rate, oxidative stress, and related histopathological injuries manifested in obesity. It was also reported that Hab El-Rashad seeds induced weight loss in test subjects along with enhancement of the lipid profile through lowering the levels of total cholesterol, triglycerides and LDL in Sprague Dawley rats exposed to high-fat ratios nutrition (L'hadj et al., 2019). This beneficial effect has been attributed by several authors to phytochemical molecules, especially the flavonoid group which has the mimetic insulin property of improving sensitivity to insulin which had a significant role in enhancing the condition of obesity making it a promising weight loss supplement (Alharbi and Hanan, 2017).

It was reported that the ingestion of natural bioactive compounds with a recognized anti-obesity effect, such as pumpkin seed extract became of high interest as it can help in replacing other medical interventions owing to its ability to control weight gain and help in weight loss (Ghahremanloo et al., 2018). Based on recent studies it was found that pumpkin seeds chloroform extract has a potent effect on adipogenesis, and downregulation of some genes related to obesity including FABP4, PPARGC1A, CEBPB, respectively (Alshammari and Balakrishnan, 2019). Moreover, this plant has been employed in the food industry for the production of purees, juices, jams, and alcoholic beverages. Pumpkin seeds as a rich source of bioactive compounds have been used frequently as functional foods or medicines. Moreover, pumpkin seed has gained attention not only as edible seed, but also as a potential nutraceutical supplement (Montesano et al., 2018).

Fenugreek seeds were reported to be one of the most used seeds all over the world, due to the rich nutritional content and various phytochemical active compounds. The seeds are used as dietary supplement as it has shown to promote lean body mass and decrease cholesterol in recent research. Moreover, enhance digestion, glycemic profile and lipid profile. The seeds have high potential as functional food to be also a weight loss agent (Yao et al., 2020).

8. Role of dietary seeds in food industry

Quinoa seeds are abundant in fiber, which adds to the product's health advantages. Furthermore, quinoa's overall nutritional content has aided in increasing demand in the global market. Quinoa rice and flour recently replaced the traditional white rice and flour, also quinoa pasta is used recently replacing the pasta

done with white flour, it is also used in bakery and cakes owing to the nutritional content of the quinoa seed, offering a better and healthier option that can be used in daily diet. Moreover, quinoa seeds are rich in saponins which can be utilized in a variety of commercial applications in the agricultural (e.g., as a bio-insecticide), food, cosmetic, and pharmaceutical sectors due to their physicochemical and biological characteristics (Angeli et al., 2020).

Chia seeds is used in the market in many categories including food, beverage, bakery, morning cereals, soups, and gravies. The seeds provide a healthier and gluten free option for many bakeries and cereals in the market. Moreover, chia seeds have a high concentration of gum and mucilage, making them potentially helpful in the food and cosmetics business. Bread, spaghetti, cookies, and cakes are just a few of the goods that contain chia seeds in them. Chia seeds can also be added to drinks, snacks, and other foods (Katunzi-Kilewela et al., 2021). A lot of interest is invested in the incorporation of the seeds in food industry owing to its high nutritional value and health benefits.

Hab El-Rashad is used in many food products especially in Saudi Arabia, it is used in cereals, bread, cookies, muffins, and noodles as a healthier option. Hab El-Rashad seeds may be utilized to make a variety of functional and nutritionally enhanced foods including dahiwala bread, omega-3-fatty acid-rich biscuits, iron-rich biscuits, health drinks, vegetable oils mixed with alpha-linoleic acid-rich Hab El-Rashad oil, fortified burfi, and fortified chikki. Moreover, Hab El-Rashad oil is used as a native medicine where it is used in relieving inflammation and pain (Lahiri and Rani, 2020).

Pumpkin seeds are popular to be used as a healthy snack, unsalted seeds are used as healthier option as a topping in many bakeries and used with oats to make healthy and nutritionally rich snacks. The seeds in raw or roasted form are used in cakes, breads, cereals, and salad dressings, while seed oil is used as a cooking oil. Also, pumpkin seed oil may be used in a variety of dishes, including pasta, salads, and soups. Moreover, the seeds powder is used in cookies preparation for a healthier recipe (Adsul and Madkaikar, 2021).

Fenugreek is widely used as a spice in many food products, whereas its seeds are used in many bakery products including bread, cookies and cakes. Also, it is used as an infusion powder or added to black and green tea. Moreover, seed powder is used in capsules to increase breast milk (Yao et al., 2020).

9. Technological perspective and future trends

Dietary seeds rich in phytochemicals show great promise as a natural source of nutrients and additives for contributing to modern diets. Moreover, seeds have a promising role in the production of functional foods and nutraceuticals in the forthcoming years. The nutritious and bioactive components have the potential to be used in food, cosmetics, and pharmaceutical manufacturing. Nowadays, many technologies are utilized for extraction of bioactive compounds from the seeds, also the seeds are used in different forms such as flour and seed powder to be incorporated in dietary supplements and food industry owing to the fact that these seeds are rich in bioactive compounds and nutritional components (Akbar, 2020). Seeds include a variety of components, including proteins, omega 3 fatty acids, dietary fiber, minerals, flavonoids, and polyphenols, all of which are appealing to the food industry and customers seeking healthy meals.

10. Conclusions

In conclusion, alternative medicine and dietary supplements are required to address the most pressing issues linked with obesity. In the past years dietary seeds have been used widely against the battle of many chronic diseases including obesity and related diseases. There is growing evidence everyday about the usage of dietary seeds and natural products either in food industries or in drugs and supplement products.

Food industries around the world is now moving towards more healthy options and widely using medicinal plants in the daily diet along with food supplements to help decrease the worldwide catastrophe of obesity. These seeds showed a promising potential to be used as a weight reducing nutraceutical. The exact mechanism by which these seeds exert their action against obesity is still not fully discovered, therefore, further research and clinical trials should be conducted to assess their efficiency and mode of action.

This review contributes to a deeper understanding of the cellular and molecular processes that mediate the anti-obesity impact of some dietary seeds used nowadays. Furthermore, it prompts understandable knowledge to practitioners, and consumers who are seeking safe and economical natural product with anti-obesity effect. Seeds have been shown to have potent pharmacological effects with little or no toxicity, and their intake as a dietary supplement appears to lower the risk of many human health problems. Taken together, the suggested optimum strategy to achieve long-term loss in body weight seems to be the use of potential anti-obesity natural products and to be incorporated into the daily low-fat diet.

Acknowledgments

None.

Conflict of interest

The authors declare that they have no conflict of interests.

CRediT authorship contribution statement

Heba Hosny: Data analysis, Manuscript writing, Revision
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Supplementary File

None.

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Reviewed by:

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INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS
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RESEARCH ARTICLE OPEN ACCESS

Extraction of bioactive compound from *Acacia seyal* gum, *in vitro* evaluation of antitumor activity of its crude extract against leukemia

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ARTICLE INFO

Article History:

Received: 03 January 2022
Revised: 20 January 2022
Accepted: 21 January 2022
Available online: 24 January 2022

Edited by: E.S. Istifli

Keywords:
Acacia seyal gum
Sonication
FTIR spectroscopy
Raman spectroscopy
Anticancer

ABSTRACT

Today, many therapy drugs have been used to treat cancer patients. However, those drugs are not effective enough and usually have adverse side effects on human health. Different herbal medicine is rising in popularity because it is more compatible with the human body and has fewer side effects. Even while alternative herbal remedies effectively decrease symptoms in traditional medicine, many of them have yet to be scientifically proven. As a result, it's critical to keep looking for ways to recover its efficiency against cancer cells. *Acacia seyal* gum (ASG), known as Arabic gum, is a well-known traditional medicinal therapy with various restorative characteristics. In this study, the yield of ASG extract was optimized using experimental design followed by chemical characterization of a bioactive compound for the last yield, then the therapeutic potential of ASG crude extracts against leukemia cancer cells was investigated *in vitro*. The Raman Spectroscopy (RS), Fourier Transform Infrared (FTIR) Spectroscopy, and GC-TOFMS analyses were used to characterize ASG crude hydroethanolic extract bioactive components. The anti-leukemic activity of ASG crude extracts was investigated *in vitro* against tumoral Jurkat T-cell ALL, and K562 leukemia cancer cell lines, as well as nontumoral WIL2NS cells. The optimum extraction conditions resulted in a yield of 75.89% after 45 min of extraction at temperature 40 °C and solid/liquid ratio of 1:25 g/ml. The cytotoxicity assays of ASG and Taxol revealed that both treatments inhibited the growth of K562 and Jurkat T cancer cells and exhibited the lowest IC_{50} for K562 and Jurkat T cancer cell (IC_{50}=10 g/ml and IC_{50}=5.11 g/ml, respectively), and a negligible inhibition effect for WIL2NS cells (IC_{50}=80 g/ml).
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1. Introduction

Leukemia is a cancerous tissue-forming disease that triggers the excessive production of immature blood cells entering the bloodstream (Azher and Shiggaon, 2013). Leukemia cell proliferation occurs primarily in the bone marrow, with several forms in the lymphoid tissue (Naumburg et al., 2002). Leukemia has been recognized since the publication of reports of patients who died of the disease in 1845 and had elevated blood cells (Naumburg et al., 2002). According to the World Health Organization (WHO), cancer deaths will increase by 104% worldwide by 2020 (Dutta et al., 2019).

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e-ISSN: 2791-7509
doi:
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Currently, leukemia is among the most prevalent types of cancer in various parts of the world. In the US, leukemia may cause mortality of an estimated 58.100 people in 2018, and the type of cancer suffered among adolescents and young adults below 20 years in the same country is leukemia (Collins, 1987). In Malaysia, the incidence of lymphocytic leukemia in both men and women was 2.8 and 1.7 per 100.000 population, respectively, while the incidence of myeloid leukemia in both men and women was per 100.000, respectively, it was 3.0 and 2.7 (Saedi et al., 2014).

Patients with leukemia are treated with combination therapies such as chemotherapy (primary treatment), antibiotics, blood transfusions, radiation therapy, and bone marrow transplantation. While these treatments have helped increase the survival rate of leukemia patients, some of these treatments are difficult to practice. Thus, there is a need to look for alternative remedies to cure leukemia. Therefore, the use of natural products has increased

Please cite this article as: Kabbashi, N., Alawdat, T.M., Qudsieh, I.Y., Alam, M.Z., Shahbuddin, M., 2022. Extraction of bio active compound from Acacia seyal gum, in vitro evaluation of antitumor activity of its crude extract against leukemia. International Journal of Plant Based Pharmaceuticals, 2(1), 82-88.

gradually to discover a new antitumor drug that has few side effects on the immune system and is an important target in many immunopharmacological studies (Xu et al., 2017).

Hence, these natural plant products should be studied to understand their properties, protection, and effectiveness. It would result in reduced use of most common therapies for cancer such as radiation therapy, surgery, and chemotherapy. Some cancer cells showed resistance to chemotherapy treatment; as a researcher, we need to discover new cytotoxic drugs that function through distinctive mechanisms.

In developing countries, using therapeutic herbs as cures for leukemia is gaining popularity. About 35.000 plant samples were collected by National Cancer Institute from 20 countries, and around 114.000 extracts were screened for anticancer activity (Prakash et al., 2013).

Plants are known to be one of the most important sources of bioactive substances. Various modules of studies showed that constituents of plants demonstrated various biological and pharmacological activities. For thousands of years, plants were utilized to treat various diseases. Different plant part like leaves, barks, fruits, leaves due to their bioactive compounds acted as anticancer following different mechanism of action like an inhibitor of carcinogen formation, blockers of carcinogen interaction, and suppressor of tumor progression according to the chemical compound in the plant (Saedi et al., 2014).

In Australia, northern tropical Africa, and Egypt (Awad et al., 2018), *Acacia seyal* gum (ASG) is popular due to its medicinal use and is known as the second most important source of Arabic gum product (Nie et al., 2013) (Figure 1) after *Acacia senegal*. Numerous studies showed that constituents of ASG have various biological and pharmacological activities where it has a lot of dietary fibers and polyphenolic compounds, which can improve human health due to its antioxidant activity (Elnour et al., 2018b). It was used in traditional medicine as an astringent against colds, diarrhea, hemorrhage, ophthalmia, bronchitis, rheumatism, leprosy, also used against cancer due to its powerful anticarcinogenic effect on specific cancer cell lines (Ahmed, 2018). Much research has been found that ASG has been found in many applications in medicine and healthcare due to the increased quantities of alkaloids, flavonoids, glycosides, and saponins found in the ASG. The traditional practice and medical application of using bark juice to cure wounds were observed (Mbaveng et al., 2014; Suriyamoorthy et al., 2014). The extracts of ASG have been reported to have other different pharmacologic effects like antipyretic, anti-inflammatory, anti-diarrheal, hypoglycemic, hepatoprotective, antioxidant, and antimicrobial activities.

Figure 1. *Acacia seyal* gum powder

Recently, consumption of ASG increased following the discovery of phytochemical constituents such as phenolic compounds and flavonoids that are associated with a variety of pharmacological activities (Elmi et al., 2020). At present, more attention is being focused on the anticancer properties of ASG, and they tested its cytotoxicity against various kinds of cancer cell lines.

Acacia seyal (Del.) tree belongs to the family Mimosaceae of the genus *Acacia* and is usually distributed in tropical Africa, mainly found in West Africa, East Africa, and the Arabian Peninsula (Awad et al., 2018). *A. seyal* tree grows up to 17 m tall and 60 cm in diameter, with a flat top crown. Like most Acacias, it grows vigorously, coppice readily, and withstand heavy browsing. The leafy branches can be cut for fodder within the growing season without significant damage to tree performance (El Mahi and Magid, 2014).

It has a characteristic smooth powdery rind that varies from white to greenish-yellow or orange-red, with a layer of green beneath it. Some populations have both red and yellow bark trees. The gum is used for biotechnology research and the food industry because of its clarity and solubility. The bark leaves and gums of the *A. seyal* tree are used for many medicinal purposes, for example, as a pharmaceutical ingredient in medication for throat and stomach inflammation and as a film-forming agent in peel-off skin masks, colds, hemorrhage, jaundice, headache, burns and chronic renal failure (Glover, 2012). However, exposure to smoke is believed to relieve rheumatic pains; the bark is used against leprosy, skin lesions, and dysentery, is a stimulant, and acts as a laxative for humans and animals.

Various chemical compounds have been isolated and characterized from ASG like flavonoids, particularly flavonols quercetin which bark showed cytotoxic activity *in vitro* (Murakami et al., 2008). ASG has been primarily composed of complex polysaccharides and contains a small amount of protein. Traditional African system medicine is used to treat many illnesses such as infertility, skin diseases, and cancer.

2. Materials and methods

Extraction using the Ultrasound-Assisted Extraction (UAE) method of the bioactive compounds from ASG and optimizing the condition of extraction to get high yield to depend on a Face-Centred Central Composited Design (FCCCD) under Response Surface Methodology (RSM) following the method of Fan et al.(2020). Next, the qualitative identification of the most bioactive compounds available in ASG was carried out using Raman Spectroscopy, FTIR Spectroscopy, and GC-TOFMS analysis. Finally, the crude extract was tested *in vitro* for antiproliferative effects against WIL2NS, K562, and Jurkat -T human leukemia cell lines using the MTT assay, with Taxol as a positive control.

2.1. Sample preparation, gum extraction

ASG nodules were taken from Blue Nile State, Sudan. The samples were cleaned from impurities such as bark and sand. For homogeneity of the sample, the ASG nodule was selected randomly from the other nodules. Then it was ground to powder and sieved by U.S.A standard testing sieve (Fisher Scientific, Massachusetts, USA) with a 1.40 mm mesh size. The finished ASG powder was packed and stored in a polyethylene ziplock bag at 4 °C until further analysis. Sampling was performed only one time.

The bioactive metabolite extraction from plant materials was performed according to the method of Adwan et al. (2010) and Esmaeili et al. (2021) with some adjustments. The optimum extraction parameters were systematically examined in this study. The gum powder samples (3 g) were mixed with 30 ml of 60% ethanol (v/v) (ethanol was diluted with distilled water), *n*-hexane, and acetone mixed in a 200 ml beaker for OFAT experimental design (Table 1), then covered with aluminum foil and sonicate using 40 kHz, power level 5, at room temperature as a standard parameter. The presence of different organic solvents was attempted to assess their capability to get the best yield of extract from ASG solvents, including acetone, 60% ethanol, and hexane. The extraction process followed the standard protocol.

2.2. Ultrasound-Assisted Extraction (UAE) optimization

The experimental design was intended to study and observe the effects of the extraction protocol, extraction procedure on ASG extraction yield (%), and the content of bioactive compounds obtained. During the preliminary test, it was found that in addition to the type of extraction solvent, several factors such as extraction temperature, time, and solid-liquid ratio influence ASG extraction (Norshazila et al., 2017). All experiments were performed 3 times. Extraction yield (%) was defined as reaction (Y).

Table 1. Experimental design and levels of independent process variables

Symbol	Independent variables	Low level	High level
A	Extraction time (min)	30.00	60.00
B	Extraction temp (°C)	30.00	50.00
C	Solid-liquid ratio (gm/l)	15.00	25.00

2.3. Statistical optimization of experiments

The design of the experiment has been applied to improve the quality of the extraction processes and the extract yield. Additionally, to make these products healthy to extreme conditions. The application of statistical optimization experiments using various factors related to the manufacturing process is well documented. The RSM is employed to significantly improve extraction processes and the extract (Pandey et al., 2018). These procedures were performed for analyzing different factors at once. We also reduced the number of related experiments, improved the interpretation of the data, and reduced the time required for the experiments. In this study, Expert Design Software v.12.0.0 (Stat-Ease Inc., USA) proved remarkably useful in establishing the optimal ASG extraction parameters that optimize the extraction yield.

A traditional One-Factor Data Time (OFAT) test design was used to determine the optimal solvent for maximizing the yield of ASG extract. The earlier literature showed that the solvent used for extraction affects extraction yield, phytochemical contents, and other factors like extraction time, temperature, and solid-liquid ratio (Abolmaesoomi et al., 2019; Che Sulaiman et al., 2017).

The design of experiment findings, which was determined from the FCCCD, contains 20 runs of experiments; each experiment was performed 3 times. Answer (Y) was the percentage of extraction yield. Analysis of variance (ANOVA) was evaluated by performing statistical analysis of the model. The relationship between the response (dependent factor) and the experimental level of each variable in this study was described from the viewpoint of contour lines and response surface graph, and an approximate polynomial was confirmed.

2.4. Preparation of solutions and reagents

Due to the possibility of contamination from reagents and materials, all apparatus was sterilized at 121 °C and 15 psi for 15-20 minutes using the autoclave method. This effect was important because the cell culture was naturally insensitive to contamination. Cell lines were maintained in complete cell culture or growth medium RPMI 1640 (Sigma Aldrich, USA). The medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were cultured at 37 °C. under 5% CO_2 conditions in a cell culture laboratory at IIUM's Department of Biotechnology Engineering, Malaysia. The culture medium with 10 % (v/v) FBS was freshly prepared to ensure sterility and extend the medium's shelf life. Firstly, FBS was filtered inside the biosafety cabinet using a 0.45 µm filter to ensure sterility. Then 100 ml of FBS (GIBCO, USA) was added to 900 ml of cell culture medium, and the solution was mixed gently to avoid the formation of a bubble. The remaining liquid medium was placed inside the chiller at 4 °C. Each time this medium was used, the temperature changed to 37 °C in a water bath.

3. Results and discussion

3.1. Optimisation of solvent using OFAT

There is limited study on the effect of polar solvent in ultrasound extraction on the yield and the bioactive compound of ASG crude extract. In contrast, a survey conducted by (Elnour et al., 2020) reported that using methanol as solvent of extraction explained that the maximum yield of extract was 11.10% - 15.56% for ASG. While ethanol is more desirable because it is a commercial solvent widely used for the extraction process, it is non-toxic, has a suitable polarity, and can dissolve bioactive compounds. It has been suggested that ASG could be more soluble in methanol and ethanol than other organic solvents. Therefore, Elnour et al. (2018a) explored that methanol for extraction of raw ASG was found to be more effective in all the assays than the chloroform, hexane, acetone fractions. Lower extraction yield when using acetone and hexane compared to 60% ethanol could be due to the low solubility of gum in acetone and hexane. In contrast, ethanol can dissolve both polar and non-polar substances. The polar character of ethanol is higher than acetone.

3.2. Significance of the regression

The regression coefficient R^2 values demonstrated the effect of each variable on the experimental response, while the predicted R^2 (0.89) was in reasonable agreement with the adjusted R^2 (0.96) (Table 2). The higher values of R^2 (0.90) and the closest to the adjusted R^2 (0.96) for extraction yield also revealed the model's efficacy. The Lack of Fit F-value of 0.38 indicates that the Lack of Fit is insignificant ($p > 0.05$) in comparison to the pure error for extraction yield. Thus, they proved that the model fits the data and demonstrated its apparent adequacy (Table 2). In addition, the answer is that the linear coefficients time (A) and temperature (B) and quadratic coefficients (A, B, A2, and B2) are ($p < 0.05$), and since the extraction yield, an important model term for this study. It shows that it is. A minimal p-value ($p < 0.05$) indicates that extraction time and temperature correlate with extraction yield.

Table 2. ANOVA for yield (%) fitted quadratic model of extraction conditions

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	
Model	431.23	9	47.91	53.20	< 0.0001	significant
A-Time	0.5429	1	0.5429	0.6028	0.0045	
B-Temp	3.21	1	3.21	3.57	0.0088	
C-S/L	3.59	1	3.59	3.98	0.0739	
AB	2.73	1	2.73	3.03	0.1125	
AC	0.2211	1	0.2211	0.2455	0.6310	
BC	0.0561	1	0.0561	0.0623	0.8079	
A^2	99.96	1	99.96	111.00	< 0.0001	
B^2	44.64	1	44.64	49.57	< 0.0001	
C^2	0.1090	1	0.1090	0.1210	0.7351	
Residual	9.01	10	0.9006			
Lack of fit	5.14	5	1.03	1.33	0.3808	not significant
Pure error	3.86	5	0.7728			
Cor total	440.24	19				

Model terms with p-values < 0.05 were significant. The p-value of 0.38 indicated that the lack of fit was insignificant.

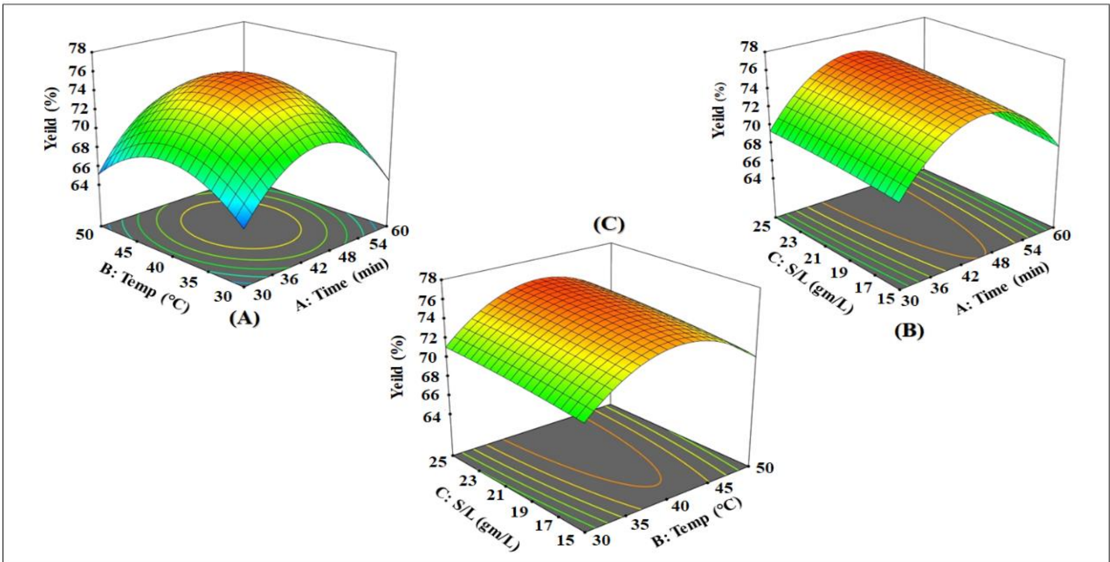

Figure 2. 3D plots representing the intersection response effects

3.3. The interaction response effects

The three-dimensional (3D) surface plots as presented in Figure 2 indicated extraction time affected, most significantly. The ASG extracts yield increased along with the increase of extraction time followed by extraction temperature effect, factor B considering that the prolonged heating resulted in a decrease in extraction yield due to the sample's thermal degradation and polymerization. The optimal conditions for ASG by UAE suggested by the model to gain high yields were specified: an extraction time of 45 min, temperature 40 °C, solid/liquid ratio of 1:25 g/ml, and the optimum yield of 75.89%. These parameters were selected for maximizing yield, with the highest overall desirability of D = 0.9. The mean values of the investigated response obtained from actual

experiments at this point were: 75.88. Figure 2 shows the interactions between the experimental levels of the tested variables and their impact on the response. You can see that the different shapes of the contour plots have different interactions between the variables. Figure 2A shows the effects of extraction time and temperature and their effect on the percentage of extraction yield. The yield of ASG extract increased as the extraction time increased, and it took a certain amount of time for the ultrasonic waves to stimulate cell wave interference before releasing the extract. A similar effect of extraction temperature on the yield of ASG extract was observed. Similar interactions between extraction temperature and solid-liquid ratio in Figure 2C were observed with ASG Ultrasonication Assisted Extraction "UAE". The results showed that increasing the extraction temperature increased the solubility of ASG and also improved the extraction yield (%). Yields on ASG extracts also increased for other reasons such as increased solvation, increased material porosity, and mass transfer. The study's results were confirmed by Maran et al. (2013).

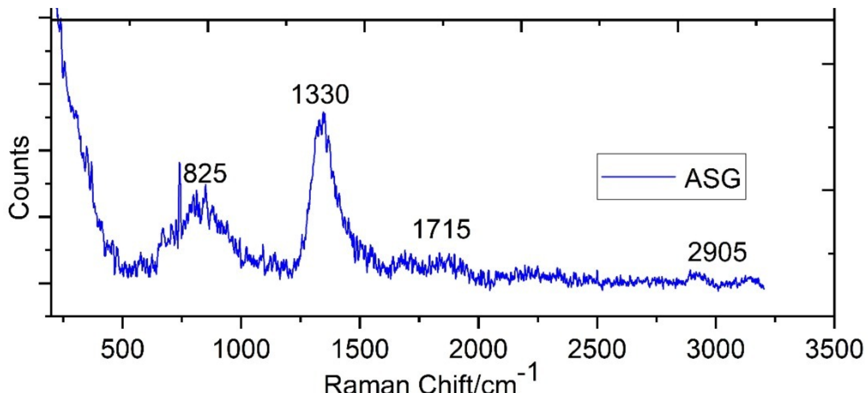

Figure 3. Typical Raman spectrum of *A. seyal* gum powder sample

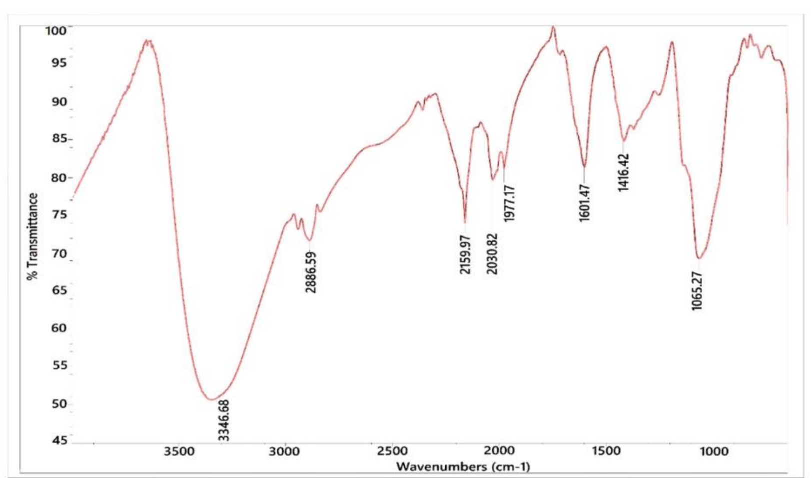

Figure 4. FTIR spectra for *A. seyal* gum powder sample

3.4. Phytoconstituent identification of ASG

The ASG Raman spectrum of ethanol extract is nearly identical to raw gum. The observed spectrum wavelength identification is shown and assigned peaks in Figure 3. The Raman spectrum of the optimized extracts revealed that the ethanol ASG extracts had three primary peaks at 1330 and 825 cm^{-1}. ASG spectra showed bands at around 1715 cm^{-1}.

The present study used FTIR analysis to recognize the functional groups of ASG powder based on their peak values. The spectroscopic characterization of the ASG sample and its components by FTIR is shown in Figure 4. The findings suggested the existence of functional groups like alkane, imine, amine, aromatic compounds or ketone, and phenol stretching. The FTIR spectrum of the ASG ethanol extract exhibited few significant differences from the native ASG FTIR spectrum. The FTIR spectrum was obtained with an ASG extract of 4000-400 cm^{-1}. The resulting spectra showed significant overlap between each component's absorption spectrum. Each band describes the absorption peaks associated with the functional groups isolated from the ASG extract. The maximum absorbance peaks of ASG were detected at 1747 cm^{-1} in the spectral band 1200-824 cm$^{-1,}$ corresponding to the ASG carbohydrate group. Skeletal stretch vibrations of the side chains of polysaccharides create this so-called carbohydrate fingerprint area.

3.5. Cytotoxicity assay against K526 cells

Growth-inhibiting action of ASG crude ethanol extracts on K562 cells after exposure to different concentrations (10, 5, 2.5, 1.25, 0.625, 0.3125 µg/ml) suggested that the ASG treatment induced a dose-dependent inhibition of cell growth in K562 cells. The IC_{50} value for ASG in K562 cells was 10 µg/ml simultaneously. Taxol was also evaluated for its cytotoxicity resulting in a value of IC_{50}=6.3 µg/ml (Figure 5). The ASG extract concentration required to reduce cell viability by half was10 µg/ml.

The organic ASG crude extract was cytotoxic in a dose-dependent manner on two different leukemic cell lines, K562 and Jurkat T cancer cells. Cell growth inhibition was more pronounced in Jurkat cells than in the K562 cell line. These studies recommended ASG as an antitumor agent, although the mechanism of action behind it needs to be elucidated.

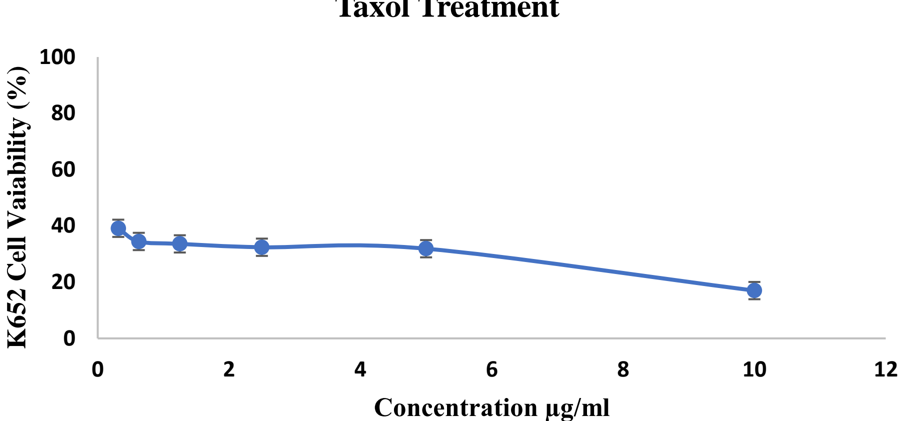

Figure 5. The cell viability percentage of K652 cell lines vs. different concentrations of Taxol treatment (R^2 = 0.9009, IC_{50}=6.3 µg/ml)

4. Conclusions

This study has proven that *Acacia* gum is an important plant genus widely used due to its ethnomedicinal therapeutic benefits as an anti-inflammatory, anti-ulcerative anticancer, immunostimulant, anti-obesity, anti-chronic renal failure, anti-diarrhea, and antitoxic effects. Treatment for leukemia can be a complex combination with many sides effects. Hence, searching for a safer and more effective treatment is considered important. This study was successfully added value to investigate the antiproliferation effect of the crude ASG extract on leukemia cancer cell lines after optimizing its metabolites yield. The ASG extraction was successfully applied to optimize the extraction conditions of gum using response surface methodology to maximize the extraction yield. Validation of the optimized condition at temperature 40 °C, with a solid-to-solvent ratio of 25 g/ml, and an extraction time of 45 min exhibited an extraction yield of 75.89% ± 0.52. Moreover, comparing the UAE extraction method with conventional extraction methods proved to be more efficient regarding extraction efficiency, shorter extraction time, and the small quantity of liquid used, making it environmentally friendly.

Acknowledgments

The author would like to acknowledge those directly or indirectly involved in this research.

Conflict of interest

The authors confirm that there are no known conflicts of interest.

CRediT authorship contribution statement

Nassereldeen Kabbashi: Conceptualization, Investigation, Data analysis, Supervision
Tahani Maher Alawdat: Writing-original draft; Resources, Conceptualization, Visualization, Formal analysis, Investigation, Methodology
Isam Y. Qudsieh: Resources, Formal analysis, Investigation
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Supplementary File

None.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 89-97

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

https://ijpbp.com

RESEARCH ARTICLE

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Fatty acid composition, antioxidant, antifungal activities, and functional group analysis of *Corylus jacquemontii* seeds grown in Kashmir

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ARTICLE INFO

Article History:

Received: 11 January 2022
Revised: 26 January 2022
Accepted: 27 January 2022
Available online: XX Xxxxx 2022

Edited by: B. Tepe

Keywords:
Corylus jacquemontii
Soxhlet extraction
Fatty acid composition
Antifungal activity
Antioxidant activity
Functional group analysis

ABSTRACT

Corylus jacquemontii (Decne.) is an important aromatic plant possessing nutritional and various therapeutic properties. This plant has got wide abundance in the Kashmir region with very low care cost. In this study, Soxhlet extraction was used to obtain different seed extracts. The highest yield observed was 32.25% and 30.27% in petroleum ether and acetone extracts, respectively. Gas chromatography coupled with a flame ionization detector was used to determine the fatty acid profile of petroleum ether extract. Unsaturated fatty acids were found in the dominant amount, notably 79.33% oleic acid. The antifungal activity against *Aspergillus niger*, *A. fumigates*, and *Penicillium marneffei* and antioxidant assays such as CAT, APx, SOD, DPPH were observed in petroleum ether, ethyl acetate, acetone, and methanol extracts. The dominant inhibition against *A. niger* and *A. fumigates* was displayed by methanol extract with 16.78 mm and 19.23 mm inhibition zone, respectively, while P. marneffei methanol (20.98 mm) acetone (20.27 mm) extracts were most effective. Moreover, all extracts displayed good antioxidant activities. These results increased the attention towards the importance of the present study.

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1. Introduction

Corylus jacquemontii (Decne.), commonly known as hazelnut, belongs to the Betulaceae family and is distributed worldwide, mostly in the coasts of the Black sea region of Turkey, Northern Europe (Italy, Spain, France, and Greece), and in some other parts of the world, especially Iran, Azerbaijan, China, and India. Hazelnut fruit is a nut connate with its tightly adherent fruit coat. This dry coat provides an average of 40% of a nut weight, and the remaining 60% constitutes a nut itself. These hazelnuts are mainly produced in Turkey (79.19%), Italy (11.18%), Spain (6.47%), and the United States (2.47%) (Demir and Beyhan, 2000). Traditionally, due to the presence of a high quantity of fats, hazelnuts have been prescribed by the general public, but recent epidemiologic and clinical studies have concluded that frequent consumption of nuts has nutritional and health benefits, especially in the reduction of coronary heart diseases (Alphan et al., 1996; Durak et al., 1999; Elvevoll et al., 1990; Garcia et al., 1994). Various research has confirmed the benefits of introducing hazelnuts in human diets due to the presence of fat (around 60%), most of which are rich in monounsaturated fatty acids (MUFA) (primarily oleic acid), tocopherol (α-tocopherol), phytosterols (β-sitosterols), polyphenols, and squalene (Di Nunzio, 2019; Mercanligil et al., 2007). Besides the nutritional activities, these hazelnuts are also used as a unique and good flavor ingredient in various foods (Alasalvar et al., 2003, 2004).

The oxidants and free radicals play a dual role in toxic and beneficial compounds. They can be either useful or harmful to the human body. However, an overload of free radicals causes oxidative stress, which leads to various chronic and degenerative diseases. Certain

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e-ISSN: 2791-7509
doi:
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Please cite this article as: Nengroo, Z.R., Azeem, M., Parveen, M., 2022. Fatty acid composition, antioxidant, antifungal activities and functional group analysis of Corylus jacquemontii seeds grown in Kashmir. International Journal of Plant Based Pharmaceuticals, 2(1), 89-97.

synthetic antioxidants such as butylated hydroxytoluene (BHT) are used to prevent the oxidation of lipids in various foods. However, it has been found that the overuse of synthetic antioxidants leads to harmful human effects on human health (Valentão et al., 2002). Moreover, synthetic antioxidants are stable under particular conditions.

Pathogenic fungi are the main infectious agents for the plants, causing alterations during development as well as in the post-harvest stage. The genus *Alternaria* nees is the most common fungi on the phyllosphere (Lopes and Martins, 2005). It includes both plant-pathogenic and plant-saprophytic species that may damage the crop and causes post-harvest decay (Griffin and Chu, 1983), which ultimately decreases the economy of farmers and the food industry. These fungi also decrease the quality of fruits and seeds. In certain cases, due to the production of mycotoxins or allergens by fungi, these are indirectly related to toxic or allergic disorders among consumers. Synthetic fungicides can control fungi, but these are restricted to use by the cause of harmful effects of pesticides on human health and the environment (Harris et al., 2001; Hayes and Laws, 1991). The latest trend is searching for antimicrobial and antioxidant agents of plant origin because of their safe, eco-friendly, and cost-effective nature (Amadioha, 2000; Cheijinna, 2006; Tauchen et al., 2015). In this study, we took *C. jacquemontii* seeds from Kashmir because of their high abundance, low care cost, and tremendous traditional applications. Hence, in continuation of determination of the fatty acid composition of seed oils (Nengroo and Rauf, 2019, 2020). This work has investigated hazelnut seeds extracts for fatty acid composition, antioxidant activity, and functional group analysis. In addition, *in vitro* antifungal activity of seed extracts was also screened against *Aspergillus niger*, *A. fumigates,* and *Penicillium marneffei*.

2. Materials and methods

2.1. Sampling

C. jacquemontii (locally known as Virin) seeds were collected from Lehenwan, Vailoo, and Lisser areas of Kokernag, Anantnag district, South Kashmir (India). Sampling was made in different orchids; in October 2019, hazelnut samples were placed inside a polystyrene box. In their unshelled state, samples were kept at room temperature (25 °C) for two weeks until analyses were performed. A series of fatty acid methyl esters (FAMEs), including *trans*-9C18:1, *trans*-10C18:1, and *trans*-11C18:1 isomers, were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA) and Sigma Aldrich (USA). All the reagents and internal standards used for the gas chromatography (GC) were of analytical grade and purchased from Fischer Scientific (UK).

2.2. Extraction of seed extracts

The nuts of seeds were manually cracked, and the kernels were ground under a dried air to a fine powder in a coffee grinder. A powder weight of 50 g was successively extracted by petroleum ether b.p. 40-60 °C (150 ml) followed by ethyl acetate, acetone, and methanol in a Soxhlet apparatus up to 6 h. The extraction percentages were determined as the difference in weight of dried samples before and after extraction. The extracts were dried over anhydrous sodium sulfate (Na_2SO_4) and were kept at 4 °C until further analyses.

2.3. Preparation of fatty acid methyl ester (FAME)

The hazelnut oil (200 mg) was treated with 3 ml of sodium methoxide in methanol (0.5 mol/l) at 100 °C in a water bath for 10 min. The solution was cooled at room temperature, and 2 ml of 12% (w/w) boron trifluoride (BF_3) in methanol was added. The mixture was heated in a water bath for 10 minutes and cooled to room temperature. After cooling, 1 ml of hexane was added, and the mixture was shaken vigorously. Then, 1 ml of 0.6% (w/v) sodium chloride was added. The organic layer was transferred into another screw-capped test tube with the help of a Pasteur pipette and finally dried by anhydrous sodium sulfate and filtered. The filtrate was concentrated under a gentle stream of nitrogen (Figure 1).

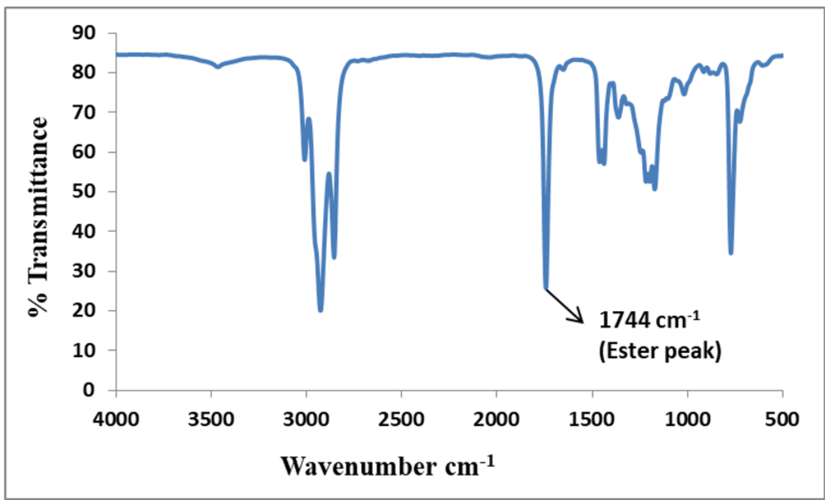

Figure 1. FT-IR analysis of fatty acid methyl ester (FAME) of *C. jacquemontii*

2.4. Gas chromatographic analysis

FAMEs were analyzed using gas-liquid chromatography (GLC) with a flame ionization detector (FID). The sample (1 µl) was injected into the GC, a Hewlett-Packard (HP) 5890 series 11 (Little Falls, Wilmington, DE, USA) equipped with a 60 m Supelcowax-10 capillary column (Supelco, Bellefonte, PA, USA) coated with poly-(ethylene glycol) (0.25 mm I.D., 0.25 µM film thickness). The oven

temperature was programmed as follows: 180 °C for 2 min, then raised to 200 °C at 2 °C/min, held at 200 °C for a further 10 min, then raised to 215 °C at 2 °C/min, and kept there for 10 min. The carrier gas used for the analysis was helium at a 0.5 ml/min flow rate. The injector and detector temperatures were maintained at 200 and 250 °C, respectively. Samples were injected into the column inlet using a Hewlett-Packard 7673 automatic injector. FAME identification was based on retention times compared to those of standard FAMEs.

2.5. Antifungal assay by disc diffusion technique

The petroleum ether, ethyl acetate, acetone, and methanol seed extracts of *C. jacquemontii* were screened for antifungal against *A. fumigatus*, *A. niger,* and *P. marneffi* by disc methods per the guidelines of the NCCLS on filamentous fungi diffusion (Bayer et al., 1966). The concentration of extracts, i.e., 10 µl, 15 µl, and 20 µl/disc, were used for analysis. The fungal cultures were grown on czapexdox broth (diffco). Twenty ml of agar media was poured into Petri dishes and allowed to solidify. The lawn of a particular fungal strain was made on the surface of agar media around the disc. The sterile discs (6mm diameter, Whatman filter paper no:42) were soaked in added concentrations (10 µl, 15 µl, and 20 µl) of extracts. A disc without extract was used as the negative control, while standard antibiotic nystatin was used as the positive control in this study as its efficacy for combating fungal infections has been proven in previous studies. The mycelia mat of *A. fumigatus*, *A. niger,* and *P. marneffi* of 7-day old culture were washed, suspended in normal saline solution, and then filtered through glass wool aseptically. The colony-forming units per ml of a suspension of the test fungi were determined, and inoculum was adjusted 1-5 X 100 ml. The appearance of conidia was used for *in vitro* antifungal assay tests. 0.1 ml of inocula were applied on the surface of the Czapek's dox agar (Diffco) plate and spread by using a sterile glass spreader. The test was performed in triplicate. These dishes were incubated for 48 h at 28 °C. The zone of inhibition in mm was determined after 48 h.

2.6. Antioxidant activity

2.6.1. Activity of catalase (CAT)

The activity of CAT was measured by the method of (Aebi, 1984) with slight modification by observing the disappearance of H_2O_2 at 240 nm through UV-vis spectrophotometer. The reaction mixture consists of 22.5 µl seed extracts, 100 mM phosphate buffer (pH 7.8), and 10 Mm H_2O_2. The activity was obtained by using the extinction coefficient of 0.03 $mM^{-1}.cm^{-1}$.

2.6.2. Activity of ascorbate peroxidase (APx)

The activity was done by Nakano and Asada (1981) procedure in a 3 ml reaction mixture containing phosphate buffer (50 mM, pH 7.0), 0.1 mM AsA, 0.1 mM H2O2, and seed extract. The AsA oxidation was observed by a decrease in absorbance at 290 nm (extinction coefficient 2.8 $mM^{-1}.cm^{-1}$). One unit of APx was defined as the amount of enzyme oxidizing 1 µmol of AsA per minute.

2.6.3. Activity of superoxide dismutase (SOD)

The SOD was determined according to the method described by Ramiro et al. (2006). Briefly the reaction mixture, containing 50 mM phosphate buffer (pH 7.8), 20 µM riboflavin, 75 µM nitroblue tetrazolium (NBT), 130 mM methionine, and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The reaction mixture was irradiated under fluorescent light tubes (40 µmol $m^{-1}.s^{-1}$) for about 10-15 min. The absorbance was measured at 560 nm by a UV-visible spectrophotometer. The samples for blanks and standards were run in accordance. The SOD activity, which gives half of the maximum inhibition of NBT reduction, was defined as one unit of SOD activity.

2.6.4. Radical scavenging activity (DPPH assay)

The radical scavenging activity of seed extracts of *C. jacquemontii* extracts was performed by following the procedure of Shimada et al. (1992) with minor modifications. Briefly, 200 µl of each extract (25-100 µg/ml) with 3.8 ml DPPH solution was kept in the dark for about 1 h at room temperature. The absorbance was monitored at 517 nm. The scavenging activity of each extract was obtained by comparing its absorbance with that of a blank solution (no sample). The ability to scavenge the DPPH radical was calculated using the following equation:

$$DPPH\ radical\ scavenging\ activity\ I\ (\%) = \frac{(\text{A standard} - \text{A sample})}{\text{A standard}} x\ 100$$

where, A standard is the absorbance of DPPH radical (without the test sample), and A sample is the absorbance of DPPH radical with the different extract samples of various concentrations. Synthetic antioxidant drug BHT was used as the positive control.

2.7. Functional group analysis

Absorbance spectra of hazelnut seed extracts were obtained using a Perkin–Elmer Spectrum One FT-IR spectrometer (UK) fitted with an Attenuated Total Reflectance (ATR) crystal of zinc selenide. The temperature of the ATR crystal was maintained at 65 °C. A very small amount (50-100 µl) of the sample was required to cover the surface area of the ATR crystal. All the samples were measured in duplicate. The spectra were collected continuously over a wavelength range of 500–4000 cm^{-1} with a data resolution of 4 $cm^{-1,}$ and the air was taken as a reference background material. The solvent taken was chloroform and acetone, depending on the polarities of the particular extract. After each scan, the ATR crystal was cleaned with tissue paper wet with ethanol, and then dried.

2.8. Statistical analysis

Estimation of treatment results was conducted in triplicate, and the values were reported as average along with their standard deviation. Duncan's multiple range tests were conducted at 5% significance using analysis of variance (ANOVA) while using the statistical software (IBM SPSS Statistics 20 New York, USA).

3. Results and discussion

3.1. Physiochemical properties of seed extracts

The yield of various extracts of *C. jacquemontii* after Soxhlet extraction is depicted in Table 1. The percentage yield ranges from 15 to 33%. However, extract obtained through petroleum ether showed a 32.25% yield followed by acetone 30.27% methanol 23.65%, while ethyl acetate extract showed an average yield (p < 0.05). Saponification value (SV) is an index of the molecular weight of triglycerols. Higher SV is related to the high proportion of shorter carbon chain length of the fatty acids (Kirk and Sawyer, 1991), and the SV is also used to check the adulteration of oils. Higher the SV better is the oil's soap-making ability (Nielson, 1994). As given in Table 1, the SV of *C. jacquemontii* was found to be 177.32. This value was comparably less than its related species (*Corylus avellana*), which has 200.5 SV (Sharma et al., 2008). The iodine value

(IV) is an important tool to know the degree of unsaturation. As given in Table 1, the IV of *C. jacquemontii* was 92.98, which is comparably more than *Corylus avellana* (Sharma et al., 2008). This is mainly due to the presence of a high degree of unsaturated fatty acids compared to this species. However, it has been found that the IV of various hazelnut species usually falls in the range of 90-95 (Crews et al., 2005), which supports our study on *C. jacquemontii*. Moreover, the SV above 90 helps us categorize *C. jacquemontii* in non-drying oils (Chang et al., 2019).

Table 1. Extracted yield (%, w/w) of seed extracts, saponification, and iodine values of petroleum ether extracts of *C. jacquemontii*

Petroleum ether	Ethyl acetate	Acetone	Methanol	SV	IV
32.25 ± 0.55[a]	15.62 ± 0.51[d]	30.27 ± 0.43[b]	23.65 ± 0.26[c]	177.32 ± 1.68	92.98 ± 1.42

SV: saponification value, IV: iodine value
Values are arranged as mean ± S.D. (n = 3). In each row, different letters in superscript are significantly different at ($p < 0.05$) by Duncan's test.

3.2. Fatty acid composition

The total fat content was determined according to the Association of Official Analytical Chemists (Horwitz, 2010). The distribution of fatty acids (Table 2, Figure 2) showed that the *C. jacquemontii* is made of fatty acids with 14-22 carbons. The unsaturated fatty acid predominated (91.86%), mainly of oleic acid (C18:1) 79.33%, was the most dominant fatty acid found. The second, third, and fourth dominancy was shown by linoleic acid (C18:2), palmitic acid (C16:0), and stearic acid (C18:0) with the contribution of 12.21%, 4.95%, and 2.10% to the total fat, respectively (Table 1). Several other FAs were also detected but less in quantity (< 1%). Moreover, a very minute amount of 0.60% *trans*-fat (*t*-C18:1) was found. These results were in good agreement with the previous results reported on the FA profile of other species of hazelnuts (Savage et al., 1999; Xu et al., 2007; Xu and Hanna, 2010).

Table 2. Fatty acid composition of *C. jacquemontii*

S. No	Common and systematic names	Carbon numbers	Molecular formula	Area (%)
1	Myristic acid	C14:0	$C_{14}H_{28}O_2$	0.04 ± 0.02
2	Pentadecanoic acid	C15:0	$C_{15}H_{30}O_2$	0.02 ± 0.01
3	Palmitic acid	C16:0	$C_{16}H_{32}O_2$	4.95 ± 0.12
4	Palmitoleic acid	C16:1	$C_{16}H_{30}O_2$	0.17 ± 0.02
5	Heptadecanoic acid	C17:0	$C_{17}H_{34}O_2$	0.06 ± 0.01
6	Heptadecenoic acid	C17:1	$C_{17}H_{32}O_2$	0.03 ± 0.01
7	Stearic acid	C18:0	$C_{18}H_{36}O_2$	2.10 ± 0.08
8	Oleic acid	C18:1	$C_{18}H_{34}O_2$	79.33 ± 1.04
9	Elaidic acid	C18:1	$C_{18}H_{34}O_2$	0.60 ± 0.03
10	Linoleic acid	C18:2	$C_{18}H_{32}O_2$	12.21 ± 0.82
11	Linolenic acid	C18:3	$C_{18}H_{30}O_2$	0.12 ± 0.01
12	Eicosanoic acid	C20:0	$C_{20}H_{40}O_2$	0.05 ± 0.02
13	Eicosenoic acid	C20:1	$C_{20}H_{38}O_2$	0.14 ± 0.03
14	Docosanoic acid	C22:0	$C_{22}H_{44}O_2$	0.02 ± 0.06
15	[a]Σ SFA			7.24 ± 0.62
16	[b]Σ TUFA			91.86 ± 1.05

[a]Total saturated fatty acids (TSFAs)
[b]Total unsaturated fatty acids (TUFAs)

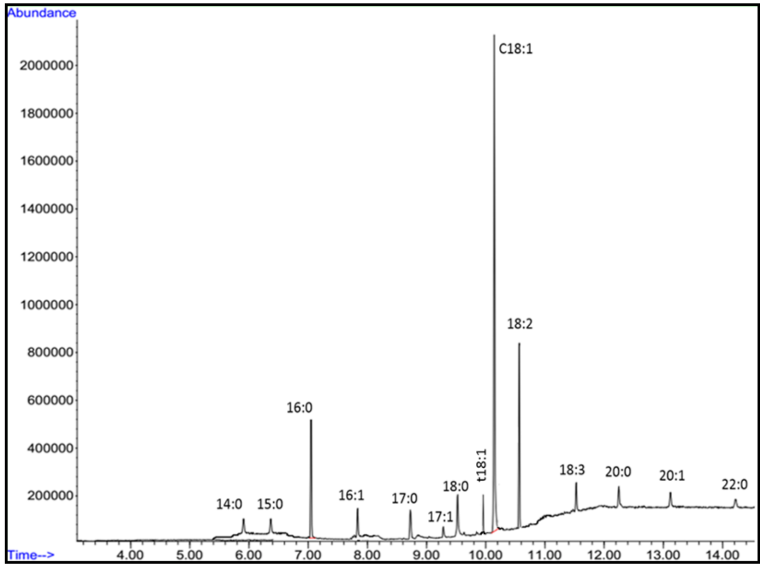

Figure 2. Gas chromatogram of FAME of *C. jacquemontii*

As mentioned, the distribution of fatty acids in *C. jacquemontii* oil is similar to widely consumed Almond used in the pharmaceutical and cosmetic industry, which contains an average of 60-80% oleic acid, 7-30% linoleic acid, 0.1-1% linolenic acid, 4-9% palmitic acid, 2.5%

max stearic acid and 0.6% palmitoleic acid (Álvarez and Rodríguez, 2000). This fatty acid reveals that *C. jacquemontii* can be an excellent source of oleic acid, which performs nutritional benefits and affords oxidative stability to the oil. Moreover, its composition, similar to almond oil, could be used for cosmetic purposes (Álvarez and Rodríguez, 2000). The hazelnuts are used as an excellent food product besides various other applications, mainly due to their diverse unsaturated fatty acids. The results were comparably similar on correlating the result of fatty acids to other Corylus species of the Betulaceae family (Chang et al., 2019). Hence, the nuts of this species could act as an excellent source of fatty acids and could be used as an alternative to some important nuts such as almonds, groundnuts, walnuts, etc.

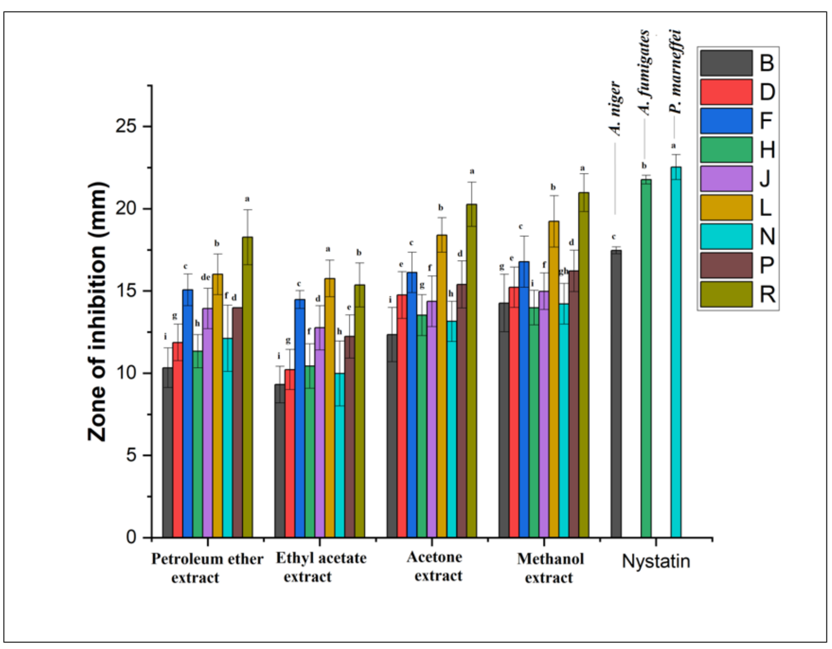

Figure 3. Antifungal activity of petroleum ether, ethyl acetate, acetone, and methanol extracts of *C. jacquemontii*
Values in different letters are significantly different at ($p < 0.05$).
(Concentrations of the extracts: B, H, N: 10 L; D, J, P: 15 L; F, L, R: 20 L)

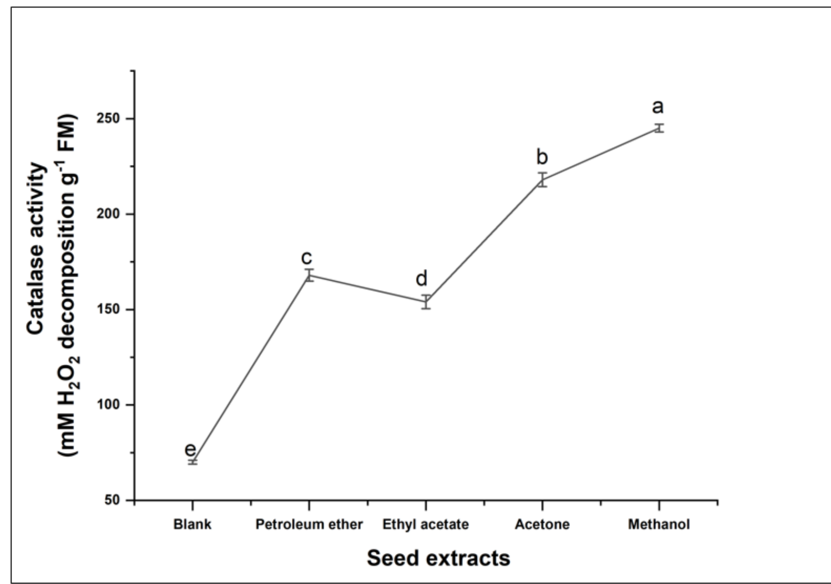

Figure 4. Catalase activity of petroleum ether, ethyl acetate, acetone, and methanol extracts of *C. jacquemontii*
Duncan's test shows that means with different letters are significant at ($p < 0.05$).

3.3. Evaluation of the antifungal activity of extracts

The control of infection caused by fungal has become a major problem due to resist of fungus against a series of commercially developed antibiotics. Thus the search for natural antifungal agents from natural sources has expanded (Webster et al., 2008; Nengroo et al., 2020). This study assessed the antifungal activity of petroleum ether, ethyl acetate, acetone, and methanol seed extracts of C. jacquemontii against three fungal strains, including *A. fumigatus*, *A. niger*, and *P. marneffi*. All the extracts showed good antifungal activity (Figure 3). However, dominant inhibition was shown at a 20 µl concentration of extracts. Against *A. niger*, the methanol extract

showed 16.78 mm (inhibition zone diameter) followed by acetone 16.12 mm concerning standard nystatin 17.47 mm at $p < 0.05$. In the case of *A. fumigates*, methanol extract showed predominant inhibition of 19.23 mm, followed by acetone extract 18.40 mm The petroleum ether extract showed 16.01 mm and ethyl acetate extract 15.76 concerning nystatin 21.77 mm. Against *P. marneffei*, methanol and acetone extracts showed almost similar inhibition of fungi with 20.98 mm and 20.27 mm, respectively, followed by petroleum ether extract 18.27 mm with nystatin showed 22.53 mm (Figure 3). These results were comparatively better compared to some of the plant extracts of related *Corylus* species against various microbes (Ceylan et al., 2013). Moreover, it has been found that the effect shown by the pure seed extracts of *C. jacquemontii* in this study was even better than silver nanoparticle extracts of *C. avellana* leaves (Eshghi et al., 2021).

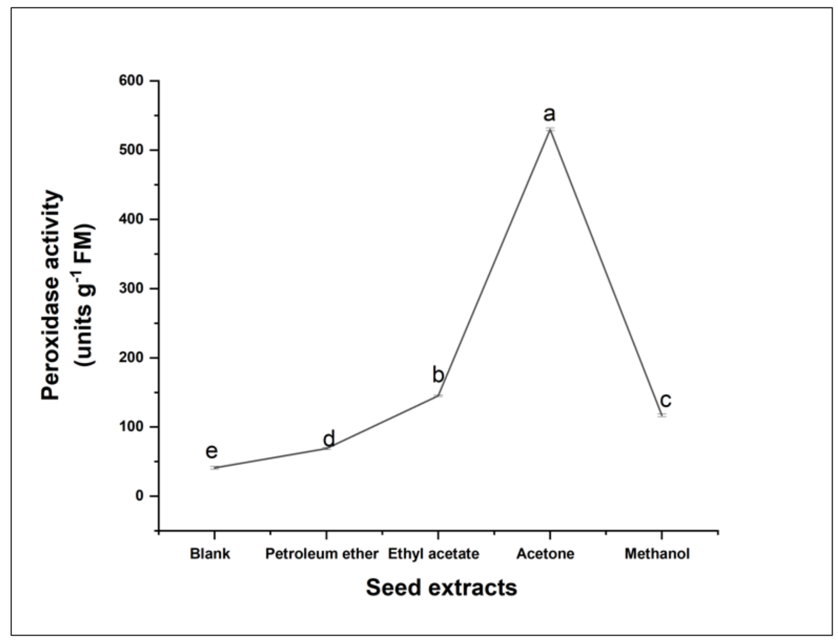

Figure 5. Peroxidase (Apx) activity of petroleum ether, ethyl acetate, acetone, and methanol extracts of *C. jacquemontii* Means with different letters are significant at ($p < 0.05$).

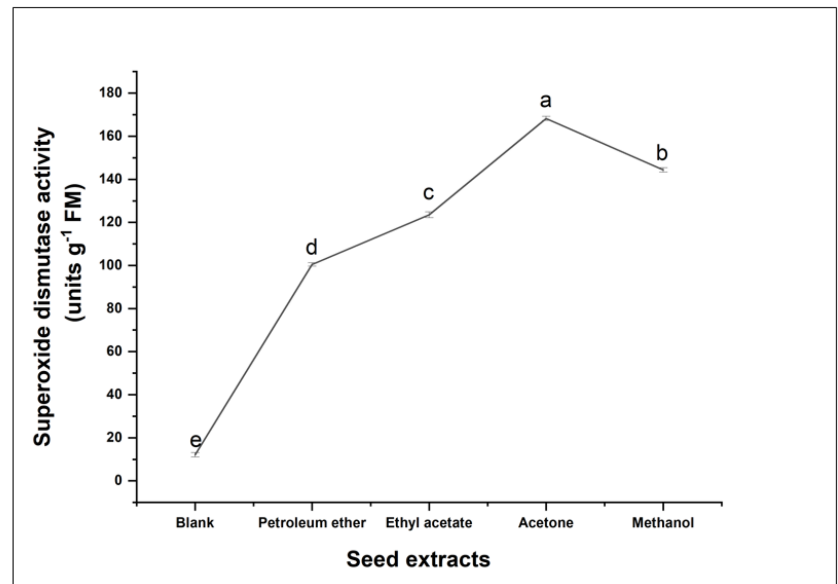

Figure 6. Superoxide dismutase activity of petroleum ether, ethyl acetate, acetone, and methanol extracts of *C. jacquemontii* Means with different letters are significant at ($p < 0.05$).

3.4. Antioxidant activity

The CAT enzyme activity is wholly related to the decomposition of H_2O_2 radicals, in which the higher the decomposition of H_2O_2 radicals is, the higher the CAT activity is. As shown in (Figure 4), the data indicated the highest CAT activity was observed in the situations of methanol and acetone extracts, followed by petroleum ether extract, while ethyl acetate extract showed moderate CAT activity concerning standard ($p < 0.05$).

The highest APx activity was shown by the acetone extract (530 Ug^{-1} FM) as depicted in Figure 5, while the ethyl acetate (145 Ug^{-1} FM)

and methanol (117 Ug^{-1} FM) extracts of *C. jacquemontii* showed moderate activity at ($p < 0.05$). The lowest effect was shown in petroleum ether (69 Ug^{-1} FM) to blank (41 Ug^{-1} FM).

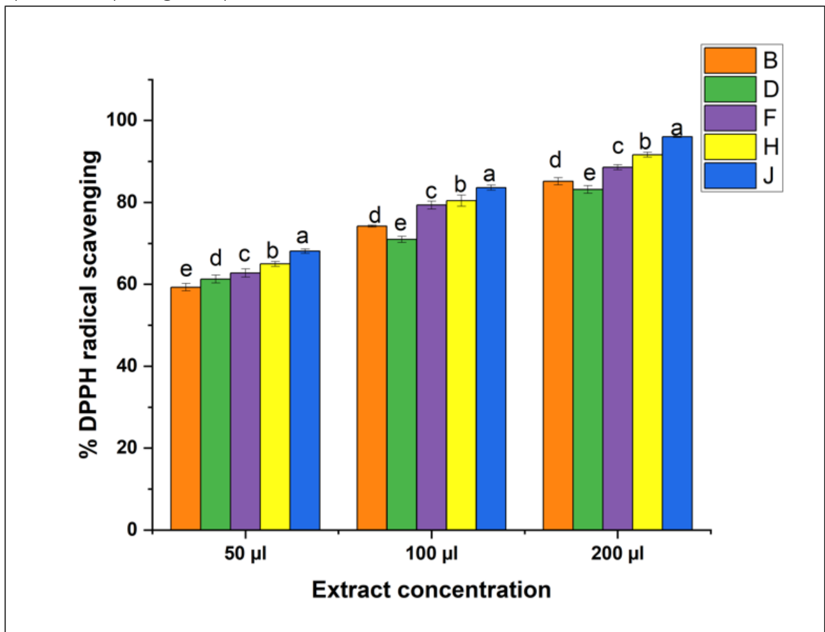

Figure 7. Scavenging activity (DPPH) of seed extract of B: petroleum ether; D: ethyl acetate; F: acetone; H: methanol of *C. jacquemontii,* and J: butylated hydroxytoluene (BHT) as standard
Different letters on each concentration are significantly different at ($p < 0.05$).

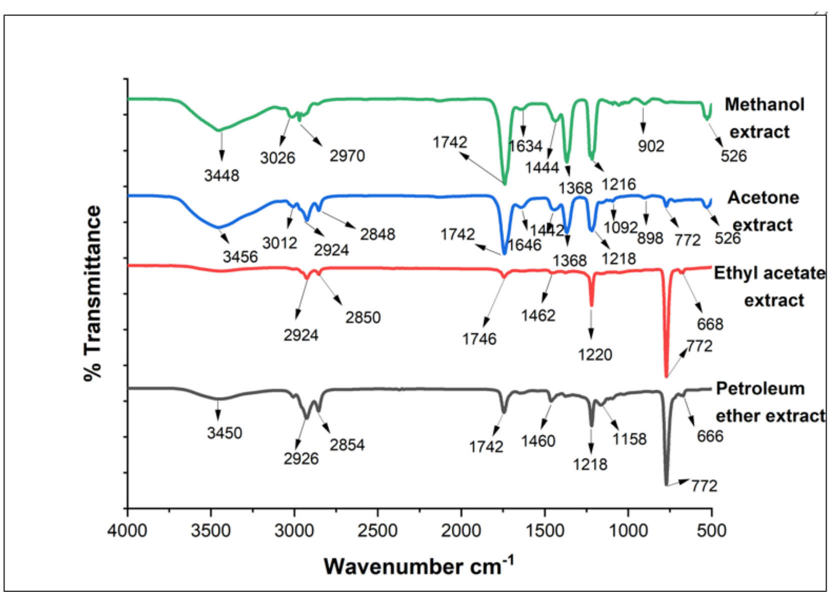

Figure 8. FT-IR analysis of petroleum ether, ethyl acetate, acetone, and methanol extracts of *C. jacquemontii*

As displayed in (Figure 6), the dominant SOD radical scavenging effect was shown by acetone (168.25 Ug^{-1} FM) followed by methanol (144.34 Ug^{-1} FM). Ethyl acetate (123.53 Ug^{-1} FM) and petroleum ether (100.43 Ug^{-1} FM) extracts showed average inhibition compared to other extracts.

The antioxidant potential of the petroleum ether, ethyl acetate, acetone, and methanol extracts was quantified as the concentration in (µg/ml) required to scavenge 50% of DPPH radicals. This method is commonly used to determine plants' antioxidants and their various extracts because it is simple, easy, and quick to be performed. Figure 7 shows the DPPH radical scavenging properties of *C. jacquemontii* seed extracts. All the extracts show good radical scavenging activity. At 50 µg/ml, dominant inhibition was displayed in methanol extract (64.98%) followed by acetone (62.75%) and ethyl acetate (61.29%) extracts to BHT (68.09%). At 100 µg/ml, methanol extract showed the highest scavenging activity (80.44%) followed by acetone extract (79.36%), while the least was observed in ethyl acetate extract (70.98%) as compared to BHT (83.63%). However, the predominant effect was observed at 200 µg/ml concentration of extracts with the order of decreasing radical scavenging methanol (91.66%) > acetone (88.58%) > petroleum ether (85.15%) > ethyl acetate (83.15%) extracts with BHT showed (96.04%). The inhibitions shown by seed extracts are comparatively

better than different extracts of bark, roots, and leaves of *Betula utilis* (Betulaceae family) from Kashmir (Wani et al., 2018). Moreover, the DPPH radical scavenging activity shown by *C. jacquemointii* seed extracts were comparatively similar with some important plant species from Kashmir viz., *Ulmus wallichiana*, *Celosia argentea*, *Sisymbrium irio*, *Aesculus indica*, and *Abies pindrow* (Nengroo and Rauf, 2019) and some other plants in various environments (Tatari et al., 2012). These results could categorize this plant not only as a good food source but as an excellent source of the natural drug upon further modifications.

Table 3. FT-IR of petroleum ether, ethyl acetate, acetone, and methanol extracts of *C. jacquemontii*

Petroleum ether extract		Ethyl acetate extract		Acetone extract		Methanol extract	
Wavenumber (cm^{-1})	Functional group	Wavenumber (cm^{-1})	Functional group	Wavenumber (cm^{-1})	Functional group	Wavenumber (cm^{-1})	Functional group
3450	Alcohols	2924	$-CH_2-$	3456	Alcohols	3448	Alcohols
2926	$-CH_2-$	2850	$-CH_2-$	3012	*cis*-RHC=CHR	3026	*cis*-RHC=CHR
2854	$-CH_2-$	1746	−C=O (ester)	2924	$-CH_2-$	2970	$-CH_2-$
1742	−C=O (ester)	1462	$-C-H$ ($-CH_2$)	2848	$-CH_2-$	1742	−C=O (ester)
1218	$-C-H$ ($-CH_2$)	1220	$-C-H$ ($-CH_2$)	1742	−C=O(ester)	1634	C=C (*cis*)
1158	−C–O	668	Non-assigned	1646	C=C (*cis*)	1444	$-C-H$ ($-CH_2$)
666	Non-assigned			1442	$-C-H$ ($-CH_2$)	1368	$-C-H$ ($-CH_3$)
				1368	$-C-H$ ($-CH_3$)	1216	$-C-H$ ($-CH_2$)
				1218	$-C-H$ ($-CH_2$)	902	−HC=CH− (*cis*)
				1092	−C–O		
				898	−HC=CH− (*cis*)		
				772	Halides		

The table was constituted according to References (Guillen and Cabo, 1997; Silverstein et al., 2005; Vlachos et al., 2006).

3.5. FTIR analysis

FTIR spectroscopy is an important, sensitive, fast, non-destructive, and accurate technique in which a sample is needed in very little amount. This technique has found wide applications in grape oil as an adulterant (Nurrulhidayah et al., 2011), various edible oils used in frying (Zhang et al., 2012), and vegetable oils (Rohman et al., 2011). The FTIR transmission spectra of *C. jacquemontii* extracts (prepared in chloroform and acetone depending on particular extract's solubility) are given in (Figure 8). The data of peak values and probable functional groups are presented in Table 3. The peak in the range of 3500-3400 cm^{-1} in the case of petroleum ether, acetone and methanol extracts is mainly of hydroxyl group, which could be due to the presence of any phenolic compounds as natural antioxidants or any antimicrobial agent (Robbins, 2003). The transmission spectra in the range of 3100-300 cm^{-1} are mainly because of alkenes which may include naturally occurring compounds such as carotenoids, quinoline, etc. The sharp peak in the 1450-1440 cm^{-1} is the ester functional group peak. This peak could depict the presence of ester bearing antimicrobial or antioxidant agents. The peak at 1630-1646 cm^{-1} in the case of acetone and methanol extracts could be mainly the presence of z-stilbene, cis-beta-carotene, etc. In short, FTIR analysis gives general information regarding the functional group, which could be useful in isolating and purifying a particular bioactive compound responsible for various biological activities.

4. Conclusions

This work demonstrated that *C. jacquemontii* seed extracts could be a good source of oleic acid, as food ingredient with high antioxidant activities such as CAT, Pox, SOD, and DPPH and good antifungal activities against *A. niger*, *A. fumigates*, and *P. marneffei*. The present study provides a base for further work, particularly the isolation and purification of individual bio-actives responsible for antioxidant and antifungal activities and the use of the C. jacquemontii seeds in the future.

Acknowledgments

The authors are thankful to UGC for the award of Non-Net fellowship and Department of Chemistry, and Instrumentation Facility (USIF), Aligarh Muslim University, for providing the requisite facilities to complete this study. Moreover, Prof. Sajad Gangoo is duly acknowledged for helping collect and identify this plant.

Conflict of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Zubair Rehman Nengroo: Conceptualization, Methodology, Antimicrobial activity, Software and writing
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Supplementary File

None.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 98-110

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS
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REVIEW

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Plants based materials as the antifungal and antibacterial agents

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ARTICLE INFO

Article History:

Received: 30 November 2021
Revised: 27 January 2022
Accepted: 28 January 2022
Available online: 01 February 2022

Edited by: B. Tepe

Keywords:
Antimicrobials
Antifungal
Phytochemistry
Medicinal plant materials
Primary and secondary metabolites
Pharmaceutical importance

ABSTRACT

The medicinal plants are a distinguished source of our earth, which cannot be replaced even though many developments in science and technology have been. Plants are very rich in bio-medicinal properties, as well as fabrication applications. Natural product materials play a vital role in curing many diseases without having many side effects; that's why many researchers were working on phytochemistry. Plant organo-compounds such as quinine, alkaloids, polypeptides, lectins, coumarin, terpenoids, flavones, flavonoids, flavonols, fatty acids, tannins, and essential oils are metabolites for biological activities. In this review, plant materials and plants part, which are responsible for antimicrobial activity, have been discussed.

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1. Introduction

1.1. Historical perspective

Plants are a fundamental part of our universe; it has shown their potentiality since primordial time. Human beings are so connected to the natural resource that life cannot exist on the earth. Plant-based materials have been used as a classical resource for traditional medicine and pharmaceutical drugs for a long time; they have played a vital role in treating all kinds of diseases that infect humans and farm animals. Pesticides traditionally used at large scales are synthetic chemicals that have non-target action as well along some of them have persistence in the environments.

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e-ISSN: 2791-7509
doi:
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To overcome these problems for the last two decades, intensive effort has been made by agricultural and botanical researchers to discover chemical compounds from plant origin having an antibacterial and antifungal activity (Sofowora, 1993; Egamberdieva et al., 2017). Most chemically synthesized compounds are halogenated, hazardous, and toxic to the environment and living organisms. It is very indispensable to have naturally occurring compounds be used as drugs. Synthetic pesticide, along with fungicides, has been used to control diseases and harmful organisms; however, most of these synthetic compounds exhibit teratogenicity, mutagenicity, carcinogenicity, phytotoxicity, and residual effects (Bajaj and Ghosh, 1975).

"Evolution" is a process by which all living organisms live here and flourish with many modifications adaptations are the biggest boon that the mother earth has blessed us with. As rightly said in Bhagavad-Gita, that "Every flora must be seen as the incarnation of God". There are different tales in many civilizations that are close to the plant kingdom and its uses to the human race. Ancient people knew the wisdom of nature and its uses. Plants and plant products have proved to be the biggest resource next to air and water, and of course, all three are interconnected within. The plant kingdom has not only given the food resources but they have been used as the

Please cite this article as: Bhovi, V.K., 2022. Plants based materials as the antifungal and antibacterial agents. International Journal of Plant Based Pharmaceuticals, 2(1), 98-110.

medicine for curing many diseases innumerable times. “Sushruta Samhita” is considered the olden and golden medicinal book, which contains major works in the field of Ayurveda chiefly deals with different sides of fundamental principles and the concept of surgery. These ancient books describe the more than 100 types of surgical apparatus, including scissors, forceps, scalpels, specula, etc. were explicated with their uses and applications. Dissection and operative procedures were explained, making use of vegetables and cadavers. It contains an explanation of nearly 650 drugs and confers different facets related to other surgery-related topics such as embryology, anatomy, toxicology, and applied therapeutics. Maharshi-Patanjali used the amalgamation of natural products, yoga, and the spiritual mind as the best medicine for disease-less life and bliss. Same has been defined today as a definition of health “health is not merely the absence of disease but a social, intellectual, spiritual well-being,” which reiterates the old concept of Ashtanga yoga which Patanjali prescribed.

1.2. Use of medicinal plants, strategies, and challenges

Medicinal plants have attained major importance in medical care, diverse ailments in different civilizations of the world (Shikhsamani et al., 2011). World Health Organization (WHO) has published a well-sorted strategy for the advancement and promoting traditional medicinal wisdom in four major areas (Naseri, 2004) which include,

- Identification and classification of plants of medicinal value, presentation of suitable policy and proposals,
- Development and funding of research and education, particularly at the level of university and higher,
- Establishment of harmony and cooperation between stakeholders of traditional medicine and researchers of modern medicine,
- Cultivate the herbs of medicinal importance and check the annihilation of natural resources and, many times, protect the unique ecosystem in which it habilitates.

Table 1. List of some plants and their active parts used

Sl No	Examples of common plants	Active parts used
1	Turmeric	Rhizome
2	Sandalwood	Heartwood region of the stem
3	Neem	Whole plant
4	Ginger	The rhizome (root)
5	Chinchona	Bark
6	Holy basil(tulsi)	Whole plant
7	Cloves	Unopened buds
8	Pepper	Seeds
9	Coconut	Endosperm part
10	Periwinkle	Flowers and leaves

These plans have depicted the vitality of the phyto-resources for the treatment and prevention of many dreadful diseases. Nowadays, there is a renaissance in the consumption of herbal formulations in standardized crude extracts. These are measures of their manifested side effects, the high cost of chemical drug synthesis, and intellectual property rights were less concerned. Many-medicinal plants such as garlic, onion, and ginger have gained admiration from the masses to treat and prevent numerous chronic disorders. The influence of various journals on publishing data on medicinal plants is getting lots of attention towards plant materials' phytochemicals and biomedicines activities. Mounting drift can include phytotherapy and plant-based medicines in the school curriculum of medicine, which is also considered the revival of Ayurvedic methods in modern outfits. Nowadays, more than 70% of German physicians prescribe plant extracts, and St. John’s Wort is more generally used to treat depression than any other modern chemical drug. Phyto-therapy is now being accepted as one of the safest treatment methods as it does not contain any specific drug but a mixture of phytochemicals of natural origin which act as effect-multiplier and side effect nullifying, although which is still debatable. Natural available medicinal plants demonstrate the varieties of antioxidants properties and are extensively used to treat many infections around the globe. On the other hand, these plant materials also display other activities such as antimicrobial, anticancer agents, anti-diabetic agents (Chaleshtori et al., 2011), anti-atherosclerosis and immuno-modulatory (Shahrani et al., 2009), and many times may also act asreno-protection (kidney protector) or hepatoprotective effects (liver protective) (Baradaran et al., 2013).

1.3. Biological activities of plants

Extensive research work was done on medicinal plants, giving you an idea about the significant antioxidant activity properties (Chaleshtori et al., 2011). Incidentally, a different category of animal models comprising of diabetes, autoimmune diseases, encephalo-myelitis, inflammation of bowels, ischemia in rat striated muscle tissues and kidney, renal toxicity hepatotoxicity, hyperlipidemia, radiation-induced necrosis, and cataracts for recording antioxidative effects of traditional plants have been worked and are published in different journals worldwide. Incidentally, medicinal plants have proven to be a novel source for preparing new drugs. One astonishing advantage over the isolated drug is that the crude plant extract of the plant containing the designated drug has a very little affinity towards antibiotic resistance over the isolated drug delivery. The crude extract of a plant that contains numerous phytochemicals has a cumulative effect on the harmful pathogens than a specific drug. It is also assumed that the cumulative effect on the pathogens confuses the immune system of the pathogen, and antibiotic resistance is checked. Nowadays, researchers are forcefully reliant on traditional medicinal plants to discover new potential drugs with minimal side effects. Focus is now given to plant-mediated drug therapy and drug discovery. Multilayered teamwork, including ethnobotanists, physicians, pharmacologists, and phytochemists, is essential for the successful results of medicinal plant research. Vitally, stringent policy making is also needed to enhance the efficacy quality without compromising the safety when delivering the drugs. Various nations define herbal medicines differently. Besides, countries have adopted vivid licensing, manufacturing, marketing, dispensing, and medicinal produces. In most countries in the west, herbal medicines are either licensed as medicines with proven efficacy by significant clinical trials. However, few underdeveloped and developed countries such as the Islamic Republic of Iran, China, and the United States of America were treated medico-herbal products are considered food supplements and, thus, are not required to meet the international drug standards.

1.4. Plant active parts (seeds, fruits, barks, leaves)

It is astonishing to know that we use the root of ginger as medicine (Mashhadi et al., 2013) but not the leaves. Similarly, in Santalum's album (sandalwood), the oil extracted is used as a product of economic value but not any other part. So, it is to be observed that the phytochemicals present in the plant are not present in all the regions of the plant but present in a specific part of the plant like stems, roots, leaves, flowers, and fruits. Some of the important active compounds and their active site are listed in Table 1.

This review sheds light on the antimicrobial and other pharmacological aspects of the commonly used plants of day-to-day life. The biological and pharmacological properties are illustrated in Figure 1. One part of the plant may not hold good for the other part of the same plant; hence proper literature survey and perfection are very much needed for the analysis in phytochemistry.

Figure 1. Schematic representation of bioactivity of plant materials

2. Neem (*Azadirachta indica*)

2.1. Systematic position

Kingdom: Plantae
Clade: Angiosperms
Order: Sapindales
Family: Meliaceae
Genus: *Azadirachta*
Species: *A. indica*

2.2. General description of the plant

The neem tree (*A. Indica*) is one of the most common trees in the Indian sub-continent, which has many ethnopharmacological uses and ethical properties attributed to it. Neem plants are generally regarded as possessing powerful health-promoting properties for ages. Indeed, many shreds of evidence show that the neem leaves and their bark were used as medicine for wound ailments 4500 years ago (Bhardwaj and Rajput, 2010). Maharshi Patanjali documented reveals that neem plant materials are medicine for Vata-related diseases (vataja). Even today, the *A. indica* tree is admired as the “Rural Dispensary” as every part of it shows distinctive therapeutic properties (Figure 2).

2.3. Distribution of neem on the basis of geography and climate

Neem belongs to the family of the Meliaceae family (WCSP, 2016). The Meliaceae family has naturally grown rapidly with less special treatment, a perennial, evergreen tree with a property of drought resistance. The neem tree grows into a large shady tree with a round canopy and has a life span of 150-200 years. Neem possesses the characteristic properties by its strong repulsive odor (Subapriya and Nagini, 2005), which is seen extensively in its oil; its flowers have a specific honey-like aroma that can be smelled from miles flowering season is from April to May. This is a fast-growing tree that needs scarce water; neem is also renowned as a remarkable renewable resource as it gives a very good substitute for the costly trees like teak rosewood, etc.

2.4. Ethnomedical importance

During the Ayurvedic era, neems were used to balance the vata. Its anti-cold and dry qualities tend to intensify vata. Therefore, neems are habitually recommended with other herbs that help soothe its vata-intensifying nature. Ethnically, people have used neem to clean teeth with neem twigs. Juice of neem is again considered a noble tonic to increase appetite and antipyretic eradicate helminths present as hosts in the intestine. Therapeutically, its aqueous crude extracts of bark and fresh leaves have been used for centuries in ethnic medicines to control diseases such as leprosy, ascariasis, and chronic respiratory disorders (Prieto et al., 1999).

Figure 2. Photography of neem plant

2.5. Phytochemical constituents

The dominant phytochemical constituents in ethyl acetate crude extracts reported are hexahydrofarnesyl acetone, 9,12,15-octadecatrienoic acid, and (9E,12E,15E)-9,12,15-octadecatrien-1-ol. One of the leading chemical compounds which were extracted in aqueous chloroform extracts are methyl-1,4-methylpentadecanoate, lineoleoyl chloride, phytol, methyl isoheptadecanoate, (2E)-3,7,11,15-tetramethyl-2-hexadecen-1-ol, and nonacosane. Lastly, the butyl alcohol (butanol) extracts of the fresh leaves contain (2E)-3,7,11,15-tetramethyl-2-hexadecen-1-ol, lineoleoyl chloride, levoglucosenone, benzaldehyde,2-methyl, methyl-14-methylpentadecanoate, and hentriacontane (Hossain and Nagooru, 2011; Hossain et al., 2011). The different compounds extracted from neems include hydrocarbons, terpene, terpenoids, phenolic compounds, alkaloids, long-chain fatty acids, and their derivatives. Literature survey shows that most of the compounds identified in neem are tabulated in Tables 2 and 3 (Cock et al., 2009; Kauroo et al., 2021).

Table 2. The phytochemicals recorded in neem

Phytochemical constituents	Methanol extract	Ethanol extract	Chloroform extract
Alkaloids	+	+	+
Saponin	+	+	+
Steroid	+	+	+
Tannin	+	+	+
Anthocyanin	+	+	+
Phenol	+	-	+
Terpenoids	+	+	-
Anthroquinone	+	+	+
Gums	+	+	-
Resins	+	+	+

\+ = Present; - = Absent

Table 3. The phytochemicals constituent present in neem (Alzohairy, 2016; Hossain et al., 2013)

Sl No	Chemical Name	Common name	Chemical structure
1	6,10,14-Trimethyl-2-pentadecanone	Perhydrofarnesyl acetone	
2	Octadeca-9,12,15-trienoic acid	9,12,15-Octadecatrienoic acid	
3	(2E,7R,11R)-3,7,11,15-tetramethyl-2-hexadecen-1-ol	Phytol *trans*-Phytol	
4	Methyl 14-methylpentadecanoate	Methyl isohexadecanoate	
5	(9Z,12Z)-Octadeca-9,12-dienoyl chloride	Linoleoyl chloride	

SI No	Chemical Name	Common name	Chemical structure
6	Methyl 15-methylhexadecanoate	Methyl isoheptadecanoate	
7	(1S,5R)-6,8-Dioxabicyclo[3.2.1]oct-2-en-4-one	Levoglucosenone	
8	Hentriacontane	*n*-Hentriacontanone	

2.6. Antifungal and antibacterial activities

Anti-fungal and antibacterial properties of extracts from neem leaves were reported; they have been studied in both methods, which are intricate *in vitro* or *in vivo*. The study reveals the presence of different antimicrobial active components in leaves of neem trees, such as quercetin, desactylimbin, and sitosterol. On the contrary, the researcher explains the presence of other phytochemical components which held responsible for enhancement active constituents like triterpenes or the limonoids such as meliantriol, azadirachtin, desactylimpin, quercetin, sitosterol, nimbin, nimbinin, nimbosterol, nimbidin, and margisine, and different bitter-tasting substances such as gums, alkaloids, terpenes, resins, glycosides, and phenols (Lucantoni et al., 2010; Grewal and Grewal, 1988). Lyer and Williamson (1991) have credited the antifungal properties of neem extracts to the reduction in protease activity of dermatophytes induced by the organic neem extract. A crude extract of neem juice acts as an antibacterial agent and is useful in inhibiting vibrios in marine shrimp. Neem may likely have acted as an adjuvant in using nature-antibiotics auxiliary antibiotics, which have dominated the current market. The effectiveness of neem in inhibiting bacteria is a well-known Ayurvedic medicine in rural India. The decoction prepared from the leaves of neem has been used to treat skin problems, especially measles. Aqueous and organic extracts from the neem leaves showed their reductional effect in all the standardized concentrations against the sex pathogenic fungi. These human pathogenic fungi are four *Aspergillus* species (*A. terrues*, *A. flavus*, *A. niger*, and *A. fumigatus*) which are generally known to cause aspergilloses, adding to this, *Microsporum gypseum* (a dermatophyte) and *Candida albicans*, the causative agent of dreadful dermatophytosis and candidiases. It is also recorded that all standard concentrations of the aqueous extract effectively suppresses the growth of mycelia of these fungi, and its effect was recorded to have increased with increasing concentration (Kannusamy et al., 2016). These human pathogens (*Aspergillus* and *C. albicans*) are the causal agent of dermatophytosis and candidiases (Okemo et al., 2001).

Figure 3. *Z. officinale* (ginger) rhizome (Ayodele et al., 2018)

3. Ginger (*Zingiber officinale*)

3.1. Systematic position

Kingdom: Plantae
Clade: Angiosperms
Order: Zingiberales
Family: Zingiberaceae
Genus: *Zingiber*
Species: *Z. officinale*

3.2. General description

Ginger is one of the frequently found common spices used extensively in different parts of the world because of its medicinal properties. It has made its revered regards in the kitchen of India and the European continent. Rhizome or root part of ginger (genus *Zingiber*) is the active part of the plant used in medicine for the treatment of various diseases like cough, cold, nausea, vomiting, diarrhea, gastrointestinal ulcers (peptic ulcers), diabetes, fever, reducing cholesterol levels of the arteries, rheumatoid arthritis, dry

mouth/xerostomia, sore throat, cancer, migraine headache, minor respiratory disorders. *Z. officinale*, commonly called ginger, belongs to the Zingiberaceae family. Gengibre Ancoas is the most commonly recognized trivial Spanish name of ginger (GRIN, 2017), but in Hindi and Urdu, it is known as "Adrak". The horizontally solid underground stem/rhizome of ginger is the most extensively used cooking ingredient and spice in daily household cooking activities (GRIN, 2017). Ginger is also esteemed in ayurvedic, Unani, allopathic, and household medicines despite its use as a food and flavoring agent. The rhizomes of the ginger can be utilized in the form of a paste, ginger juice in preparing tea (flavoring), dried powder for preparing medicinal tonics (Kashaya), and preserved slice its juice and decoction (Figure 3).

3.3. Distribution of neem on the basis of geography and climate

Ginger has wildly seen in southern China, Asia, and West Africa. In ancient times, ginger cultivation was exceptionally common in different countries like Japan, China, Indonesia, Nigeria, India, Brazil, Sri Lanka, and the Philippines. It is ethnically called a fixed deposit of farmers as it can even spread its rhizomes during drought conditions (GRIN, 2017).

Table 4. The phytochemicals recorded in ginger

Bioactive principle	Chloroform extract of ginger	Methanol extract of ginger
Alkaloids	+	+
Tannins	+	+
Glycosides	+	+
Saponins	+	+
Steroids	-	-
Flavonoids	+	+
Terpenoids	+	+
Pholobotannins	+	+

+ = Present; - = Absent

Table 5. The most common phytochemical constituents present in ginger (Rahman, 2014; Liu et al., 2019)

SI No	Chemical Name	Common name	Chemical structure
1	*(E)*-1,7-bis(4-hydroxy-3-methoxyphenyl)hept-4-en-3-one	Gingerenone-A	
2	4-(4-Hydroxy-3-methoxyphenyl)butan-2-one	Zingerone Zingiberone	
3	*(E)*-1-(4-Hydroxy-3- methoxyphenyl)dec-4-en-3-one	Shagoals	
4	1-(4-Hydroxy-3-methoxyphenyl)decan-3-one	Paradol	
5	(S)-5-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone	Gingerol	
6	1-(4-hydroxy-3-methoxyphenyl)tetradec-1-ene-3,5-dione	1-Dehydro-10-gingerdione	

3.4. Phytochemical constituents

Documentary of ginger's phytochemical constituents of roots (rhizome) was tabulated below. Cumulative effects of alkaloids, tannins, and saponins have been attributed to acting as the ginger's antifungal and antibacterial activities (Ayodele et al., 2018) listed in Tables 4 and 5.

3.5. Antibacterial and antifungal activities

It is recorded that diverse bacterial strains exhibit various levels of sensitivity gradient against the extract of ginger. The biggest havoc of today's world is the development of antibiotic resistance by bacteria (pathogenic microorganisms). To overcome this worrying menace, finding new novel active compounds against new targeted microorganisms and reducing their overall side effects is the utmost matter of urgency. Most of the available spices extracted in an

aqueous medium or organic solvents have active biological compounds, mainly used in the formulation, extraction, and synthesis of potent drugs. Thus, ginger, a normal ingredient in Indian daily food delicacies, can provide a natural defense against certain natural foes like bacterial pathogens. It is also to be noted that the single bioactive compound extracted and treated to certain diseases has a high risk of getting resistance, whereas the natural extract, which is a mixture of many phytochemicals, is notably the novel method to treat the bacterial diseases without the fear of antibiotic resistance. The diversified significant statistics of the effects of ginger on different strains of fungi are recorded. *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. were predominant mycotoxigenic fungal contaminants of Pearl millet grains. Methanolic extracts of ginger (*Z. officinale*) roots possess good antifungal activity against *A. flavus*, *A. oryzae*, *A. fumigatus*, *Penicillium italicum*, *P. chrysogenum*, *P. oxalicum*, *Fusarium oxysporum*, *F. subglutinans*, and *Trichoderma harzianum* (Kim et al., 2009; Ravindran and Babu, 2016). This confirms the presence of biologically active compounds such as alkaloids, tannins, flavonoids, and saponins, which, when purified, can be used as a biocontrol agent in the prevention of deterioration in agricultural products and could also be explored as a therapeutic agent in the control of mycotoxigenic fungi associated diseases in humans (Naveena et al., 2004).

Figure 4. *C. longa* (turmeric) rhizome

4. Turmeric (*Curcuma longa*)

4.1. Systematic position

Kingdom: Plantae
Clade: Angiosperms
Order: Zingiberales
Family: Zingiberaceae
Genus: *Curcuma*
Species: *C. longa*

4.2. General description

Turmeric (*C. longa*) is a perennial herb of the ginger family. Ginger's physiological properties were reported elsewhere (Ajav and Ogunlade, 2014; Funk et al., 2016). It grows up to 3 feet long with aromatic green leaves. The rhizome part of the plant has a characteristic aroma, a bitter taste, and stains deep orange-yellow color. The color changes to red in higher alkaline pH. Turmeric is an active ingredient that adds colors and flavors to most foods (like curry, relishes, pickles, and spiced kinds of butter for vegetables, poultry, fish and egg dishes, rice, and pork). Due to its medicinal and cosmetic properties, it has a wide range of applications (Figure 4).

4.3. The distribution of neem on the basis of geography and climate

Southern India and Indonesia have grown larger and most common spice. India itself owes 40 different species of turmeric having a different flavor, color, and shape of the rhizome (Prasad and Aggarwal, 2011).

4.4. Ethnomedical importance

C. longa (turmeric) is a rhizome part of the plant that has been used as an antimicrobial and antiseptic agent and an insect repellant for a very long time in India. It has also been adored as the symbol of marriage in south Indian culture. Several studies have reported that a vibrant spectrum of antimicrobial activity attributed to curcumin includes antiviral, antibacterial, antifungal, and antimalarial activities. It is a safe food additive as its antimicrobial activity, and negligible toxicity are well pronounced even at high doses (12 g/day), which was accessed by clinical trials on humans; that's apart, it is also used as a key to designing the new antimicrobial agents. Curcumin was used as a standard drug for synthesizing many modified and developed drugs for the synthesis and various derivatives relating to curcumin. It also has a greater application in textile industries as an antimicrobial agent suitable for textile materials. Turmeric is the active substance in herbal remediation and the spices of food value. The bright yellow color imparts turmeric and curry, which are usually isolated from the rhizome part of the plant. *C. longa* is a natural, healthy, and safe product to use even without any clinical prescriptions. Turmeric has huge benefits recorded in traditional medicine, such as treating rheumatoid arthritis (Pourhabibi-Zarandi et al., 2021), jaundice, eye infections, liver cirrhosis, and dental arches (Praditya et al., 2019). Also, the exploitation of biological potency of turmeric in new medicine has been permitted, such as anti-inflammatory, hypoglycemic agent,

antioxidant, necrosis healer, and antimicrobial activities. In India, it is common that turmeric powder is smeared on the wounds and cuts for a quicker healing process. The mechanism of its antifungal activity is not properly analyzed and recorded, but it has been recommended that its fungicidal mechanism would involve the accumulation of chitin over the outer layer of the cell wall. However, curcumin has been advised as a wonderful antibacterial and antifungal agent. Some antiviral activities are also recorded in turmeric traditionally (Zorofchian Moghadamtousi et al., 2014).

Table 6. The phytoconstituents in *C. longa*

Phytoconstituents	Methanol extract	Ethanol extract	Chloroform extract
Alkaloids	+	+	+
Saponin	+	+	+
Steroid	+	+	+
Tannin	+	+	+
Anthocyanin	+	+	+
Phenol	+	-	+
Phlobatannin	+	+	-
Anthraquinone	+	+	+

+ = Present; - = Absent

Table 7. The phytochemicals present in *C. longa* (turmeric) (Oghenejobo and Bethel, 2017; Niranjan and Prakash, 2008)

Sl No	Chemical Name	Common name	Chemical structure
1	(6S)-2-methyl-6-(4-methylphenyl)hept-2-en-4-one	*(+)-(S)-ar*-turmerone	
2	2-methyl-6-(4-methylidenecyclohex-2-en-1-yl)hept-2-en-4-one	Curlone	
3	2-Methyl-6-*p*-tolyl-2-heptene	*aryl*-Curcumene *alpha*-Curcumene	
4	*(1E,6E)*-1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione		

4.5. Phytochemical constituents

The major phytochemicals of turmeric oil were curlone, *ar*-turmerone, and *ar*-curcumin. It is said that the major photo-constituent *ar*-turmerone would be responsible for turmeric's antioxidant activity. Due to the presence of phenyl ring portion and a 1,3-unsaturated ketone functions of *ar*-turmerone, *in vitro,* antioxidant properties of turmeric oil are highly pronounced (Chanda and Ramachandra, 2019). Increased levels of superoxide dismutase, glutathione, and glutathione reductase enzyme levels in blood and glutathione-S-transferase and superoxide dismutase enzymes in the liver were reported in mice after the oral administration of turmeric oil for one month (Rudrappa and Bais, 2008). Saponins have been reported in turmeric, which acts as an antimicrobial agent added to which they also act as a key precursor for steroidal substances. These steroidal substances have recorded a vibrant spectrum of pharmacological activities (Liju et al., 2011). The terpenoids and sesquiterpenes isolated from the oils of turmeric also exhibit anti-inflammatory and antimicrobial effects. Preliminary phytochemical screening of methanolic extract of turmeric shows tannins, phenolic compounds, alkaloids, terpenoids, phytosterols, saponins, and flavonoids. The scientific method of phytochemical screening offers a comprehensive scientific validation for its use in treating spectra of disorders and diseases. The phytochemical constituents are listed in Tables 6 and 7.

4.6. Antibacterial and antifungal properties of turmeric

Various works have been reported on turmeric in exploring the antimicrobial potency against infectious bacteria and different strains of the dreadful fungi which cause various types of diseases in many studies on aqueous extract of *C. longa* rhizome revealed that MIC (minimum inhibitory concentration) value, MBC (minimum bactericidal concentration) is found to be 4-6 g/l and 16-32 g/l respectively, against *Staphylococcus epidermidis* ATCC 12228, *S. aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 10031 and *Escherichia coli* ATCC 25922 (Niamsa and Sittiwet, 2009). The antibacterial studies of ethanolic extracts and essential turmeric oil have shown degrees of sensitivity on microbes. This may be due to the active phytochemical compounds present in the extracts. The phytochemical analysis confirms the presence of flavonoids, glycosides, and phenols in varieties of turmeric. This result is comparable with the results of Luthra et al. (2001), who proved that the ethanol and methanol extracts of *C.longa* were active against different strains of fungi. The leaf extract was significantly effective ($p < 0.05$) against the test pathogens. Of all the extracts, the maximum zone was exhibited by methanol extract against *E. coli*

and the least one by ethanol leaf extract against *Serratia marcesens* (6.5 mm). The leaves of *C. longa* showed a zone of inhibition against most test microbes in a comparable quantity with the rhizomes (Singh et al., 2002). Antifungal activities about curcumin are attributed to the most significant effect recorded against *Candida* spp. and *Paracoccidioides brasiliensis*, although curcumin showed fungicidal effects on various fungal strains. Although different biological activities have been reported on curcumin, no proper clinical literature have been reported so far for this compound, and satirically, clinical trials are still under examination for different diseases, namely, multiple myeloma (type of cancer), colon and pancreatic cancers, myelodysplastic syndromes, Alzheimer, and psoriasis (Hashemi et al., 2008). Until 2013, over 65 clinical trials on curcumin have been reported, and still, more are underway.

Figure 5. *O. sanctum* (Tulsi or Holy Basil)

5. Tulsi or Holy basil (*Ocimum sanctum*)

5.1. Systematic position

Kingdom: Plantae
Clade: Angiosperms
Order: Lamiales
Family: Lamiaceae
Genus: *Ocimum*
Species: *O. sanctum*

5.2. General description

O. sanctum (L.) is well known for its sacred uses and holiness more than its pharmacological properties (Prakash and Gupta, 2005; Pattanayak et al., 2010). It has a very special status in the Indian traditional families where its presence is very important for the prayers and is predominantly grown for religious practices, ethnomedicinal purposes, and its essential oil (Siva et al., 2016). It is largely known across the Indian subcontinent as a common household medicinal plant, and its addition to the tea makes it a delicious beverage. An herbal decoction (Kashaya), commonly used in Ayurveda, also has appreciated the use of Tulsi. Tulsi is considered the goddess and has an important role in the Vaishnavite cult of Hinduism (madhwa and srivaishnava philosophy), in which devotees perform worship involving holy basil plants or leaves as the favorite plant of the Lord. The other traditional names for the *O. sanctum* are Tulsi or Holy basil (Figure 5).

5.3. Distribution of neem on the basis of geography and climate

It is an aromatic herb belonging to the family Lamiaceae. It is native to India and found predominately in Southeast Asia. It is a perennial herb growing 3-4 feet with aromatic seeds and leaves. There are many species and subspecies in *Ocimum,* seen in India. It is extensively used in ancient Ayurvedic medicine and the Siddha system of medicine to cure various infirmities. It is regarded as one of the holiest and sacred herbs grown in the Indian subcontinent.

5.4. Ethnomedical importance

This plant has uppermost medicinal properties, which exhibit biomedicinal applications such as analgesic, anti-inflammatory, antistress, antimicrobial, hypoglycemic, immunomodulatory, antiseptic, and hypertensive activities (Sofowora, 1993; Devendran and Balasubramanian, 2011). The Indian subcontinent has used this medicinal plant for ages to manage different diseased conditions from ancient times better. But, in contrast, very less is known about the mode of action at different molecular mechanisms that need to be studied. Tulsi plants parts are generally used on the common cold, cough, fever as a stimulant and antihelminthic (Buchineni et al., 2015). It is also used as a mosquito and insect repellent (Singh et al., 2012). *O. sanctum* leaf extract has been shown to stimulate biochemical pathways of insulin production and release into the bloodstream. The ethanolic extract substantial reduction in tumor cell size and an increased life span of mice suffering from sarcoma-180 solid tumors (Yamani et al., 2016); on the other hand, benzene extract fraction in the albino rat has been shown to reduce the total sperm count and sperm motility and leading to the condition called oligospermia (Sood et al., 2005). *In vivo* studies on different animals have shown that extracts of Tulsi leave showed a reduction in plasma level of the stress hormone-corticosterone induced by both acute and chronic noise stress. This is in good accordance with the traditional use of eating Tulsi leaves during acute headaches, and even today, many ayurvedic practitioners prescribe Tulsi as a chief drug for treating migraine.

5.5. Phytochemical constituents

The Tulsi is mainly composed of different active biomolecules such as terpene, terpenoids, alkaloids, tannin, saponin, and steroids are very complex and contain many different types of phytochemicals. Qualitative phytochemical analysis of the extracts from the leaves of *O. sanctum* divulged the presence of phytochemicals such as steroids, flavonoids, terpenoids, tannins, saponins, and cardiac glycerides. Similarly, the phytochemical constituents like phlorotannin, which, as expected, were found to be nil. The phytochemical compounds present in the hydroalcoholic extracts of *O. sanctum* were identified by GC-MS analysis. The active principles were analyzed with their retention time (RT), molecular formula, MW (molecular weight), and concentration (%) are recorded (Sethi et al., 2003). The leaves contain eugenol and caryophyllene, dominantly responsible for various antimicrobial activities. Eugenol, largely seen in Tulsi, is mainly responsible for its mosquito repellent properties. The same eugenol is also considered an anti-oxidant drug. This property enables us to maintain proper health conditions and prevent the occurrence of the change of heart diseases by reducing the accumulation of various free radicals in the body and triggering many other biochemical reactions. Eugenol is the major compound found in the whole plant of *O. sanctum,* which is being used for pharmacological work. Saponins, which are seen in huge amounts in O. sanctum, are generally regarded as an anti-nutrient but are also believed to be useful in the human diet for controlling cholesterols. Therefore, its presence in this plant could suggest that the plant is of medicinal value. Saponins confirmed by the foam test was done in ancient days to identify the presence of saponins so that the given plant extract could be administered for stomach ailments. Tannins (Table 8) are also severe, tasting that precipitate and bind to different protein structures and destabilize them and is seen in various plants of Plantae kingdom. Tannins have usually considered non-nutritional phytochemical, but they may be applied pharmacologically in hemostatic, antidiarrheal, and anti-hemorrhoidal compounds in treating the various ailments. The chemical composition and structure are listed in Table 9.

Table 8. Phytochemicals recorded in Holy Basil

Phytoconstituents in *O. sanctum*	Presence or absence
Alkaloids	+
Tannin	+
Saponin	+
Steroid	+
Phlobatannin	+
Terpenoid	+
Flavonoid	+

+ = Present; - = Absent

Table 9. Phytochemicals present in *O. sanctum* (Chaudhary et al., 2020; Singh and Chaudhuri, 2018; Jacob et al., 2016)

SI No	Chemical Name	Common name	Chemical structure
1	2-Methoxy-4-(prop-2-en-1-yl)phenol	Eugenol	HO, O
2	(1R,4E,9S)-4,11,11-Trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene	Caryophyllene	H, H
3	(4aS,6aR,6aS,6bR,8aR,10S,12aR,14bS)-10-hydroxy-2,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydropicene-4a-carboxylic acid	Oleanolic acid	OH, H, O, HO, H
4	(1S,2R,4aS,6aR,6aS,6bR,8aR,10S,12aR,14bS)-10-Hydroxy-1,2,6a,6b,9,9,12a-heptamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid	Ursolic acid	OH, H, O, HO, H
5	(2*R*)-3-(3,4-dihydroxyphenyl)-2-{[(2*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}propanoic acid	Rosmarinic acid	OH, OH, O, HO, O, OH, HO, O
6	3,7-Dimethyl-1,6-octadien-3-ol	Linalool	HO

Sl No	Chemical Name	Common name	Chemical structure
7	2-Methyl-5-(propan-2-yl)phenol	Carvacrol	HO
8	(1E,5E,8S)-1,5-dimethyl-8-(prop-1-en-2-yl)cyclodeca-1,5-diene	Germacrene	

5.6. Antifungal and antibacterial properties of *O. sanctum*

Methanolic extract of *O. sanctum* possesses antimicrobial potential against both gram-negative and gram-positive bacterial strains (Yamani et al., 2016). Due to its vibrancy in the efficacy of both types of bacteria, it is considered the efficient drug of antimicrobial properties. The study shows a solid indication that extracts of *O. tenuiflorum* contain bioactive compounds that are of medicinal significance and thus justify using plant species as traditional medicine for the treatment of various diseases. It is also known for its antibacterial properties against gram-positive (*S. aureus* and *Bacillus subtilis*) and gram-negative (*Pseudomonas putida* and *K. pneumoniae*, *E. coli*) bacterias (Mallikarjun et al., 2016). A well-diffusion assay used to test the sensitivity of bacterial strains towards antibiotics with a clear zone around the well depicts the bacterial sensitivity towards antibiotics which showed *Ocimum* leaves extract exhibited significant inhibition levels against the five selected bacterial strains. According to Baliga et al. (2013), plant extracts of Tulsi were notably toxic against some fungal and gram-positive microorganisms and exhibited mild toxicity against *E. coli*. Basil is also considered one of the home remedies for simple fungal infections. The leaf juice was applied on the older days on the infected area for the ailment. On literature survey, the effect of basil leaves is studied on ten different strains, namely *Rhizoctonia solani*, *R. batticaloa*, *Phomasorghina*, *Colletotrichum gloeosporioides*, *F. pallidorosem*, *F. oxysporum* (ciceri), *Sclerotium rolfsii*, *Sclerotinia sclerotiorum*, *Alternaria solani,* and *A. alternate* and all the ten strains of fungi multiplication was reduced significantly by the different concentrations of the extract, but not even the single form could critically inhibit the growth on any one of these fungi strains. The boiled extract at 10% is far more effective than powdered, crude and ethanol extracts against five fungal pathogens, i.e., on *R. solani*, *R. batticaloa*, *S. rolfsii*, *S. sclerotiorum*, *A. solani*, and *A. alternata*. The efficacy of boiled, crude, and powdered extract against the respective fungal strains was progressively improved with the increase in the concentration level from 15 to 20%, but the total inhibition of test fungus was not recorded even at a maximum standard concentration of 20%. This may be because, in the boiled extract, the protein denaturation is done by tannins, and saponins are in the activated state, which directly acts on the cell membrane of the fungi and inhibits the growth of cancer in different cell types (Gurib-Fakim, 2006).

6. Prospects, challenges, and limitations

The world is nourished with innumerable types of different plants and animals all playing their unique role in maintaining the ecological balance of the natural system. Therefore, Ruskin Bond once says "every organism is the rightful inheritor of this earth". Plants have their unique properties which are of economical uses and very essentially, possess medical values. If exploited rightly, they are the cure for almost all diseases that mankind is suffering from today. Researchers must center their exploration in identifying the right plant for its medicinal properties which is used as medicine for the ailments of various disorders.

This review has enlightened on traditional medicinal plants, which are commonly used in the day-to-day life of every human being irrespective of continental barriers. These plants have been used as a medicinal substitute, food additive for a very long time, yet even today, true scientific significance is not understood by most common people. Therefore, amalgamating ancient wisdom and modern techniques is the only way to make scientific research more characterized.

We have focused on a limited number of plants due to the vastness of the available data; otherwise, it would be hard to concise the content and become illegible to read and understand. Also, the different approaches in analyzing the phytoconstituents are to be considered so that the specificity of the phytochemical and its biological effect is divulged more precisely.

7. Conclusions

The modern world has modern problems with modern diseases that need a combination of technology and ancient wisdom for smart solutions. Pathogens are also evolving as we are in this nature, so pharmacology is again an ever-evolving subject, and so is the life sciences. In India, it's commonly said that "every plant has its uses and side effects," which is true to every word and opens up a huge flood of opportunities for the researchers in quest of developing many drugs which are of medicinal importance. Today the world is split in differences; the isolation of phytochemical compounds is advantageous over the crude extract. One simple answer that could be derived is that the amalgamation of two polarized contexts must be bought together as every pathogen acts unique to different drugs. Isolated compound delivery may be very accurate in curbing the pathogen, but it has a high risk of developing resistance to the drug. One instance which could be given is the quinine resistance by *Plasmodium* sp within a decade. Contrastingly the cinnamon bark extract (decoction) administered over many years never showed the resistance, but the cure rate was slowed. So, the amalgamation of these two concepts of the research must be the need of the hour which shall pave a new pathway for the modern drug delivery strategies. As cited earlier, the plant-based extraction of drugs and their efficacy on many strains of bacteria, fungi, and other microorganisms has been the hot subject for researchers nowadays but needs strict vigilance of the eco-committee as many plants of economic value are on the verge of extinction.

In this review, the medicinal plants of incredibly common needs of the home were selected to discuss their antimicrobial and antifungal activities, which enhances their use of their cultivation and its uses on a large scale. Turmeric, a rhizome, has been used in many food delicacies used as a coloring agent, food ingredient, and flavoring

agent has many antiseptic, antimicrobial, and antifungal activities. It also has the anticancer property, which is still under investigation.

Ginger (*Z. officinale*) has long been used in naturopathy, Ayurveda, and Chinese medicine due to its potential antimicrobial activity against different pathogens. The study enables us to determine the antimicrobial activity of ginger using chloroform, ethanol, acetone, and petroleum ether solvents against *F. oxysporum* and *F. lycopersici*. These strains are infecting tomato crops, due to which the farmer is under loss. Biological pest control, which is the major strategy in IPM (integrated pest management), also advocates the use of natural products and biological means as the best treatment for the pests of crops.

Neem, which has been used as an antibacterial agent for a very long time, also shows the antifungal effects on different species of fungi against *Curvularia* sp. followed by that of *Aspergillus* sp. and *Fusarium* sp. Extracts of the neem tree (*A. indica*) showed to be pejorative against *Vibrio alginolyticus,* and *V. parahaemolyticus* worked on cultured marine shrimps. The fresh neem juice showed a zone of inhibition that reported a direct linear link to the concentration of neem juice against both bacterial strains.

O. sanctum (Holy basil) is another example of a plant with antibacterial and antifungal properties. There is an expensive possibility of chemical compounds including eugenol, ursolic acid, carvacrol, linalool, caryophyllene, limatrol, methyl carvacrol, sitosterol anthocyanins, etc. is found in this plant. The therapeutical studies describe the present review of the therapeutic value of *O. sanctum;* hence, the plant's utility for human and animal consumption and disease treatment strategies reinforces the significance of the ethnobotanical approach as a potential source of biogenic substances.

Acknowledgments

The author is gratified to PG Chemistry, Vijayanagara College Hosapete, for providing well-established research facilities.

Conflict of interest

The author confirms that there are no known conflicts of interest.

CRediT authorship contribution statement

Venkatesh K. Bhovi: The author performed all parts of the study alone.

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Supplementary File

None.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 111-117

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS
https://ijpbp.com

RESEARCH ARTICLE

OPEN ACCESS

Natural phenolic compounds from *Satureja* L. as inhibitors of COVID-19 protease (Mpro): Computational investigations

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ARTICLE INFO

Article History:

Received: 30 December 2021
Revised: 31 January 2022
Accepted: 31 January 2022
Available online: 06 January 2022

Edited by: E.S. Istifli

Keywords:
Satureja L.
Phenolic compounds
COVID-19
Protease inhibitor (Mpro)

ABSTRACT

Coronavirus (SARS-CoV-2) causes a new type of severe acute respiratory syndrome that first appeared in Wuhan in December 2019; it is a very fast-spreading and deadly virus. Therefore, urgent discovery or development of “lead compounds” against this virus is crucial. Natural compounds have always served as a great source, especially the use of traditional medicinal plants, in modern drug discovery. This study aimed to investigate the SARS-CoV-2 protease inhibition potential of the phenolic compounds in the genus *Satureja* L. The affinities of the chosen natural products were understood using molecular docking simulation against the SARS-CoV-2 protease enzyme. The study proved that three different phenolic compounds namely 5,6-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-7,8-dimethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5,6-dihydroxy-7,8-dimethoxy-4H-chromen-4-one, and 5,6-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-7,8-dimethoxy-4H-chromen-4-one obtained from *Satureja* L. taxa were found as promising against SARS-CoV-2 main protease.

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1. Introduction

Coronavirus, named after the crown-like spikes on their surface, is defined as a family of enveloped, single-stranded, and positive-stranded RNA viruses containing helical nucleocapsids (Coronaviridiae family, Nidovirales line). It has been known that they cause acute and chronic respiratory, enteric and central nervous system diseases both in animals and humans (Orhan and Deniz, 2020; Weiss and Navas-Martin, 2005). As of December 21, there are 275,839,211 COVID-19 cases, and 5,377,934 deaths from 219 different countries are confirmed, and the numbers, unfortunately, keep rising (WHO, 2020).

It is important to understand the virulence mechanisms of SARS-CoV-2 in order to design an effective drug molecule. Therefore, many studies are being carried out to understand the mechanisms; since the SARS-CoV-2 pandemic begins. As a result of the studies, several points exhibit which receptor/receptors are crucial for antiviral activity. Human ACE-2 can be recognized easier than the former virus by SARS-CoV-2; thus, the transmission capacity of human-to-human is expanding. On the other hand, the main protease determined as the PDB 6LU7 protein structure of SARS-CoV-2 is another significant receptor that plays a role in viral gene replication and expression (Rauf et al., 2021).

Concurrently, another property of the SARS-CoV-2 virus is that it possesses “genomic proofreading” in extremely few viruses. Thus, the virus doesn’t become either weak or inactive by repairing the mutated RNA. That is why the old antiviral drugs used for different viruses, such as ribavirin, have no effect (Robson et al., 2020). All in all, novel drugs are required to treat the disease.

Mpro in CoV possesses a key role during viral proteolytic maturation; as a result, it might be useful as a marker or potential target protein needed to reduce infection spread by inhibiting the viral polyprotein cleavage (Wax and Christian, 2020). As a result, Mpro protease in COVID-19 serves as a potential drug target for the treatment of COVID-19 infection. But SARS-CoV-2 has mutated

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e-ISSN: 2791-7509
doi:
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Please cite this article as: Başoğlu-Ünal, F., Tufan, S., Tan, N., 2022. Natural phenolic compounds from Satureja L. as inhibitors of COVID-19 protease (Mpro): Computational investigations. International Journal of Plant Based Pharmaceuticals, 2(1), 111-117.

many times; however, the main protease that plays a crucial role in viral gene expression and replication hasn't mutated yet. Therefore, the main protease, Mpro, is chosen to propose a probable natural anti-coronavirus product (Rajagopal et al., 2020; Rauf et al., 2021).

5-desmethoxynobiletin (1)
5-desmethoxynobiletin (2)
5-desmethoxynobiletin (3)
Apigenin (4)
Xanthomicrol (5)
genkwanin (6)
cirsimaritin(7)
cirsilineol (8)
6-Hydroxyluteolin-7,3'-dimethyl ether (9)
Chlorogenic acid (10)
gellic acid (11)
Rosmarinic acid (12)
vanillic acid (13)
Protocatechuic acid (14)
carnosic acid (15)
ferulic acid (16)
caffeic acid (17)
p-coumaric acid (18)

Figure 1. Natural phenolic compounds from *Satureja* L.

Under the given circumstances and urgent need to treat COVID-19 natural products, especially medicinal plants, have gone under investigation by different research groups (Bekut et al., 2018; Orhan and Deniz, 2020). The selection of medicinal plants is a deliberate process. Plant material itself or extracts obtained from medicinal plants have been safely used by various cultures worldwide for years (Barnes et al., 2007; Heinrich, 2000; Momtaz and Abdollahi, 2010; Mukhtar et al., 2008). The efficacy, availability, and affordability of medicinal plants have always been the key point in folk medicine. Also, the use of these medicinal plants for years eliminates most of the question marks against their safety as well as their use as food (Barnes et al., 2007). Along with direct use of plant material or

extracts, approximately 25% of the synthetic active compounds in current treatment are derived from natural compounds (Momtaz and Abdollahi, 2010).

Lamiaceae is one of the most important and large plant families, which is widespread. Due to their chemical constituent profile is widely used in both pharmaceutical and food industries (Bekut et al., 2018; Raja, 2012). Moreover, taxa in the Lamiaceae family have drawn attention due to their antiretroviral properties (Abad et al., 1999; Bekut et al., 2018). Newly published studies exhibited that the extracts from Lamiaceae such as *Mentha piperita*, *Mosla* sp., *Ocimum kilim,* and *scharicum* possessed antiviral effects against coronavirus. Additionally, the extracts were reported to have high inhibitory activities on ACE2 receptors and COVID Mpro (Jalali et al., 2021).

The genus *Satureja* L. belongs to the Lamiaceae and contains terpenoids and phenolic compounds as major compounds (Davis, 1982; Tepe and Cilkiz, 2016). Especially infusions and decoctions, which are rich in phenolic compounds, prepared from the *Satureja* L. taxa, have traditionally been used against cold, flu, wound antiseptic, cough in Turkey (Chorianopoulos et al., 2006; Emre et al., 2020; Giweli et al., 2012; Güllüce et al., 2003; Ilhan et al., 2020; Ozcelik et al., 2011), a muscle pain reliever, tonic, and carminative in the treatment of stomach and intestinal ailments such as cramps, nausea, indigestion, and diarrhea (Zargari, 1990). Along with ethnobotanical uses, researches revealed that *Satureja* L. taxa have antimicrobial, antioxidant, antiviral, anti-diabetes, anti-hyperlipidemic, reproductive stimulating, expectorant, and vasodilator activities (Abdollahi et al., 2003; Amanlou et al., 2005; Momtaz and Abdollahi, 2010; Sahin et al., 2003; Tepe and Cilkiz, 2016; Vosough-Ghanbari et al., 2010).

Table 1. Natural phenolic compounds docking results based on binding energy calculations and their ADMET properties

Compound name	Docking score[a]	MW[b]	QlogPo/w[c]	Human oral absorption %[d]	PSA[e]	HBD[f]	HBA[g]	Lipinski rule of five
NativeLigand (RZS)	-5.421	147.18	1.872	93	48.57	1	3	0
1	-7.190	360.32	2.004	79	117.30	2	6	0
2	-7.182	374.35	2.715	92	103.50	1	6	0
3	-6.903	360.32	1.994	79	117.30	2	6	0
4	-6.803	272.26	1.603	74	99.25	2	4	0
5	-7.018	346.34	2.594	91	95.27	1	5.5	0
6	-6.889	284.27	2.384	87	84.81	1	3.75	0
7	-6.573	314.29	2.593	90	91.05	1	4.5	0
8	-6.533	344.32	2.655	91	98.81	1	5.25	0
9	-6.690	330.29	1.815	76	113.16	2	5.25	0
10	-7.804	354.31	-0.298	18	180.43	6	9.65	0
11	-6.870	170.12	-0.586	41	115.35	4	4.25	0
12	-6.570	372.33	1.392	40	165.92	5	7	0
13	-5.599	168.15	1.005	66	80.25	2	3.5	0
14	-4.915	154.12	0.014	53	93.92	3	3.5	0
15	-5.208	332.44	3.543	89	67.22	3	3.5	0
16	-4.872	194.19	1.268	66	82.41	2	3.5	0
17	-4.968	180.13	0.519	54	96.08	3	3.5	0
18	-8.841	164.16	1.403	67	74.65	2	2.75	0

[a]Docking score (kcal/mol).
[b]Molecular weight (g/mol) (recommended value ≤ 500)
[c]Logarithm of the octanol/water ratio coefficient of compound (recommended value < 5).
[d]Percentage oral absorption (< 25% weak and > 85% strong).
[e]Polar surface area (Å) (recommended value ≤ 140 Å).
[f]Hydrogen bond donar (recommended value ≤ 5)
[g]Hydrogen bond acceptor (recommended value ≤ 10)

Initially, it is necessary to evaluate the probable inhibitory effects of natural compounds by screening. For this reason, keeping in view the pharmacological activities based on previous literature, 18 natural compounds from *Satureja* L. were selected. ADMET property calculations were also estimated for all selected compounds, and the interactions with the active site (PDB ID: 5R82) were visualized.

In light of the information provided above, phenolic compounds of *Satureja* L. are a great "starting point" in discovering and developing lead compounds against COVID-19. In this research, molecular docking studies were performed on 18 phenolic compounds (Figure 1).

2. Materials and methods

2.1. Data set

Chlorogenic acid (1) (Silva et al., 2009; Tepe and Cilkiz, 2016), 5,6-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-7,8-dimethoxy-4H-chromen-4-one (2) (Moghaddam et al., 2007), 2-(3,4-dimethoxyphenyl)-5,6-dihydroxy-7,8-dimethoxy-4H-chromen-4-one (3) (Gohari et al., 2009), 5,6-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-7,8-dimethoxy-4H-chromen-4-one (4) (Gohari et al., 2009), gallic acid (5) (Cetojevic-Simin et al., 2004), apigenin (6) (Cetojevic-Simin et al., 2004), xanthomicrol (7) (Malmir et al., 2012), genkwanin (8) (Skoula et al., 2005), cirsimaritin (9) (Skoula et al., 2005), rosmarinic acid (10) (Cetojevic-Simin et al., 2008), cirsilineol (11) (Skoula et al., 2005), 6-hydroxyluteolin-7,3i-dimethyl ether (12) (Skoula et al., 2005), vanillic acid (13) (Palavra et al., 2011), protocatechuic acid (14) (Palavra et al., 2011), carnosic acid (15) (Kosar, 2003), ferulic acid (16) (Askun et al., 2013; Cetojevic-Simin et al., 2012), caffeic acid (17) (Cetojevic-Simin et al., 2012), and *p*-coumaric acid (18) (Askun et al., 2013; Cetojevic-Simin et al., 2012) were identified previously in *Satureja* L. taxa.

2.2. Molecular docking

The molecular docking studies of 18 phenolic compounds on COVID-19 Mpro (Hall Jr and Ji, 2020) were examined using the Schrödinger program. COVID-19 protease crystal structure was retrieved from protein data bank (PDB ID: 5R82: Resolution 1.31 Å) (Rajagopal et al., 2020; Wax and Christian, 2020). The docking calculations were carried out using the Glide SP (standard precision) module of the Schrödinger Suite (Friesner et al., 2004, 2006; Halgren et al., 2004). The RMSD value between the docked pose and the crystal

conformation of the native ligand (6-(ethylamino)pyridine-3-carbonitrile) was found as 1.595 Å.

The ADMET properties of the selected compounds such as molecular weight, HBA, HBD, logPo/w were determined by the QikProp module (Ligprep and Macromodel, 2011) of the Schrodinger suite (Table 1).

3. Results and discussion

A docking study was performed on 18 phenolic compounds from *Satureja* L. against SARS-CoV-2 protease (Mpro). Tables 1 and 2 show the docking score and amino acid residues, which form various interactions with the chosen compounds, while Figure 2 shows 2-dimensional ligand-receptor interactions of probable more active compounds. Additionally, their 3-dimensional ligand-receptor interactions are displayed in Figure 3.

In this study, 18 phenolic compounds show hydrogen bond and π- π interaction with many amino acid residues in the active region but active/inactive ranking of the compounds cannot be done precisely based on the energy values obtained as a result of primitive scoring algorithms (Başoğlu et al., 2021). Despite that, pharmacologically active conformations of the ligands and the determination of their binding modes to the receptor can be done successfully. Therefore, the binding affinities of the chosen natural phenolic compounds were determined by analyzing binding conformations and interactions of each compound with the active site of the receptor.

Table 2. Receptor interaction with chosen phenolic compounds and RZS into the binding site in the COVID-19 main protease

Compound name	Receptor amino acids
NativeLigand (RZS)	Gln189, His41
1	Thr26, Gln189, Gly143
2	Thr26, Gly143, His41
3	Thr26, Gly143, His164, His41
4	Phe140, Glu166, Arg188
5	Glu166, Gly143, Cys44
6	Thr190
7	Arg188
8	Thr26, Gly143, Glu189
9	Thr26, Gly143, Glu189
10	Gly143, Thr190
11	Gly143, His41
12	Phe140, Glu166
13	Gly143, Glu166
14	His41, Gly143
15	Glu166
16	Gly143, Gln189, His41
17	Gly143, Gln189, His41
18	His41

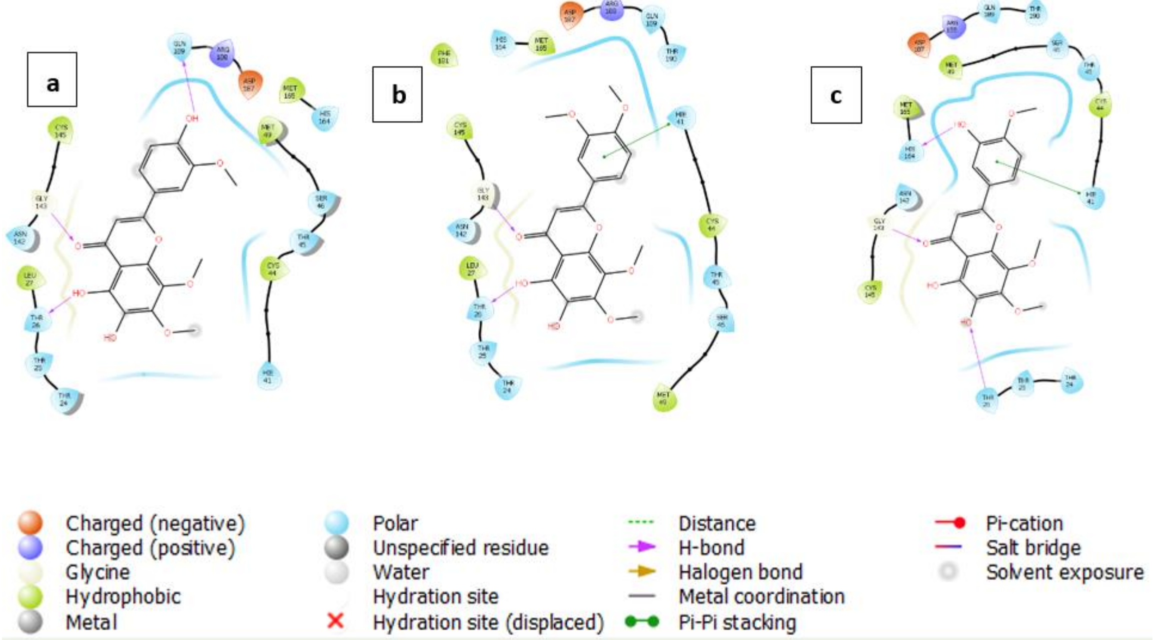
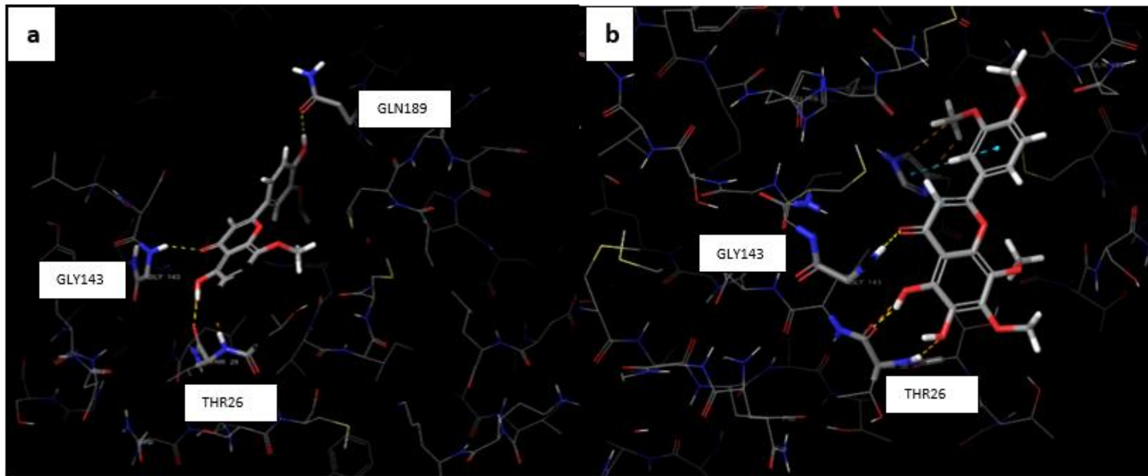


Figure 2. 2D representation of docking of compounds: compound 1 (a), compound 2 (b), compound 3 (c) into the binding site of the COVID-19 main protease

Considering the previous *in silico* studies reported in the literature, it is obvious that some amino acid residues such as Gln19, Thr24, Thr25, Thr26, Leu27, His41, Ser46, Met49, Asn119, Asn142, Gly143, His164, Met165, Glu166, Asp187, Arg188, and Gln189, might play a strong role in the inhibitory activity (Rajagopal et al., 2021; Thirumalaisamy et al., 2021).

Compounds 1, 2, 3, 8, and 9 looks similar with minor differences. Considering these differences and their binding poses, -OH at 4th position on phenyl is required for H-bond with Gln189, a critical amino acid residue. The significant H-bond with Gln189 is formed with -OH at the 4th position of the phenyl of compounds 16 and 17.



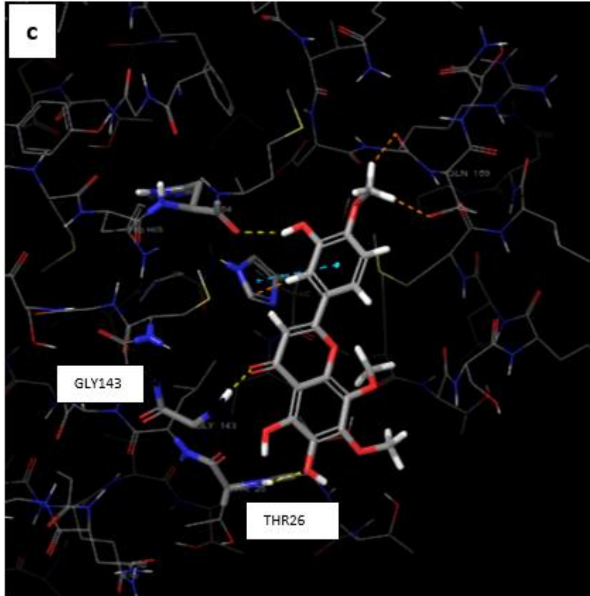


Figure 3. 3D representation of docking of compounds: compound 1 (a), compound 2 (b), compound 3 (c) into the binding site of the COVID-19 main protease
(The yellow dash bond indicates H-bond, the blue dash bond illustrates pi-pi interaction.)

Compounds 1, 2, 3, 8, and 9 generate H-bond with Thr26, which is also among one of the crucial amino acid residues against SARS-CoV-2. Flavonoids could be a significant moiety for H-bond generation since these carry hydroxy moiety at either 5th or 6th position. However, compounds 6 and 7 have an H-bond with different amino acid residues, Thr190 and Arg188, respectively. Interestingly, their structures are similar to compounds 1, 2, 3, 8, and 9. Carbonyl group and 5th position -OH of flavonoid are located at 5Å distance from the Gln189, Thr26 amino acid residue due to their positions within the receptor active site. Therefore, no H-bond is formed with these amino acids, which are predicted to be important for activity. The same state is true for compound 18. The only amino acid residue that can form H-bond with -OH at the 4th position of phenyl is His41; therefore, it's important for its activity.

Additionally, an extra pi-pi interaction is observed between compounds 2, 4, 11, 16, 17, and 18's aromatic ring and His41 amino acid residue.

Compounds 1, 2, and 3 generated more hydrophobic interactions with amino acid residues, significant for the inhibition effectiveness. As seen clearly in Figure 3, compound 1 showed hydrophobic interaction with Thr26, compound 2 exhibited hydrophobic interaction with Thr26 and His41, and compound 3 generated 3 hydrophobic interactions with different amino acids residues Arg188, Gle189, and His49. That might be why the docking scores of compounds 1, 2, and 3 are higher than the other chosen phenolic compounds.

Furthermore, chemo-informatic properties were calculated using the QikProp module in Maestro Schrödinger. The compounds' permeability and solubility must be estimated using these evaluations, especially for novel drug discovery, which will be administrated orally. Lipinski's rule of 5 foretells whether compounds have good absorption and permeation. Regarding the rule of 5, for good absorption and permeation properties, the number of hydrogen bond donors, acceptors, molecular weight, and

octanol/water coefficients should be within certain limits (Lipinski et al., 1997). The selected 18 phenolic compounds from *Satureja* L. showed acceptable values and obeyed the rule of 5 (Table 1). By considering their logPo/w values, it might be possible that the affinity for the target of protease increased when logPo/w approached two.

Moreover, PSA is another parameter used for the drug's optimization ability to permeate cells. Preferably, from the previous research point, the PSA value should be less than 90 (Başoğlu et al., 2021). But almost all the chosen 18 compounds except compounds 6, 13, 15, and 16 possess higher values than the standard.

4. Conclusions

Medicinal plants have always had an important role in modern drug discovery and may also be a pioneer for COVID-19 treatment. As a result of this study, we recommended 3 different natural phenolic compounds found in *Satureja* L. taxa as promising against SARS-CoV-2 main protease inhibitors by evaluating their affinities against SARS-CoV-2 main protease and also ADMET properties. It is anticipated that compounds 1, 2, and 3 might be promising inhibitors of the main protease, and further *in vitro* studies can be conducted based on the preliminary *in silico* study carried out in this work.

Acknowledgments

The support of Rita Podzuna, who offered a free trial of Schrödinger from Schrödinger GmbH, is greatly appreciated. Additionally, we thank Dr. Abdulilah Ece from Biruni University for his valuable comments and support.

Conflict of interest

The authors confirm that there are no known conflicts of interest.

CRediT authorship contribution statement

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Selin Tufan: Resources, Conceptualization, Data curation, Writing-original draft, Methodology
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Supplementary File

None.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 118-127

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

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REVIEW

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Anthocyanins: Plant-based flavonoid pigments with diverse biological activities

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ARTICLE INFO

Article History:

Received: 28 December 2021
Revised: 30 January 2022
Accepted: 04 February 2022
Available online: 07 February 2022

Edited by: B. Tepe

Keywords:
Anthocyanins
Natural pigments
Antioxidants
Cardiovascular protecting agents
Neuroprotective agents

ABSTRACT

Anthocyanins are flavonoid containing polyphenolic phytochemicals. They are widely present in plants and accounts for different color shades displayed by the plant organs. A broad range of health-revitalizing effects is attributed to anthocyanins, constituting a vital part of the human diet. They are also accountable for ameliorating the detrimental effects of various lifestyle diseases, including cancer, cardiovascular disorders, neurological disorders, etc. These beneficial impacts highly depend on the bioavailability of anthocyanins, governed by their absorption and metabolism in the human body. The primary goal of this review is to summarize the latest anthocyanin knowledge while focusing on the chemistry, pharmacokinetics, and various biological advantages with anti-cancer, neuroprotective, antidiabetic, antioxidant, cardiovascular protective, vision improvement, antiviral, and antimicrobial effects.

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1. Introduction

The term Anthocyan has been derived from the Greek word Anthos, which means flower, and kyanos means blue (Marquart, 1835). It was introduced by Ludwig Clamor Marquart, a German pharmacist, to distinguish the blue color pigments of various flowers. In the plant kingdom, most species contain anthocyanins (Richardson and Harborne, 1990). They exist in all tissues, including leaves, stems, fruits, flowers, and roots of the higher plants. Anthocyanins are found in the cell vacuole, which gives color shade to the plant tissue such as red, blue, and purple as per their essential structure organization. Fruit plants such as red raspberry, blackberry, bilberry, blueberry, grapes, zante currants, cherry, blood orange, red cabbage, red onion, purple sweet potato, red-skinned potato, fennel, eggplant, and radish can be found in these plants (Table 1).

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e-ISSN: 2791-7509
doi:
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Anthocyanins play a shielding role in vegetables and plants, including monosaccharide transport vehicles, the absorbance of harmful radiations, and osmotic adjuster during frost or drought (Clifford, 2000). These also play a critical role in attracting livestock facilitating pollination and dispersal of seeds (Eder, 2000; Timberlake and Henry, 1988).

Recently, anthocyanins have been the primary focus of researchers for their pharmacological effects, biological properties, and high-water solubility. Certain nutritionists are progressively suggesting the utilization of foods containing anthocyanins. They are less active compared with related drugs but show physiological results of great importance when consumed in a regular diet. In traditional medicinal items, several red flowers (clove, hibiscus, pineapple sage, and rose), purple (passionflower, lavender, and purple sage), and blue (blue rosemary and cornflower) are used (Calderaro et al., 2020).

A few exceptional reports have delineated these natural dietary phytochemicals in terms of their intake (McGhie and Walton, 2007), metabolism (He and Giusti, 2010), pharmacokinetics (Kay, 2006),

Please cite this article as: Patra, S., Makhal, P.N., Jaryal, S., More, N., Kaki, V.R., 2022. Anthocyanins: Plant-based flavonoid pigments with diverse biological activities. International Journal of Plant Based Pharmaceuticals, 2(1), 118-127.

molecular mechanisms (Li et al., 2017), and bioavailability (Fernandes et al., 2014) over the past few years. In recent years, the enthusiasm for studying anthocyanins' health and medical benefits has increased rapidly. Vanamala (2019) has summarized the effect of red and purple anthocyanins on colon cancer stem cells. Khan et al. (2019) have checked out the neuroprotective effect of anthocyanins, whereas Krga and Milenkovic (2019) have described the impact of anthocyanins on cardiovascular diseases.

Table 1. Total anthocyanin content in common fruits and vegetables (Clifford, 2000; Eder, 2000; Timberlake and Henry, 1988)

Fruit or vegetable	Total anthocyanin content (mg/kg)
Cherry	3500-4500
Cranberry	460-2000
Strawberry	127-360
Grapes (blue)	80-3880
Orange	2000
Grapes (red)	300-7500
Plum	19-250
Cabbage (red)	250
Onion (red)	Up to 250
Bilberry	4600
Eggplant	7500
Elderberry	2000-15600
Radish (red)	110-600

This review describes the chemistry of anthocyanins and recent literature about various biological benefits of anthocyanins, such as anti-cancer activity, neuroprotective activity, anti-diabetic, antioxidant, cardiovascular protecting activity, vision improvement, antiviral and antimicrobial effects (Figure 1).

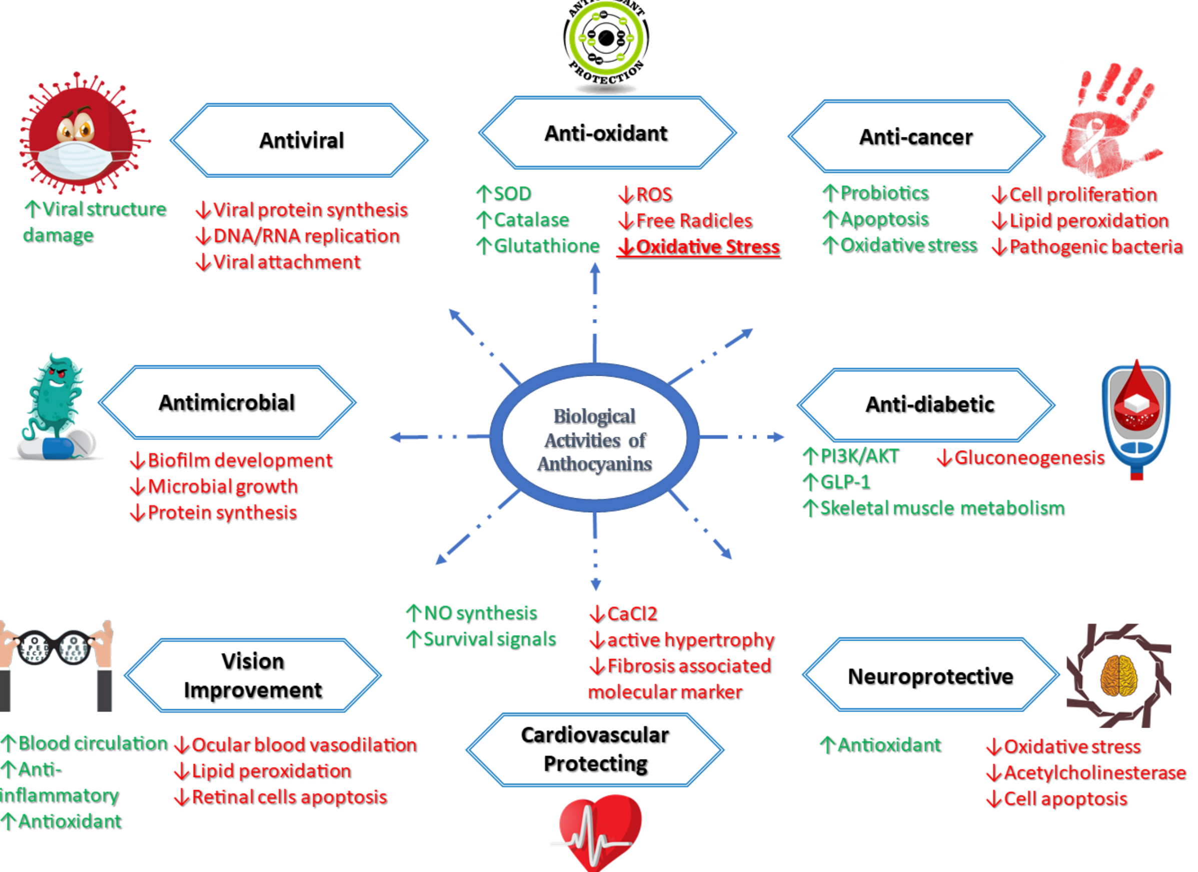

Figure 1. Biological benefits of anthocyanins

2. Chemistry of anthocyanins and anthocyanidins

Anthocyanins are anthocyanidin glycosides, have a distinctive carbon skeleton with 2 phenyl rings (A and B rings, Figure 2) and a heterocyclic pyran (C ring) fused with A ring (Castañeda-Ovando et al., 2009). Cytoplasmically-localized glycosyltransferases generally catalyze glycosylation, and the most common sugars are L-arabinose, L-rhamnose, D-galactose, D-glucose, and D-xylose. Sugars are usually bound to the 3-hydroxyl position of anthocyanidins or the 5- or 7- position of anthocyanidins (Pina et al., 2012). Anthocyanidins (aglycone) are chemically flavylium salts or (2-phenylchromenylium) ion compounds. With a molecular weight of 207.25, the molecular formula for the flavylium ion is $C_{15}H_{11}O^+$. The biosynthetic process of anthocyanins is initiated from the acetate

and shikimate pathways. Anthocyanins are transported into the vacuole by 3 possible mechanisms after completion of their biosynthesis: (i) via vesicle-mediated mass transport; (ii) via glutathione S-transferase-like protein; and (iii) via flavonoid/H^+ antiporter (Zhang et al., 2006). In the vacuole, the anthocyanins occur as the equilibrium of four molecular species, are essentially flavylium cation (I), the quinoidal base (II), the carbinol pseudo base (III), and the chalcone pseudo base (IV) (called as secondary structures, Figure 3) (Prior and Wu, 2006).

Over 600 anthocyanins have been identified, and the number continues to increase exponentially (Kong et al., 2003). Anthocyanins can be distinguished based on (i) the number and location of the hydroxyl group, (ii) the degree of hydroxyl group methylation, (iii) the number and nature of sugar (glycon) molecules linked to the aglycone portion, and (iv) the acylation of the sugar (aliphatic or aromatic acid) molecules. Caffeoylated anthocyanin, malonylated anthocyanin, acrylated anthocyanin, and coumaroylated anthocyanin can be further categorized as the acylated form of anthocyanins. Both color and hue of anthocyanins are affected by hydroxylation, glycosylation, methylation, and acylation pattern of the phenyl constituent of the anthocyanidins, and the color varies from orange-red (cyanidin, pelargonidin) to purple and pink-magenta (petunidin, delphinidin, malvidin, and peonidin) (da Costa et al., 2000; Iversen, 1996; Keppler and Humpf, 2005; Khoo et al., 2017; Lo Piero, 2015).

Glycon = D-glucose, D-galactose, D-xylose
L-rhamnose, L-arabinose

S. No.	Anthocyanidin (or aglycone)	R^1	R^2	Distribution in fruits and vegetables (%)	Major metabolic degraded products
1	Cyanidin	OH	H	50	Protocatechuic acid
2	Delphinidin	OH	OH	12	Gallic acid
3	Pelargonidin	H	H	12	4-Hydroxybenzoic acid
4	Petunidin	OCH_3	OH	7	3-*O*-methylgallic acid
5	Lanvidin	OCH_3	OCH_3	7	Syringic acid
6	Peonidin	OCH_3	H	12	Vanillic acid

Figure 2. Anthocyanidins relevance to human diet - general structure, distribution, and metabolic products (Leong et al., 2018; Turturică et al., 2015)

It is currently thought that six anthocyanins are relevant to the human diet (Figure 2). Their distribution in fruits and vegetables are 50% cyanidin, 12% delphinidin, 12% pelargonidin, 12% peonidin, 7% malvidin, and 7% petunidin (Leong et al., 2018; Turturică et al., 2015).

Due to the presence of electron-deficient flavylium cation, anthocyanins are sensitive and highly reactive, and thus easier to be degraded (Felgines et al., 2007; Yang et al., 2011). Anthocyanin's stability is affected by its molecular structure. Due to the reactive -OH group; delphinidin and cyanidin are considered more stable than peonidin, malvidin, and petunidin. Different transformations at position 4 resulting in hetero-dimers, condensation, and cycloaddition products, make anthocyanins more durable. Glycosylation at position 3 makes it stable, whereas glycosylation at position 5 leads to instability. The stability of anthocyanins increases with acylation by intramolecular pigmentation and self-association reactions (Clifford, 2000).

Since the anthocyanins' molecular structure has an ionic nature, the solution's pH determines the anthocyanins' color (Fossen et al., 1998). In acidic solution (pH 1-3), some of the anthocyanins appear red due to flavium cation formation (I), whereas in neutral pH, they appear colorless due to carbinol pseudo base formation (III). At the same time, color changes from blue to purple as pH increases to 7-8 due to the quinoidal base formation (IV) (Figure 3) (Hribar and Poklar Ulrih, 2014). It is determined that the stability of anthocyanins is directly proportional to the density of their solutions, but intermolecular interaction within the formulation often improves balance by hindering water-based anthocyanin deterioration.

3. Absorption of anthocyanins

In recent years, the study of dietary intake and health-promoting effects of anthocyanins has been increased. The aiding and therapeutic benefits of anthocyanins directly depend on the pharmacokinetic properties (absorption, distribution, metabolism, and excretion) and consequently bioavailability of individuals. The absorption mainly relies on the chemical structure, nutrient components of food, gut microbiota, and individual genetic factors (Felgines et al., 2007; Yang et al., 2011). For instance, anthocyanins based on pelargonidin or 3'-hydroxyanthocyanins are more quickly absorbed than other anthocyanins with more B-ring substituents. Usually, non-acylated anthocyanins show more absorbance capacity as compared to acylated anthocyanins.

The absorption of anthocyanin occurs within the stomach and mainly occurs in the small intestine, although it is absent in the intestinal tract. Bilitranslocase, an organic anion membrane transporter located in the gastric mucosa, produces gastric absorption (Passamonti et al., 2002). Injection of red grape wine anthocyanins into the stomach of Wistar male rats, followed by the blood from the portal vein analysis, confirmed the existence of malvidin-3-*O*-glucoside in the heart (Passamonti et al., 2003). Similarly, hexose transporters expressed on intestinal epithelium are accountable for anthocyanins' active and passive transport (Han et al., 2019). Talavéra et al. (2004) used an *in situ* perfusion procedure to examine the absorption of anthocyanins in the small intestinal

tract. The rate of anthocyanins absorption in the small intestine was determined by measuring the number of anthocyanins left in the effluent; the absorption rate for cyanidin-3-*O*-glucoside was found to be 22%, and for malvidin-3-glucoside, it was found to be 10-11%. He et al. (2009) determined that 7.5% of the black raspberry anthocyanins administered were absorbed into the small intestinal tissues of rats. In line with the above predictions, animal model studies also demonstrate that anthocyanins quickly appear in the systemic circulation (6-20 minutes) and reach a maximum blood level after 15-50 minutes (Pojer et al., 2013). However, few experimental studies demonstrate contradictory results, presenting that there is a minimal quantity of anthocyanins (i.e., < 1% of the consumed amount) in systemic blood circulation or the plasma (Prior, 2004).

Figure 3. Anthocyanins equilibria (Prior and Wu, 2006)

Temperature, intestinal pH, and the microbes influence the digestion of anthocyanins in the human intestinal tract (Hidalgo et al., 2012). Breaking the glycosidic linkage among the sugar and aglycone moieties is activated by intestinal microflora and releases aglycone. The microbes responsible for the metabolism include *Clostridium*, *Bacteroides*, *Eggertheilla*, *Ruminococcus*, and *Eubacterium* (Blaut and Clavel, 2007; De Ferrars et al., 2014; Jang and Kim, 1996). The basic conditions and microbiota further convert aglycons into phenolic aldehydes and acids (Hanske et al., 2013). When cyanidin-3-glucoside was incubated with the intestinal bacteria, a critical aggregation of metabolic degradation products including 2,4,6-trihydroxy benzaldehyde and protocatechuic acid was brought about. The reported primary metabolites from anthocyanins relevant to the human diet are protocatechuic acid, 3-*O*-methyl gallic acid, 3,5-dihydroxybenzoic acid, vanillic acid, and syringic acid contingent upon the parent anthocyanin structure (Figure 2) (Muñoz-González et al., 2013; Nurmi et al., 2009). The usual chemical transformations such as demethylation, hydrogenation, hydroxylation, demethoxylation, decarboxylation, hydrolysis, and dehydroxylation occur during the degradation of anthocyanins and their metabolites (Forester and Waterhouse, 2008; Stalmach et al., 2013).

4. Biological activities of anthocyanins

4.1. Antioxidant effects

Compounds that are easier to oxidize are often termed antioxidants. They can also be described as molecules, giving free hydrogen or electron atom to react with free radicals. Several therapeutic activities of anthocyanins are mainly contributed by their antioxidant property (Joshi et al., 2017; Lucioli, 2012). Anthocyanins' free radical scavenging property confers a superior antioxidant activity. The antioxidant activity of anthocyanins relay upon its structure, and the properties are influenced by (i) the B ring catechol moiety; (ii) the number of –OH group; (iii) the hydroxylation and methylation pattern; (iv) acylation; (v) glycosylation; and (vi) the oxonium ion present in the C ring. Glycosylation of anthocyanins lessens the antioxidant properties due to the inability of anthocyanin radicals to delocalize its electron. Acylation of anthocyanins with phenolic acids shows a prominent result of increased antioxidant activities. Diacylation of anthocyanins also shows increased antioxidant activity, whereas 5-glycosylation minimizes its activity. When comparing anthocyanins with oligomeric proanthocyanidins and other flavonoids, anthocyanins' antioxidant strength and hydrogen-donating effect are greatly

affected by the positive charged O_2 atom in the scaffold (Shaik et al., 2018; Smeriglio et al., 2016). There may be two mechanisms by which anthocyanins impose their antioxidant effect, i.e., direct and indirect pathways. These exhibit direct free radical scavenging behavior due to the hydrogen (electron) donating potential of the flavonoid molecules, which can form a bond with reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydroxyl radical species ($^·OH$), and hydrogen peroxide (H_2O_2) (Borkowski et al., 2005; Janeiro and Brett, 2007; Tsuda et al., 1996). On the other hand, anthocyanins increase the antioxidant property through various mechanisms such as: (i) increasing or restoring the superoxide dismutase (SOD), glutathione peroxidase, and the antioxidant enzymes, consequently elevating glutathione content; (ii) decreased production of DNA oxidative adducts, decreased production of endogenous ROS by obstructing xanthine oxidase and NADPH oxidase, or by altering the arachidonic metabolism and respiration of mitochondria; and (iii) activation of genes that code for the above antioxidant enzymes.

Anthocyanins-rich food mostly contains cyanidin, malvidin, peonidin, and these all play a crucial role in scavenging radicals and protecting them from DNA damage. For example, eggplant shows antioxidant activity by scavenging the oxygen-free radical with > 65% scavenging activity achieved in 2,2-diphenylpicrylhydrazyl (DPPH) assay (Li et al., 2012). Similarly, sweet cherry (*Prunus avium* L.) anthocyanins demonstrate antioxidant properties with above 70% activity on the DPPH (Sonmez et al., 2022). Likewise, blackberries exhibit the scavenging action against superoxide radicals, and the anthocyanins present in red cabbage protect it from oxidative stress (Igarashi et al., 2000; Murapa et al., 2012; Wang and Jiao, 2000; Zafra-Stone et al., 2007). Delphinidin-3-glucoside (IC_{50} values: 1.6 and 0.7 µM) and delphinidin (IC_{50} values: 2.6 and 0.9 µM) shows maximum inhibitory effect against $^1O_2$ scavenging activity and lipid peroxidation, respectively, whereas pelargonidin has the greatest inhibitory effect on the -OH radical scavenging activity with IC_{50} value of 8.5 µM (Tsuda et al., 1996). The presence of 3-OH groups on the B ring advances the antioxidant properties of delphinidin. However, oxidation of the hydroxyl group can be achieved by donating one electron (semiquinone form) or two-electron (quinone form).

4.2. Anti-cancer activity

Anthocyanins' chemopreventive effects are largely due to their anti-inflammatory and antioxidant activities. The consumption of anthocyanin-rich foods and vegetables leads to anti-cancer effects through different mechanisms like (i) anti-angiogenesis and inducing apoptosis, (ii) prohibiting oxidative DNA damage, (iii) inhibiting COX-2 enzymes (iv) inhibition of cell proliferation. 2.5-100 µM extracts from anthocyanin-containing berries of different types and eggplant and black rice tend to inhibit the invasion of cancer cell types in Matrigel (Wang and Stoner, 2008). Strong anti-proliferative activity was shown by the hydrolyzed pulp of Jamun with an IC_{50} value of 59 ± 4 µg/ml. Anthocyanins extracted from berries, especially black raspberries, help lower the epigenetic factors involved in cancer. Histone modification and DNA methylation are part of this epigenetic regulation of gene expression. They are involved in the demethylation of tumor suppressor genes and the suppression of DNA methyl transferase and thus help prevent cancer (El-Ella and Bishayee, 2019). Studies of anthocyanin extracts *in vivo* and *in vitro* (cancer cells) have shown anti-carcinogenic effects on breast cancer (Singletary et al., 2007), prostate and liver cancer (El-Ella and Bishayee, 2019; Lin et al., 2017), colon cancer (Jaganathan et al., 2014) and lung cancer. Anthocyanin rich extract of *Syzygium cumini* (Aqil et al., 2012), purple-fleshed sweet potato (clone P40) (Lim et al., 2013), raspberries, strawberry (Giampieri et al., 2018; Zhang et al., 2008), sorghum, black lentil, and red grapes exhibited anti-proliferative action on the cancer cells (Grimes et al., 2018; Mazewski et al., 2018). Anthocyanidins seem to inhibit different kinase pathways and show better antiproliferative activity than anthocyanins (Zhang et al., 2005). Delphinidin-3-glucoside and cyanidine-3-glucoside inhibit the development of colon cancer HT-29 cells (Grimes et al., 2018). Delphinidin has the best inhibitory characteristics as it possesses a hydroxyl group in its structure, which seems to show its effect by halting the MAPK pathway (Hou, 2003). Anthocyanins, gallic acid, 3-*O*-methyl gallic acid, and 2,4,6-trihydroxybenzaldehyde metabolites reduce cell viability and apoptosis of colon cells in Caco-2 cells (Forester et al., 2014). A recent study shows that the petal anthocyanins from *Crocus sativus* (saffron) have a beneficial effect on the mouse model with polycystic ovary syndrome. Saffron petal anthocyanins hinder the polycystic ovary syndrome by improving antioxidant enzymes, steroidogenic dysregulation of ovarian steroids, and inflammatory markers (Moshfegh et al., 2022). Likewise, it was found that the total anthocyanins extracted from sweet cherry (*Prunus avium* L.) inhibit human carbonic anhydrase I and II enzyme activities and thus show the anticancer effect (Sonmez et al., 2022).

4.3. Anti-diabetic activity

Diabetes (Type-2) mainly emerges because of continuous stress on the pancreas, β-cells degeneration, decreased insulin level, and expanded insulin resistance. In hyperglycemic and hyperlipidemic states, there is high oxidative stress upon pancreatic β-cells, which may lead to the execution of these cells. Consumption of anthocyanins can reduce free radicals' excessive production, which prevents the β-cells from oxidative damage (Al-Awwadi et al., 2005; Cao et al., 2019; Hossain et al., 2016). Anthocyanins inhibit alpha-glucosidase, an enzyme responsible for the hydrolysis of small intestinal carbohydrates that helps control diabetes (Zhang et al., 2019). Similarly, the anti-inflammatory effects of anthocyanins manifested by decreasing monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6) expression help in the recovery of insulin resistance and type 2 diabetes (Khanra et al., 2015; Zhu et al., 2012). Infusion of pelargonidin on streptozotocin (STZ)-induced diabetes rats brought about an increased serum level of superoxide dismutase and lowered the level of malondialdehyde and fructosamine (Roy et al., 2008). Cyanidin, a major anthocyanidin present in plants, vegetables, and fruits, helps in inciting insulin secretion by raising the level of intracellular calcium in pancreatic β-cells (Pesce and Menini, 2019). Zhu et al. (2012) examined the effect of cyanidin-3-glucoside on HepG2 cells and was found to increase the expression of glutamate-cysteine ligase, which reduces the level of ROS. Black soybean seed coat extract contains peonidin-3-*O*-glucoside, delphinidin-3-O-glucoside, and cyanidin-3-*O*-glucoside, showing protection against obesity and diabetes, reducing oxidative stress, inflammation, level of α-amylase, and lipid aggregation in adipocytes in HepG2 cells. Fasting blood glucose (FBG) and oral glucose tolerance test (OGTT) results obtained in diabetic mice showed black soybean seed coat extract (BSSCE) to exhibit the hypoglycemic effect. BSSCE could alleviate FBG levels and cause a significant decrease ($p < 0.05$) in the AUC value concerned with OGTT at a concentration of 400 and 200 mg/kg, respectively, which was comparable to positive control drug rosiglitazone (Chen et al., 2018).

4.4. Neuroprotective effects

Studies show that anthocyanins defend against several neurodegenerative diseases, including Parkinson's disease,

Alzheimer's disease, ischemia, and other neuronal injuries (Airoldi et al., 2018; Jung and Kim, 2018). The glycosylated forms of anthocyanins could cross the blood-brain barrier and reach the CNS to convey their natural and biological impacts. Collective evidence suggests that the transfer of anthocyanin takes place through a bilitranslocase transporter, mainly into the vascular endothelium and thus into the target tissue (Aqil et al., 2014; Maestro et al., 2010). The polyphenolic cationic structure of anthocyanins allows scavenging free radicals, reducing ROS formation, giving an impression of being profoundly viable in neurodegenerative diseases. Anthocyanins help boost the PI3K/Akt/GSK3 pathway and control the endogenous antioxidant Nrf2/HO-1 pathway in Alzheimer's disease. Anthocyanin (12mg/kg *i.p.* for 30 days) significantly improved memory function in APP/PS1 transgenic AD mice (Ali et al., 2018). The arrangement of soluble amyloid-β 25-35 oligomers and their neurotoxicity in the human neuronal cell line SH-SY5YY could be prevented by cyanidin-3-*O*-glucoside (Tarozzi et al., 2010). Furthermore, if malvidin-3-*O*-glucoside is tested with amyloid-β neuronal cells, it shows inhibition of the cell cycle interruption caused by the amyloid-β (Shih et al., 2011). An *in silico* study found that anthocyanins could cause conformational modifications that trigger FKPB52, a potential protein known for restricting tau accumulation (Cao and Konsolaki, 2011).

Likewise, anthocyanins inhibit inflammatory biomarker interleukin-8 and other pro-inflammatory cytokines, which may help curb neuronal apoptosis associated with the disease (Rasheed et al., 2009). When anthocyanins-rich extract of blueberry are treated with microglial cells, it reduces the expression of iNOX and COX genes, which are involved in the inflammatory process (Bensalem et al., 2015). Anthocyanins inhibit extracellular signal-regulated kinase (ERK), mitogen-activated protein kinases (MAPKs), and c-Jun N-terminal kinase (JNK), which are essential for the expression of pro-inflammatory cytokines (Bensalem et al., 2015; Pan et al., 2018; Rasheed et al., 2009; Shah et al., 2016).

4.5. Cardiovascular protecting effects

Cardiovascular disease is the leading cause of mortality globally and is the type of disease affecting the cardiovascular system. Several physiological regulations lead to cardiovascular disorder, including high plasma LDL cholesterol, hypertension, endothelium dysfunction, and platelet aggregation. Apart from all these, oxidative stress is one of the major risk factors for cardiovascular disorders. Anthocyanins play a vital role in protection against oxidative stress (Bell and Gochenaur, 2006). Delphinidin, which is present abundantly in red wine, shows a vasodilatory effect upon consumption. However, in terms of preventing atherosclerosis and improving lipid and antioxidant parameters, the juice of red grapes was more efficacious than red wine (Andriambeloson et al., 1998). Literature suggests that during a heart attack, ingestion of grape juice, which is rich in anthocyanins, could exhibit potent antioxidant activity by increasing capillary permeability and strength. This action also leads to inhibition of platelet formation and speed-up the nitric oxide (NO) production, which results in vasodilation (Andriambeloson et al., 1998; Erlund et al., 2008). Similarly, chokeberry extracts also possess vasorelaxation properties (Bell and Gochenaur, 2006; Bertuglia et al., 1995). Clinical trial studies have shown that in healthy participants, eating anthocyanin-rich strawberry for more than a month improves the lipid profile and platelet function (Alvarez-Suarez et al., 2014). Hyperlipidemia is known to be an important cardiovascular disease risk factor. The literature study shows that black currant, rich in anthocyanin, can act against the fatty acids to reduce its levels (Frank et al., 2002). Anthocyanins can carry out their cardiovascular protective action through their anti-inflammatory properties; they inhibit inflammation caused by TNF-α in the human endothelium via monocyte chemoattractant protein-1 (Garcia-Alonso et al., 2009). Free radicals are formed during ischemia-reperfusion injury, which leads to adhesion of WBC in the wall of the micro-capillary; thus, it reduces blood flow and causes capillary damage. The anthocyanins can scavenge these free radicals and prevents capillary damage (Bell and Gochenaur, 2006; Toufektsian et al., 2008).

4.6. Anthocyanins in vision improvement

Anthocyanin pigments maintain good eyes' health and are often associated with improving night vision (Kramer and Canter, 2004). Anthocyanin containing berries are beneficial for vision improvement in several ways, which includes (i) circulation increase within the retinal capillaries; (ii) improving night vision by the generation of retinal pigment; (iii) decreasing diabetic retinopathy and molecular degeneration; and (iv) preventing glaucoma, cataracts, and retinitis pigmentosa (Camire, 2000). Animal model studies show that anthocyanin-rich black currant and maqui berry have been used to improve eyesight (Matsumoto et al., 2006). Anthocyanins can be distributed through the blood-retinal and blood-aqueous barriers in ocular tissues. In 19 patients with open-angle glaucoma, anthocyanin-rich black current (50 mg/day) supplementation improved ocular blood flow for 24 months (Ohguro et al., 2012). Some literature studies indicate that anthocyanin intake may positively impact macular degeneration associated with age and diabetic retinopathy (Nabavi et al., 2015; Yang et al., 2022). These results suggest that berries rich in anthocyanins could be used as a conventional treatment for patients suffering from open-angle glaucoma and other ophthalmic disorders.

4.7. Antimicrobial activity

Polyphenol compounds like anthocyanins show antimicrobial activity against several microorganisms, especially in inhibiting food-borne pathogens. Via different mechanisms, they exhibit antimicrobial activity, such as morphological damage to the bacterial cell or degradation of the structural integrity of the cell membrane, cell wall, and intracellular matrix (Burdulis et al., 2009). Anthocyanins, such as cyanidin, pelargonidin, and delphinidin, have been effective gram-negative inhibitors of *Escherichia coli* strain CM 871. However, these results were not reported in the case of wild variety gram-positive bacteria and *E. coli* (Nohynek et al., 2006). In addition to this, anthocyanins are responsible for plasma membrane permeabilization, extracellular microbial enzyme inhibition, and cytoplasmic membrane destabilization. Cranberries may inhibit various bacteria, including both gram-positive and gram-negative bacteria (Wu et al., 2008). Similarly, extract of blackcurrant inhibits the growth of *Enterococcus faecium* and *Staphylococcus aureus* strains, whereas it shows favorable results in the case of *Saccharomyces cerevisiae* and *E. coli* (Werlein et al., 2005). Likewise, carboxypyranocyanidin-3-*O*-glucoside from the anthocyanin family inhibits the biofilm production by *S. aureus* and *Pseudomonas aeruginosa* strains in chronic wounds (Correia et al., 2021). Despite all the evidence of anthocyanins showing antimicrobial activity, it has been shown that anthocyanins positively modulate the intestinal bacterial population by enhancing the growth of *Lactobacillus enterococcus* and *Bifidobacterium* spp., so the findings seem questionable in attributing the antimicrobial effects of the anthocyanin compounds observed (Hidalgo et al., 2012).

4.8. Antiviral activity

Anthocyanins have the potential to treat viral diseases. The structure of anthocyanin plays a vital role in restricting various viral infections. Blackberry extracts play a virucidal role against *Herpes simplex* due to a large number of anthocyanins. Due to the presence of anthocyanins in the red grape pomace extract, it reduces plaque infectivity and shows antiviral activity against H5N1 (Santhi et al., 2021). Similarly, Kannan and Kolandaivel (2018) concluded that the nature-derived cyanidin-3-sabubiocide could potentially treat the H1N1 subtype influenza virus. Likewise, red-fleshed potato contains Pg-type anthocyanins, which inactivates influenza viruses A and B (Hayashi et al., 2003). Study shows that anthocyanin-enriched elderberry fruit (*Sambucus nigra* L.) extract can demonstrate activity against modified vaccinia virus Ankara. It acts by declining the secretion of cytokinin IFN-γ and TNF-α, which promotes the Th2-Helper cell response (Schön et al., 2021). Anthocyanins from small red beans (*Vigna angularis*) can be used as a novel agent in the early stage of rabies virus infection (Kawai and Fujita, 2007). Anthocyanins have been recently explored as an alternative therapeutic agent targeting COVID-19. The ongoing COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has turned out a global health concern. It has affected millions of lives to date, and researchers are still hunting for its effective treatment. Anthocyanin compounds like cyanidin-3-*O*-β-glucoside (chrysanthemin) showed antiviral effects by binding to the naphthalene inhibitor binding site and inhibiting PLpro, though only to a limited extent. Similarly, pelargonidin, an anthocyanin, interact at a fatty acid-binding pocket on the spike RBD and inhibits spike-ACE2 interaction, lowering SARS-CoV-2 spike-ACE2 interaction and viral multiplication in Vero cells (Kaul et al., 2021). Furthermore, elderberry anthocyanins have the antiviral potential for SARS CoV-2 by limiting virus multiplication via budding from the virus's host cell (Salamon, 2020). A molecular docking simulation also suggests that berry anthocyanin could be employed as a potential SARS-CoV-2 therapy. The lowest binding energy, which was shown by cyanidin-3-arabinoside, was found in the pocket through a sufficient number of hydrogen bonds with the major protease virus. Pelargonidin-3-glucoside and pelargonidin 3-rhamnoside, on the other hand, have greater binding energy with SARS-spike CoV-2's glycoprotein (Messaoudi et al., 2021).

4.9. Other uses of anthocyanins

In food industries, color plays a major role, and the acceptability of food mostly depends upon its appearance. So many industries use synthetic color for improving elegance, neglecting its side effect. But the natural colorants derived from anthocyanins shows many beneficial effects without any adverse effect. Cyanidin, delphinidin, and pelargonidin are the anthocyanins responsible for various pigmentations (Mazza and Miniati, 2018). Anthocyanin extracts from bilberry and elderberry are accountable for producing mucus in the stomach and protecting it from ulcers (Veberic et al., 2009). A recent clinical trial demonstrated that the mixture of blue butterfly pea petals, extracts of purple waxy corn, and anthocyanin complex niosomes has a beneficial effect on oral wound healing (Damrongrungruang et al., 2021). Another study shows that cyanidin-3-glucoside from purple maize, fed to an obese mouse for 12 weeks, reduced body weight and reduced brown and white adipose tissue (Tsuda et al., 2003). The ability of anthocyanins to inhibit metalloproteases like MMP-1 and MMP-9 makes it suitable for topical application as they can protect the animal skin from ultraviolet radiation (Veberic et al., 2009). Additionally, anthocyanins from red cabbage possess hepatoprotective, nephroprotective, hypocholesterolemic, and anti-obesity activity. These red cabbage anthocyanins also contain medicinal benefits against headaches, peptic ulcers, and gout (Ghareaghajlou et al., 2021). Apart from the above, much literature shows the beneficial effect of anthocyanins against diarrhea, hypertension, dysentery as analgesic and antifungal agents.

5. Conclusions

This review explains the current literature about anthocyanins' chemistry, absorption, and health benefits to give the reader a broad and updated scheme. It is believed that due to various pharmacological and beneficial activities, anthocyanins can be used as natural dietary sources in multiple diseases. Furthermore, the medical advantages of dietary anthocyanins have been shown in numerous *in vitro* and *in vivo* studies. Notably, it is evident from the above literature review that anthocyanins have been mainly explored in their extract form even with such medicinal properties. Therefore, it is time now that future studies should extensively focus on isolation and synthesis with the accurate and careful characterization of diverse anthocyanins as a sole chemical entity. This approach will allow the researchers to explore different mechanistic pathways about their biological effects and further intervene in their target-specific aspects

Acknowledgments

The manuscript bears the NIPER-Hyderabad communication number NIPER-H/2020/108.

Conflict of interest

The authors confirm that there are no known conflicts of interest.

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Supplementary File

None.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 128-135

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

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RESEARCH ARTICLE OPEN ACCESS

Phytochemistry and biological activity of *Onosma rascheyana* (Boiss.) extracts

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ARTICLE INFO

Article History:

Received: 28 December 2021
Revised: 06 February 2022
Accepted: 07 February 2022
Available online: 08 February 2022

Edited by: B. Tepe

Keywords:
Onosma rascheyana
Antioxidant
Enzyme inhibitory
Chemical composition

ABSTRACT

In recent years, it has been determined that *Onosma* species exhibit interesting biological/pharmacological activities. The aim of this study was to analyze the chemical composition, antioxidant and enzyme inhibitory activities of the methanol (MeOH), water and ethyl acetate extracts obtained from the aerials parts of *Onosma rascheyana* (Boiss.). The chemical compositions of the extracts were determined using spectrophotometric and chromatographic methods. Biological activities of the extracts were determined using antioxidant and enzyme inhibitory test systems. The MeOH extract was found to be rich in both phenolics and flavonoids (31.55 mg GAEs/g and 15.20 mg REs/g, respectively). The MeOH extract also contained higher amounts of 4-hydroxybenzoic and *p*-coumaric acids compared to other phytochemicals. The MeOH extract exhibited remarkable activity in all antioxidant test systems. However, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation (ABTS) scavenging assay resulted in superiority of water extract (88.90 mg TEs/g). The relative antioxidant capacity indices (RACI) of the extracts and the correlations between these values and antioxidant activities confirmed the high activity of the MeOH extract. In the α-amylase, α-glucosidase, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity tests, the ethyl acetate extract showed high activity, while the tyrosinase inhibitory activity assay resulted in the superiority of the MeOH extract (59.72 mg KAEs/g). It was concluded that the extracts of *O. rascheyana* could be used as alternative agents in the food, cosmetic and medical industries due to their antioxidant and enzyme inhibitory activities.

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1. Introduction

Recent studies have reported that many plants have antioxidant activity (Madsen and Bertelsen, 1995; Schwarz et al., 2001; Tanabe et al., 2002). Researchers suggest that the flavonoids and phenolic compounds of plants are determinants of antioxidant activity (Madsen and Bertelsen, 1995). Concerns by health authorities about the adverse health effects of butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT), which are synthetic antioxidant substances used as preservatives in food, have prompted researchers to investigate plants rich in phytochemicals (Sasaki et al., 2002). For this reason, there has been a great increase in the number of studies on the antioxidant activities of plants in recent years.

In addition to their antioxidant activities, plants also have a promising potential for the treatment of neurodegenerative diseases. Alzheimer's disease is a neurodegenerative disorder that causes cognitive and behavioral abnormalities in people. Today, a medical protocol that can fully treat this disease has not been developed yet. However, some hypotheses have been discovered that can partially eliminate the symptoms of the disease. According to the cholinergic hypothesis, which is one of the best-known of these hypotheses, inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) can help increase neuronal activity and therefore regress disease symptoms, since it causes an increase in the amount of neurotransmitter substances in the brain. Researchers are conducting intensive research on plants to discover new and highly effective cholinesterase inhibitors (Sarikurkcu et al., 2015).

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e-ISSN: 2791-7509
doi:
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Please cite this article as: Sarikurkcu, C., Demir, E., Ozer, M.S., Binzet, R., 2022. Phytochemistry and biological activity of Onosma rascheyana extracts (Boiss.). International Journal of Plant Based Pharmaceuticals, 2(1), 128-135.

Another disease for which plants benefit medicinally is diabetes. In both type I and type II diabetes, glucose cannot pass from the bloodstream to the cells, and the blood glucose level in these patients is above the normal level (Abesundara et al., 2004). This situation causes various tissues and organs in the body to lose their functions in the medium and long term (Funke and Melzig, 2006). One of the effective ways to keep blood glucose levels at normal levels is to reduce glucose production by inhibiting the activity of α-amylase ve α-glucosidase, which are involved in carbohydrate digestion (Kim et al., 2005). Recent studies show that plants are a potential reference source for phytochemicals that can inhibit these enzymes (Kim et al., 2005).

In addition to the biological/pharmacological activities mentioned above, plants also contain phytochemicals that have an inhibitory effect on tyrosinase, which is involved in melanin biosynthesis (El-Sayed et al., 2019). Excessive melanin production can cause the development of some diseases related to hyperpigmentation in organisms. In addition, product losses due to browning may occur during the processing of vegetables and fruits due to tyrosinase activity (Asanuma et al., 2003). Researchers have focused on discovering compounds with tyrosinase inhibitory activity for use in cosmetics, medicine, and food processing (Loizzo et al., 2012). Some studies have proven that plants contain phytochemicals with significant tyrosinase inhibitory activity (Fan et al., 2017; Gheibi et al., 2015; Kamkaen et al., 2007; Kubo et al., 2000; Saghaie et al., 2013).

The aim of this study is to determine the chemical composition of methanol (MeOH), water, and ethyl acetate extracts obtained from the aerial parts of *Onosma rascheyana* (Boiss.) and to investigate their antioxidant, anti-Alzheimer, anti-diabetic, and skin whitening activities.

2. Materials and methods

2.1. Plant material

O. rascheyana was collected from the 8th km Çaglayancerit-Kahramanmaras highway, Kahramanmaras-Turkey (1550 m, 37° 44′ N 37° 14′ E). The plant was identified and deposited by Dr. Riza Binzet from the Department of Biology, Mersin University, Mersin-Turkey. (Herbarium no: Binzet 86).

Aerial parts of the plant was used as the study material to obtain solvent extracts. Details of the extraction procedure can be found in the supplementary file.

2.2. Determination of the phenolic compositions of the extracts

Details of the spectrophotometric and chromatographic methods were given in the supplementary file (Cittan and Çelik, 2018; Zengin et al., 2015).

2.3. Biological activity

The antioxidant and enzyme inhibitory activities of the extracts were determined using the methods specified in the literature (Apak et al., 2006; Kocak et al., 2016; Ozer et al., 2018; Tepe et al., 2011; Zengin et al., 2015). Details of the methods used were included in the supplementary file.

2.4. Statistical analysis

Details of the statistical analysis were presented in the supplementary file.

3. Results and discussion

3.1. Chemical composition

Amounts of total phenolic and flavonoid compounds of the extracts isolated from *O. rascheyana* are given in Figure 1. The MeOH and water extracts were rich in phenolic compounds (31.55 and 31.13 mg GAEs/g, respectively), while the total phenolic content of the ethyl acetate extract was 17.50 mg GAEs/g. MeOH extract was also the richest extract in terms of flavonoid compounds (15.20 mg REs/g). However, water and ethyl acetate extracts were poor in flavonoids.

Our research group has previously conducted studies on the total phenolic and flavonoid content of many endemic *Onosma* species (Kirkan et al., 2018; Ozer et al., 2018; Saravanakumar et al., 2019; Sarikurkcu et al., 2018; Sarikurkcu et al., 2020a, b; Sarikurkcu et al., 2020c; Sarikurkcu et al., 2020d; Tlili et al., 2021). The results obtained from the present study were found to be compatible with the total phenolic and flavonoid contents of the mentioned *Onosma* species (6.55-69.06 mg TEs/g and 65.57 mg QEs/g, respectively).

Results of LC–ESI–MS/MS analysis are given in Table 1. According to the data in the table, the MeOH extract contained higher amounts of 4-hydroxybenzoic acid and *p*-coumaric acid compared to other phytochemicals. The amounts of these compounds in the MeOH extract were 4002 and 349 µg/g, respectively. Similarly, there was a high amount of 4-hydroxybenzoic acid in the water extract (5287 µg/g). The water extract additionally contained significant amounts of rosmarinic acid (4248 µg/g), ferulic acid (803 µg/g), vanillic acid (634 µg/g), and protocatechuic acid (202 µg/g). According to the results of LC–ESI–MS/MS analysis, the ethyl acetate extract was poorer than the other extracts in terms of phytochemicals in Table 1. As with the MeOH and water extracts, the compound with highest amount in the ethyl acetate extract was 4-hydroxybenzoic acid (125 µg/g).

There are no previously published data in the literature regarding the chemical composition of the *Onosma* species reported in the present study. However, as stated above, in many articles previously published by our research group, it has been determined that different *Onosma* species show a high similarity to each other in terms of their phytochemical compositions. In particular, 4-hydroxybenzoic acid and rosmarinic acid, which were found to be found in high amounts in the current study, are found in significant amounts in many *Onosma* species in the literature (Kirkan et al., 2018; Sarikurkcu et al., 2018; Sarikurkcu et al., 2020a, b; Sarikurkcu et al., 2020c; Sarikurkcu et al., 2020d; Tlili et al., 2021). For this reason, it was concluded that the data obtained from the present study are compatible with those in the literature.

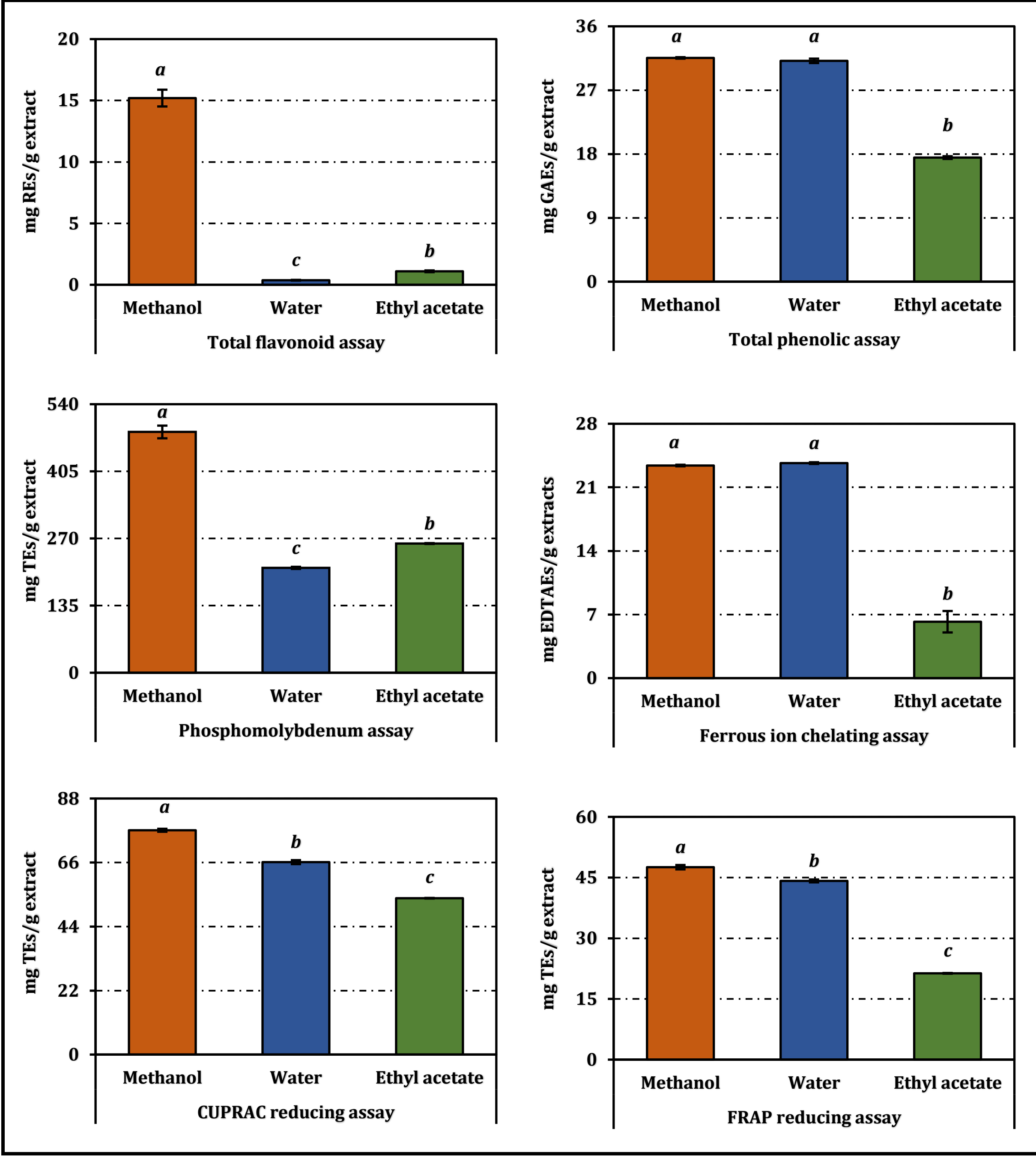
3.2. Antioxidant activity

In order to determine the antioxidant activities of the extracts, antioxidant activity tests, the details of which are given in the supplementary file, were applied (Figure 1).

MeOH extract exhibited the highest activity in the phosphomolybdenum test where total antioxidant activity was

analyzed (484.20 mg TEs/g). It was followed by ethyl acetate (259.80 mg TEs/g) and water extracts (210.90 mg TEs/g), respectively.

In the ferrous ion chelating assay, the activities of MeOH and water extracts were almost equal (23.40 and 23.67 mg EDTAEs/g, respectively). In this test system, the activity of the ethyl acetate extract was determined as 6.21 mg EDTAEs/g.



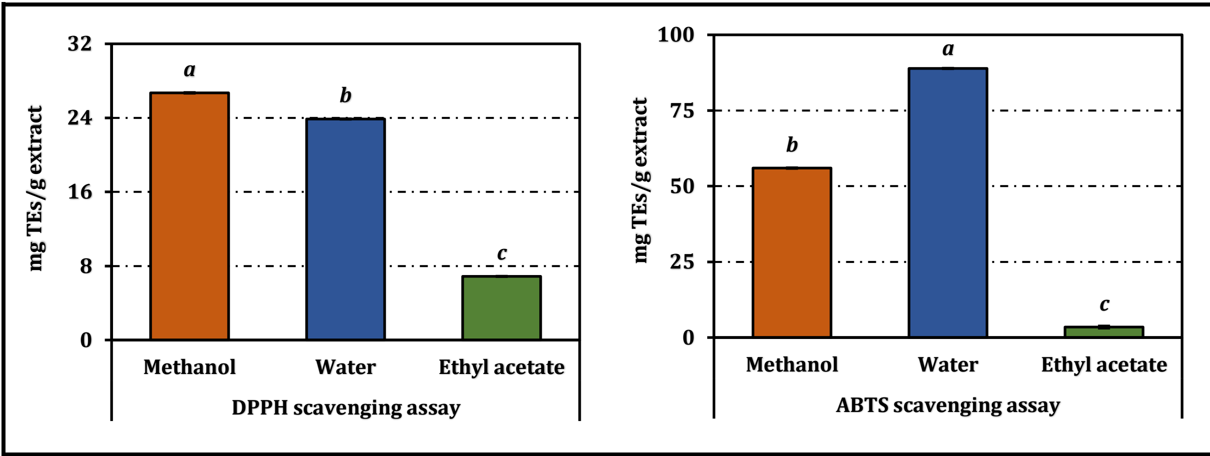


Figure 1. Antioxidant activity, total flavonoid and phenolic contents of *O. rascheyana* extracts
[REs, GAEs, TEs and EDTAEs mean rutin, gallic acid trolox and ethylenediaminetetraacetic acid (disodium salt) equivalents, respectively].
Values indicated by the same superscripts are not different from the honestly significant difference after Tukey's hoc test at 5% significance level.

Table 1. Concentration (μg/g extract) of selected phenolic compounds in *O. rascheyana* extracts[1]

Compound	MeOH	Water	Ethyl acetate
Gallic acid	6.23 ± 0.04^b	10.8 ± 0.5^a	3.94 ± 0.09^c
Protocatechuic acid	289 ± 1^a	202 ± 2^b	13.6 ± 0.5^c
3,4-Dihydroxyphenylacetic acid	16.8 ± 0.3^b	37.9 ± 0.1^a	14.2 ± 0.3^c
Pyrocatechol	27.9 ± 0.6^c	32.5 ± 0.6^b	158 ± 1^a
(+)-Catechin	18.0 ± 1.9	nd	nd
Chlorogenic acid	2.97 ± 0.10^b	102 ± 1^a	4.67 ± 0.06^b
2,5-Dihydroxybenzoic acid	15.2 ± 1.1^b	63.8 ± 2.8^a	10.4 ± 0.1^b
4-Hydroxybenzoic acid	4002 ± 7^b	5287 ± 69^a	125 ± 1^c
(-)-Epicatechin	2.22 ± 0.12^a	2.31 ± 0.19^a	2.20 ± 0.02^a
Vanillic acid	154 ± 2^b	634 ± 12^a	168 ± 5^b
Caffeic acid	14.1 ± 0.5^b	111 ± 4^a	17.8 ± 1.2^b
Syringic acid	81.7 ± 2.4^b	173 ± 9^a	4.20 ± 0.25^c
3-Hydroxybenzoic acid	34.5 ± 0.3^b	40.0 ± 1.9^a	12.3 ± 1.2^c
Vanillin	13.3 ± 0.8^c	155 ± 1^a	19.31 ± 0.49^b
Verbascoside	5.67 ± 0.04^a	6.17 ± 0.26^a	6.11 ± 0.04^a
Taxifolin	7.40 ± 0.09^b	10.3 ± 0.5^a	7.77 ± 0.31^b
Sinapic acid	9.41 ± 0.18^b	46.7 ± 1.0^a	5.15 ± 0.26^c
p-Coumaric acid	349 ± 7^b	2377 ± 8^a	27.0 ± 0.1^c
Ferulic acid	84.1 ± 0.1^b	803 ± 40^a	19.1 ± 0.8^b
Luteolin 7-glucoside	nd	38.0 ± 0.4	nd
Hesperidin	1.91 ± 0.02^b	2941 ± 5^a	6.78 ± 0.14^b
Rosmarinic acid	7.65 ± 0.13^b	4248 ± 11^a	15.7 ± 1.4^b
Hyperoside	1.40 ± 0.06^b	76.5 ± 1.2^a	1.21 ± 0.14^b
Apigenin 7-glucoside	nd	7.24 ± 0.07	nd
2-Hydroxycinnamic acid	4.16 ± 0.10^a	2.73 ± 0.10^b	3.86 ± 0.18^a
Eriodictyol	9.59 ± 0.07^a	10.0 ± 0.4^a	9.73 ± 0.07^a
Pinoresinol	nd	nd	164 ± 8
Quercetin	1.62 ± 0.06^b	6.65 ± 0.61^a	1.55 ± 0.05^b
Luteolin	nd	4.86 ± 0.10	nd
Kaempferol	nd	13.1 ± 1.4	nd
Apigenin	nd	82.0 ± 0.5^a	6.11 ± 0.07^b

[1]The values indicated by the same superscripts within the same row are not different according to the Tukey's honestly significant difference post hoc test at 5% significance level.
nd: Not detected

The reducing power potentials of the extracts were determined by CUPRAC and FRAP tests. The activities exhibited by the extracts in the CUPRAC test were higher than those in the FRAP test. In both test systems, the MeOH extract exhibited the highest activity (77.09 and 47.56 mg TEs/g, respectively). The ethyl acetate extract exhibited the lowest activity in both the CUPRAC and FRAP test (53.74 and 21.33 mg TEs/g, respectively).

DPPH and ABTS radical scavenging tests were applied to determine the scavenging activity of the extracts on free radicals. While MeOH extract showed high activity in DPPH radical scavenging assay (26.70 mg TEs/g), water extract showed the highest activity in ABTS radical scavenging test (88.90 mg TEs/g).

Relative antioxidant capacity index (RACI) data of extracts are given in Figure 2. The RACI value of the MeOH extract was calculated as 0.84, according to the data obtained as a result of the evaluation of the data obtained from all antioxidant activity tests together. It was followed by water and ethyl acetate extracts (0.23 and -1.06, respectively).

The correlation values between the RACI values of the extracts and their antioxidant activities are given in Figure 3. According to the data in the figure, the antioxidant activities of the extracts in all test systems, except the phosphomolybdenum test, were correlated with their RACI values.

According to literature data, the antioxidant activity of *O. rascheyana* has not been studied before. However, it is possible to

compare the data obtained from the current study with the antioxidant activities of other *Onosma* species previously published by our research group. Antioxidant activity data from the present study are generally consistent with antioxidant activity data from other *Onosma* species in the literature. (Kirkan et al., 2018; Ozer et al., 2018; Saravanakumar et al., 2019; Sarikurkcu et al., 2018; Sarikurkcu et al., 2020a, b; Sarikurkcu et al., 2020c; Sarikurkcu et al., 2020d; Tlili et al., 2021). There are also some data in the literature showing that 4-hydroxybenzoic acid and rosmarinic acids, which are the main components of MeOH extract with high antioxidant activity, can exhibit strong antioxidant activity (Babaei et al., 2020; Kim et al., 2014; Park et al., 2008; Ying et al., 2009).

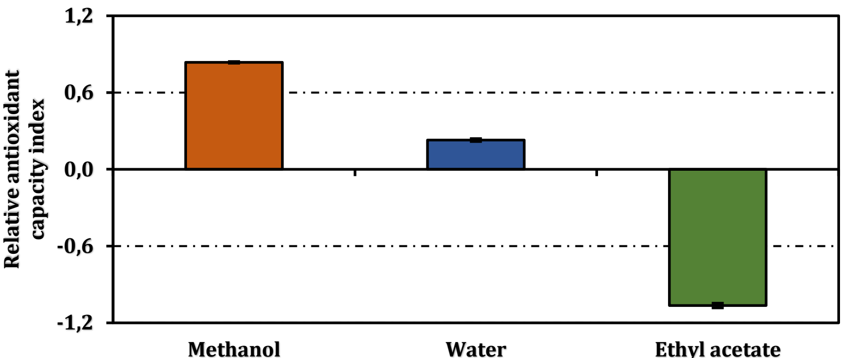


Figure 2. RACI of *O. rascheyana* extracts

Figure 3. Correlation between RACI (dashed red line with triangle) and antioxidant activity (solid dark blue line with circle) of *O. rascheyana* extracts

3.3. Enzyme inhibitory activity

The inhibitory activities of the extracts of *O. rascheyana* on AChE, BChE, tyrosinase, α-amylase and α-glucosidase are given in Figure 4.

In the inhibitory activity tests on digestive enzymes, the ethyl acetate extract exhibited the highest activity. The inhibitory activity of this extract on α-amylase and α-glucosidase was determined as 421.93 and 1568.92 mg ACEs/g, respectively. On the other hand, the water extract exhibited the weakest activity on both enzymes (44.68 and 122.46 mg ACEs/g, respectively).

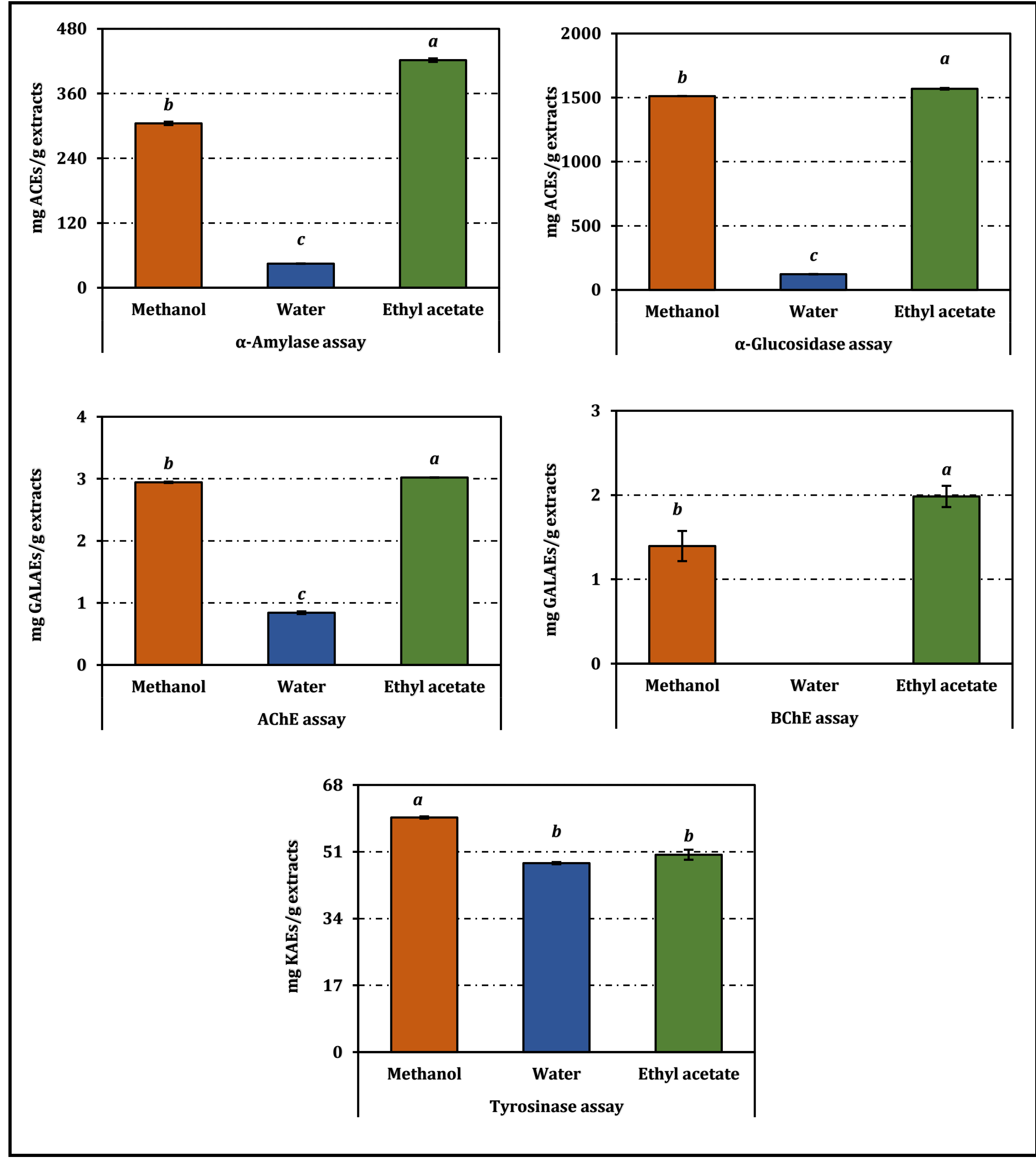


Figure 4. Enzyme inhibition activity of *O. rascheyana* extracts

ACEs, GALAEs and KAEs mean acarbose, galanthamine and kojic acid equivalents, respectively.

Values indicated by the same superscripts are not different from the honestly significant difference after Tukey's hoc test at 5% significance level.

The ethyl acetate extract also showed higher activity on cholinesterases than the others. The inhibitory activity of this extract on AChE and BChE was 3.02 and 1.98 mg GALAEs/g, respectively. Just as in the test system where digestive enzymes were inhibited, the MeOH extract ranked second after the ethyl acetate extract. The water extract, on the other hand, provided an

inhibition of 0.84 mg GALAEs/g on AChE, but was ineffective on BChE.

A different activity profile was detected in the tyrosinase inhibitory activity test than those obtained from the test systems detailed above. In this test system, the inhibitory activity of the MeOH extract was higher than the other extracts (59.72 KAEs/g). It was followed by ethyl acetate and water extracts, whose inhibitory activity values were quite close to each other (50.24 and 48.10 mg KAEs/g, respectively).

No data on the inhibitory activities of *O. rascheyana* on the enzymes discussed in the current study could be found in the literature. The literature data on the enzyme inhibitory activities of *Onosma* species generally consists of the articles published as a result of the researches of our study group (Kirkan et al., 2018; Ozer et al., 2018; Saravanakumar et al., 2019; Sarikurkcu et al., 2018; Sarikurkcu et al., 2020a, b; Sarikurkcu et al., 2020c; Sarikurkcu et al., 2020d; Tlili et al., 2021). Data from the present study indicate that *O. rascheyana* has similar enzyme inhibitory activity potential as other *Onosma* species.

3.4. Correlations among phenolics and assays

Since the determination of the compounds responsible for the activity via bioassay-guided fractionation could not be performed in the current study, correlation analysis was applied to determine to what extent the main components of the extracts contribute to the activities (Table 2).

Table 2. Correlations among phenolic compounds and assays

	TAP	DPPH	ABTS	CUPRAC	FRAP	FICA	AChEIA	BChEIA	TIA	AAIA	AGIA
RACI	0.998	0.999	0.999	0.999	0.999	0.998					
Total phenolic	0.999	0.999	0.999	0.999	0.999	0.998	-0.989	-0.938	0.993	-0.999	-0.996
Total flavonoid	0.994	0.999	0.999	0.999	0.999	0.996	-0.995	-0.949	0.987	-0.999	-0.992
Protocatechuic acid	0.998	0.999	0.999	0.999	0.999	0.998	-0.989	-0.937	0.990	-0.999	-0.994
Pyrocatechol	-0.998	-0.999	-0.999	-0.999	-0.999	-0.998	0.990	0.941	-0.992	0.999	0.995
Chlorogenic acid	-0.998	-0.999	-0.999	-0.999	-0.999	-0.999	0.990	0.943	-0.995	0.998	0.997
4-Hydroxybenzoic acid	0.998	0.999	0.999	0.999	0.999	0.998	-0.990	-0.938	0.991	-0.999	-0.994
Vanillic acid	-0.932	-0.900	-0.900	-0.940	-0.940	-0.960	0.959	0.980	-0.971	0.935	0.969
Caffeic acid	-0.935	-0.950	-0.950	-0.950	-0.950	-0.963	0.965	0.985	-0.972	0.941	0.971
p-Coumaric acid	0.997	0.999	0.999	0.999	0.999	0.997	-0.992	-0.943	0.990	-0.999	-0.994
Ferulic acid	0.998	0.999	0.999	0.999	0.999	0.998	-0.989	-0.939	0.992	-0.999	-0.995
Hesperidin	-0.998	-0.999	-0.999	-0.999	-0.999	-0.995	0.988	0.932	-0.986	0.999	0.990
Rosmarinic acid	-0.984	-0.986	-0.985	-0.987	-0.986	-0.972	0.972	0.895	-0.954	0.990	0.962

Data show the Pearson Correlation Coefficients between the parameters. TAP: total antioxidant activity by phosphomolybdenum method. AAIA. AChEIA. BChEIA. AGIA. and TIA: α-amylase. acetyl cholinesterase. butyryl cholinesterase. α-glucosidase. and tyrosinase inhibition activities. respectively. ABTS and DPPH: ABTS and DPPH radical scavenging activities. respectively. CUPRAC and FRAP: CUPRAC and FRAP reducing power potential; respectively. RACI: relative antioxidant capacity index. FICA: Ferrous ion chelating activity

According to the data in the table, it was determined that there was a high correlation between the phenolic and flavonoid compound contents of the extracts and their antioxidant activities. In addition, protocatechuic, ferulic, *p*-coumaric and 4-hydroxybenzoic acids also appear to contribute significantly to antioxidant activity. According to the correlation coefficients in the table, it is thought that some phenolic compounds (pyrocatechol, chlorogenic acid, vanillic acid, caffeic acid, hesperidin and rosmarinic acid) contribute significantly to the enzyme inhibitory activities of the extracts.

4. Conclusions

In this study, the chemical compositions, antioxidant and enzyme inhibitory activities of MeOH, water and ethyl acetate extracts obtained from the aerial parts of *O. rascheyana* were analyzed. It was concluded that MeOH and water extracts exhibited considerably higher antioxidant activity than ethyl acetate extract. It is thought that this situation may be caused by the highly polar phenolic compounds or flavonoids in the extracts in question. Correlation analyzes confirm this idea. On the other hand, as in many previous studies carried out by our research group, it was concluded that the enzyme inhibitory activity of ethyl acetate extract was higher than that of other extracts. It is anticipated that the remarkable enzyme inhibitory activity of the extract may be due to low polarity compounds. However, in all test systems presented here, it is thought that fractionation studies accompanied by quantitative chromatographic techniques should be performed to determine the phytochemicals responsible for the extracts activities.

Acknowledgments

The authors would like to thank the Scientific Research Council of Afyonkarahisar Health Sciences University, Afyonkarahisar-Turkey, for financial support of this study (Project Number: 21.KARIYER.002).

Conflict of interest

The authors confirm that there are no known conflicts of interest.

CRediT authorship contribution statement

Cengiz Sarikurkcu: Conceptualization, Investigation, Data curation, Writing - original draft, Supervision
Ersin Demir: Resources, Conceptualization, Visualization, Formal analysis, Investigation, Methodology
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Supplementary File

The supplementary file accompanying this article is available at https://dergipark.org.tr/en/download/journal-file/25005.

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Int. J. Plant Bas. Pharm. (2022) 2(1), 136-144

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

https://ijpbp.com

RESEARCH ARTICLE

OPEN ACCESS

Comparative study on phenolic content, flavonoid content, and antioxidant activities of five species of the genus *Phaseolus*

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ARTICLE INFO

Article History:

Received: 10 December 2021
Revised: 09 February 2022
Accepted: 09 February 2022
Available online: 10 February 2022

Edited by: B. Tepe

Keywords:
Fabaceae
Phaseolus
Phytochemicals
Antioxidants
Radical scavenging
Correlation

ABSTRACT

The current study was designed to assess five species of the genus *Phaseolus* for phenolic content, flavonoid content, and antioxidant ability. The antioxidant capacity of the sample extracts was assessed using different antioxidant models such as ferric reducing antioxidant power (FRAP), DPPH free radical scavenging, phosphomolybdenum reducing power, ferrous ion chelating activity, hydrogen peroxide radical scavenging, hydroxyl radical scavenging, deoxyribose degradation, and β-carotene bleaching assays. The results obtained discovered that the concentration of phenolics and flavonoids in the studied species ranged from 1.11 to 4.01mg TAE/g plant material and 0.11 to 1.16 mg QE/g plant material. The antioxidant activity of the extracts varied in a wide range in the different antioxidant assays depending on the genotype as well as the polarity of the solvents used to obtain the extracts. Ethanolic and aqueous extracts exhibited the maximum amount of phenolics and flavonoids among the solvents. The species studied exhibited a significant range of phenolics, flavonoids, and antioxidant capacity. Hence, the present investigation can provide a new direction by utilizing *Phaseolus* species to formulate cost-effective, eco-friendly, and value-added therapeutic products.

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1. Introduction

Phaseolus (Fabaceae) is one of the most important genera encompassing herbaceous to woody annuals and perennial vines comprising about 70 species all indigenous to the Americas, mainly Meso America. The species are a rich source of carbohydrates and proteins and a significant source of vitamin B complexes such as riboflavin, thiamine, niacin, and folic acid. It also provides zinc, copper, iron, phosphorous, calcium, potassium, and magnesium additionally has a high fiber content (Rocha-Guzman and Gallegos-Infante, 2007). It is also an imperative source of polyphenols such as flavonoids, isoflavones, lignans, and tannins. These compounds offer a protective role in humans owing to their strong tendency to scavenge free radicals (Câmara et. al., 2013; Bezuhla et. al., 2018).

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e-ISSN: 2791-7509
doi:
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The seeds of *Phaseolus* species were expended by humans worldwide as a dynamic source of proteins and fiber (Onyilagha and Islam, 2009). The consumption of seeds has previously been associated with a reduced risk of cardiovascular diseases, diabetes, and even certain types of cancer (Curran, 2012; Hayat et al., 2014). Some researchers reported that legume seeds are medicinally important due to their antioxidant, anticancer, antimicrobial, antiobesity, cardioprotective, hepatoprotective, and antiproliferative activities (Zhu et. al., 2012; Guajardo-Flores et al., 2013; Zou and Chang 2014). Several articles have appeared in the literature focused on the antioxidant potential of common beans (Amarowicz et al., 2008; Aknod et. al., 2011). Some studies evaluated the seeds of the most economically important species, *P. vulgaris*, for the presence of seed coat anthocyanin glycosides (Choung et al., 2003) and polyphenols (Espinosa-Alonso et al., 2006). The findings of Suárez-Martínez et al. (2016) reported *P. vulgaris* as a nutraceutical source for human health with favorable effects against cancer because of the antimutagenic and antiproliferative properties of their phenolic compounds, lectins, and protease inhibitors. The accessible literature revealed that *P.*

Please cite this article as: Kolar, F.R., Nirmanik, V., Kagawad, A., Angadi, L., Lamani, B.R., 2022. Comparative study on phenolic content, flavonoid content and antioxidant activities of five species of the genus Phaseolus. International Journal of Plant Based Pharmaceuticals, 2(1), 136-144.

vulgaris was explored pharmacologically and largely overlooked at the biological and biochemical levels among the various *Phaseolus* species. The beneficial effects of other species are not well investigated. Therefore, the current research was designed to evaluate five different species of the genus Phaseolus for the presence of phenolics, flavonoids, and their potential antioxidative effect.

2. Materials and methods

2.1. Plant material collection

The *Phaseolus* species, namely *P. vulgaris*, *P. aureus*, *P. mungo*, *P. trilobus*, and *P. aconitifolius,* were procured from the local market of Vijayapura district of Karnataka and authenticated by Dr. Sidanand Kambhar.

2.2. Preparation of extracts

The seed extracts of different species of *Phaseolus* were prepared in four different solvents (distilled water, ethanol, methanol, and acetone). 2.5 grams of dried seeds were treated with 25 ml of distilled water, and then the extract obtained was agitated and left overnight in a shaker. The extracts acquired were then centrifuged at 8.000 rpm for 20 minutes, and the supernatant was collected. The final volume of the collected supernatants was attuned to 25 ml by adding more distilled water. A similar technique was used to prepare ethanol, methanol, and acetone extracts. All the prepared extracts were preserved in a refrigerator at 4 °C, and for the tests, one percent extracts were used.

2.3. Total phenolic content (TPC)

The total phenolic content in the seed extracts was examined by the Folin-Ciocalteu method (Wolfe et al., 2003). The reaction mix was prepared by adding 125 µl of Folin-Ciocalteu reagent and 1.25 ml of Na_2CO_3 solution to an aliquot of the sample extracts (0.125 ml). The reaction mixture was then placed at normal temperature for 90 min, and the absorbance of each sample was recorded at 760 nm. All the samples were analyzed in 3 replicates, and the average value of the absorbance was attained. A calibration graph was plotted using tannic acid reference compound (10 µg/ml to 100 µg/ml, R^2 = 0.992). The results were interpreted as mg tannic acid equivalents (TAE)/g of sample.

2.4. Total flavonoid content (TFC)

The total flavonoid content in the extracts was studied following Luximon-Ramma et al. (2002). The reaction mix was devised by adding 1.5 ml of extract to 1.5 ml of methanolic $AlCl_3$ (2%), and the samples were then retained at room temperature for 10 min. After that, the absorbance was recorded at 420 nm. The samples were prepared in triplicates, and the average value was calculated. A similar technique was followed for the reference compound quercetin, and the standard graph was plotted (10 µg/ml to 100µg/ml, R^2 = 0.920). The data obtained was articulated as milligram of quercetin equivalents (QE)/g of sample.

2.5. Ferric reducing antioxidant power assay (FRAP assay)

Each extract's ferric ion reducing ability was assessed following the method defined by Pulido et al. (2000). The reaction blend was prepared by adding an aliquot (100 µl) of seed extracts to 3 ml of FRAP reagent (1 part of 20 mM $FeCl_3.6H_2O$ solution, 1 part of 10 mM TPTZ solution, and 10 parts of 300 mM sodium acetate buffer at pH 3.6). After 15 min of reaction time at 37 °C, the absorbance was measured at 595 nm. Quantification was expressed by recording the absorbance in the calibration curve of the reference compound ascorbic acid (10 µg/ml to 100 µg/ml, R^2 = 0.980). The results were interpreted as milligrams of ascorbic acid equivalent per gram of the sample.

2.6. DPPH free radical scavenging assay

The extracts' DPPH free radical (1,1-diphenyl-2-picrylhydrazyl) scavenging ability was measured by using the technique devised by Aquino et al. (2001). The reaction medium containing seed extracts (25 µl) and 3 ml of 25 mM DPPH solution was incubated for 30 min in the dark at room temperature, and the absorbance was recorded at 515 nm against a blank. The DPPH radical scavenging ability as percent inhibition was calculated as Scavenging activity (%) = [(Ac-As)/Ac] x 100, where Ac is the absorbance of the control and As is the absorbance of the sample.

2.7. Phosphomolybdenum reducing power assay

The phosphomolybdenum reducing activity of the extracts was assessed by Prieto et al. (1999). A mixture was prepared by mixing 300 µl of the extract with 3 ml of the phosphomolybdate reagent (0.6 M sulphuric acid, 4 mM ammonium molybdate, and 28 mM sodium phosphate) and placed in a water bath at 90 °C for 90 min. The mixture was chilled to normal temperature, and the absorbance was recorded at 695 nm against a blank. A calibration curve was obtained using ascorbic acid as a positive control (10 µg/ml to 100 µg/ml, R^2 = 0.953), and the results were reported as ascorbic acid equivalents (AAE) per gram of sample.

2.8. Ferrous ion chelating assay

The ferrous ion chelating activity was evaluated by following the procedure devised by Dinis et al. (1994). For the assay, 100 µl of 2 mM $FeCl_2$ and 300 µl of 5 mM ferrozine were mixed with sample extracts, and the blend was then equilibrated for 15 min at normal temperature. The absorbance of the reaction mixture was measured at 562 nm on a spectrophotometer. The tendency of the extracts to chelate transition metal ions was estimated by using the formula of inhibition percentage as employed for DPPH· free radical scavenging activity.

2.9. Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging capacity of the extracts was measured as per Zhao et al. (2006). An aliquot of 200 µl of sample and 200 µl of 40 mM H_2O_2 were mixed, followed by the addition of 200 µl of ammonium molybdate (3%), 200 µl of 2 M H_2SO_4, and 1.4 ml of 1.8 M KI. The reaction mixture was titrated against 5.09 mM NaS_2O_3 till the yellow color disappeared. The ability of the extracts to scavenge hydrogen peroxide was quantified as percentage (%) of the titer volume change [(Volume of control-Volume of the sample)/Volume of control] x 100.

2.10. Hydroxyl radical scavenging assay

The ability of the extracts to scavenge hydroxyl radicals was estimated following the method devised by Sadasivam and Manikam (1992). The reaction mix was prepared using 60 µl of 1.0 mM $FeCl_3$, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 0.15 ml of 0.17 M H_2O_2, 0.09 ml of 1mM 1,10-phenanthroline and 1.5 ml of extracts. The tubes were placed at room temperature for 10 min,

and the absorbance of the mixture was recorded at 560 nm. The hydroxyl radical scavenging capacity of the extracts was calculated using the equation: (Ac-As)/Ac x 100, where Ac is the absorbance of the control and As is the absorbance of the sample.

2.11. Deoxyribose degradation assay

Deoxyribose degradation activity of the extracts was evaluated by Halliwell et al. (1987) procedure, with some modifications. The assay mixtures, containing the samples, 50 µl of deoxyribose (50 mM), 50 µl of Na_2EDTA (1 mM), 300 µl of phosphate buffer (0.2M, pH 7.4), 50 µl of H_2O_2 (50 mM) and 50 µl of $FeCl_3$ (3.2 mM). The reaction was started by adding 50 µl of ascorbic acid (1.8 mM), and the final volume of the reaction medium was attuned to 800 µl with buffer. The reaction was completed by adding 250 µl of TCA (10%, w/w) after 20 min incubation at 50 °C. The color was then developed by adding 150 µl of TBA (5%, in 1.25% NaOH) and heating in an oven at 105 °C for 15 min. The reaction mixture was then allowed to cool at normal temperature, and the absorbance was measured at 530 nm against blank. Inhibition of deoxyribose degradation was estimated using the inhibition percentage formula as employed for DPPH· free radical scavenging activity.

2.12. β-carotene bleaching assay

β-carotene bleaching activity of the sample was evaluated following the method described by Wettasinghe and Shahidi (1999). A stock solution of 1ml of β-carotene and linoleic acid was prepared with 0.2 mg of β-carotene in 1 ml of chloroform, 20 µl of linoleic acid, and 200 µl of 100% Tween 20. The chloroform was then vaporized at 40 °C for 10 min using a rotary evaporator, and then 100 ml of distilled water was added to the remainder. The distilled water was added gently to the mix to form a suspension. 3 ml of the suspension was taken out in separate tubes with 200 µl of samples, and immediately, the zero time absorbance was recorded at 470 nm (t = 0). Then, the tubes were retained in a water bath at 50 °C for 2 h; the absorbance was measured again at 470 nm (t = 120 min) against the blank. Inhibition percentage of bleaching was estimated using the following formula (Miller, 1971):

$$\text{Inhibition \%} = 1 - \left[\frac{A1_{(t=0)} - A1_{(t=120)}}{A0_{(t=0)} - A0_{(t=120)}} \right] \times 100$$

Where:
A1: Absorbance of the sample
A0: Absorbance of the control
t = 0: Absorbance at zero time
t = 120: Absorbance after 120 min

2.13. Statistical analysis

The experimentations were executed in triplicates, and the results were interpreted as average ± standard deviation. The One-way ANOVA test was employed to elucidate the mean difference for the different species, and the $p < 0.05$ values were considered significant. The results were further subjected to Pearson's correlation coefficient of phenolics and flavonoids with different antioxidants using the GraphPad InStat and MS Excel.

Table 1. Total phenolics and flavonoid content in different species of *Phaseolus*

Species	Total phenolic content (mg TAE/g plant material)				Total flavonoid content (mg QE/g plant material)			
	Aqueous	Ethanol	Methanol	Acetone	Aqueous	Ethanol	Methanol	Acetone
P. aconitifolius	2.12 ± 0.09	4.01 ± 0.06	1.41 ± 0.07	1.11 ± 0.06	0.17 ± 0.005	0.26 ± 0.008	0.17 ± 0.005	0.13 ± 0.004
P. aureus	2.27 ± 0.06	2.34 ± 0.11	1.90 ± 0.10	2.06 ± 0.04	0.65 ± 0.002	0.61 ± 0.003	0.60 ± 0.002	0.65 ± 0.005
P. mungo	3.48 ± 0.07	3.58 ± 0.11	2.16 ± 0.61	2.20 ± 0.04	0.98 ± 0.003	0.74 ± 0.005	0.54 ± 0.004	0.52 ± 0.005
P. trilobus	1.37 ± 0.10	3.27 ± 0.06	3.21 ± 0.06	2.09 ± 0.04	0.13 ± 0.003	0.36 ± 0.004	0.27 ± 0.002	0.11 ± 0.004
P. vulgaris	1.44 ± 0.35	1.73 ± 0.06	1.48 ± 0.06	1.18 ± 0.12	0.45 ± 0.004	1.16 ± 0.671	0.26 ± 0.003	0.51 ± 0.002

Values are expressed as mean ± SD of triplicate measurements.
mg TAE/ g plant material: Milligram tannic acid equivalent per gram plant material.
mg QE/g plant material: Milligram quercetin equivalent per gram plant material.

3. Results and discussion

3.1. Phenolic and flavonoid content

The phenolics and flavonoids are responsible for the antioxidant properties of many plants. These highly reactive compounds act by scavenging free radicals, preventing the activation of procarcinogens, or by binding carcinogens to macromolecules (Krishnaswamy, 1996). Hence, it is imperative to study different genotypes of *Phaseolus* for total phenolics and flavonoid content. The concentration of total phenolics in the sample extracts is inferred as tannic acid equivalent/g of sample (TAE), and the content of total flavonoids is articulated as quercetin equivalent/g of sample (QE). The content of phenolic compounds in the species ranged from 1.11 to 4.01 mg TAE/g (Table 1), and the significant results were observed in the relative order *P. aconitifolius* > *P. mungo* > *P.trilobus* > *P. aureus* > *P. vulgaris*. Comparison among the species displayed that the species *P. aconitifolius* (4.01 mg TAE/g) showed higher content of phenolics than that of other species, and among the solvents, ethanolic extracts exhibited the maximum content of phenolics as compared to other solvent extracts.

Correspondingly, the content of flavonoids in the species ranged from 0.11 to 1.16 mg QE/g (Table 1). The species *P. vulgaris* (1.16 mg QE/g), *P. trilobus* (0.36 mg QE/g), and *P. aconitifolius* (0.26 mg QE/g) exhibited a maximum concentration of flavonoids in the ethanol extracts. In *P. mungo* (0.98mg QE/g) and *P. aureus* (0.65 mg QE/g), the content was observed to be maximum in the aqueous extracts. Among the species, *P. vulgaris* exhibited the highest flavonoids content compared to the other species. Among the solvents, the ethanol and aqueous extraction systems were the most suitable extraction systems as they exhibited the highest flavonoid contents compared to the other solvent systems. Thus, the results of the examined species for the phenolics and flavonoid content displayed the presence of an ample amount of phenolics and flavonoids contributing to its antioxidant activities. Certain reports have revealed that the main flavonoids in some varieties of *P. vulgaris* are kaempferol and quercetin, and both the compounds have been shown to decrease the risk of cardiovascular diseases and lung cancer (Espinosa-Alonso et al., 2006; Chávez-Mendoza and Sánchez, 2017). Various authors have reported that differences in phenolics and flavonoid composition among the species could be related to the color of the seed. However, detailed studies advocate that variability in phenolic content is more due to genotype than

seed color. In addition, environmental conditions can also affect the phenolics and flavonoid content of the seeds (Bezuhla et. al., 2018; Pitura and Arntfield, 2019; Rodríguez Madrera et al., 2020).

3.2. Ferric reducing antioxidant power (FRAP)

As estimated by FRAP assay, the ferric reducing ability of the *Phaseolus* species discovered that the activity varied among the species (Figure 1) and was found to be highest in *P. aureus* (22.0 mg AAE/g). However, a comparison of solvent extracts showed that all the species except *P. aureus* possessed the highest antioxidant activities in ethanol extracts compared to other solvent extracts. While *P. aureus* showed the maximum activity in the aqueous extract, the highest ferric reducing power of *P. aureus* is possible because of the presence of hydroxyl groups in the phenolic compounds, presumably acting as an electron donor. The antioxidant activities of phytochemicals comprising phenolics and flavonoids testified in the current study specified that the occurrence of reductants in the plant extracts of *Phaseolus* species triggered the reduction of the Fe^{3+}-TPTZ complex to the ferrous (Fe^{2+}) form, clearly suggesting that the extracts are significantly contributing to the observed antioxidant activity and the species possessed variable but considerable antioxidant and antiradical activities.

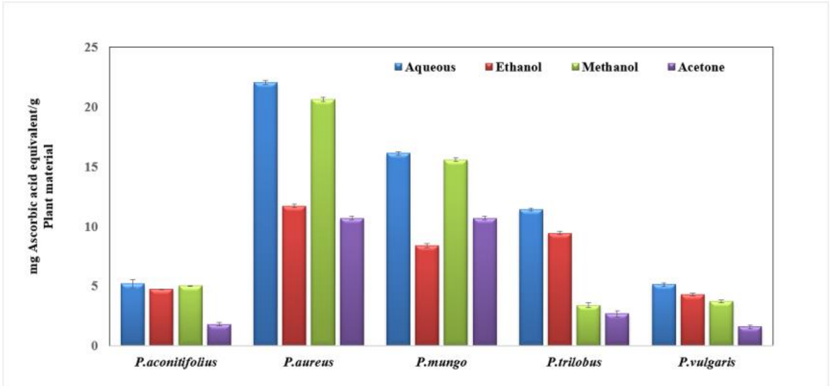

Figure 1. Ferric reducing antioxidant power (FRAP) of various *Phaseolus* species

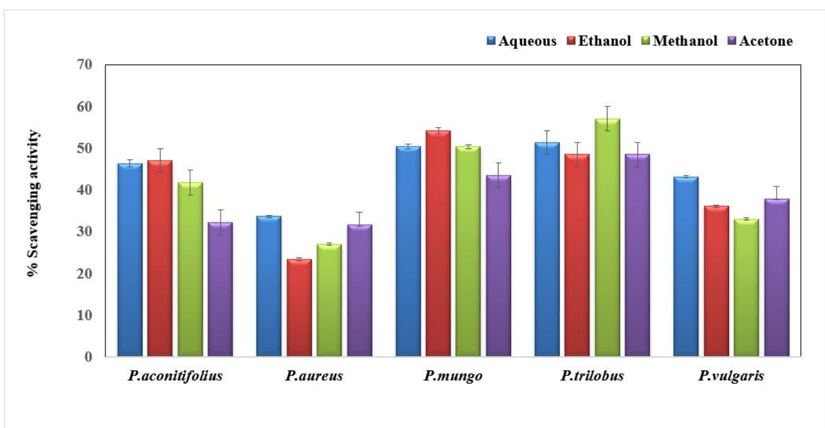

Figure 2. DPPH free radical scavenging activity of various *Phaseolus* species

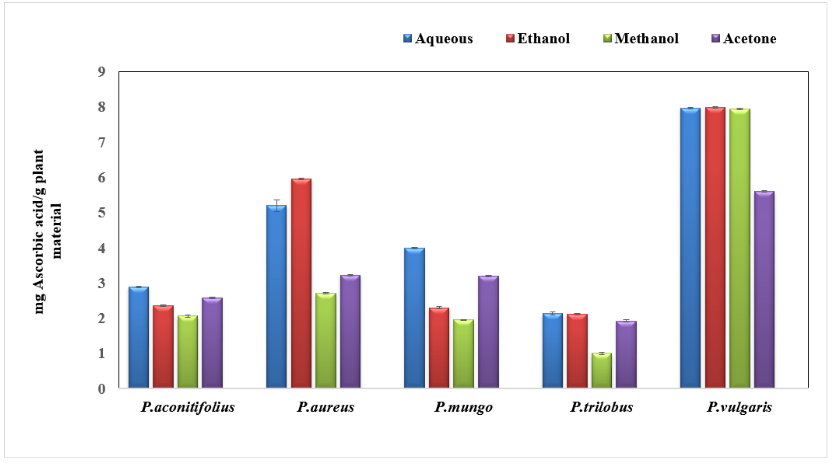

Figure 3. Phosphomolybdenum reducing power of various *Phaseolus* species

3.3. DPPH free radical scavenging activity

The DPPH free radical scavenging activity of the examined extracts displayed that *Phaseolus* species showed varied free radical scavenging activity (Figure 2). The scavenging effect of the species was found to be in the order: *P. trilobus* (57.0 %) > *P. mungo* (54.1 %) > *P. aconitifolius* (46.9 %) > *P. vulgaris* (43.1 %) > *P. aureus* (33.6 %). However, all the samples displayed the radical scavenging activity, yet, the methanol extract of *P. trilobus* exhibited relatively higher scavenging capability than other solvent extracts. Further, the ethanol and methanol extracts exhibited the highest activities among the solvents. Thus, the results indicated that all the extracts were facilitated as radical scavengers to some extent. Among the species, *P. trilobus* exhibited the highest scavenging activity; this high scavenging property of *P. trilobus* is presumably due to the presence of hydroxyl groups in the phenolic compounds that can offer the essential components as a radical scavenger (Gyamfi et al., 1999). Phytocompounds capable of implementing this response can be regarded as antioxidants and so radical scavengers (Dehpour et al., 2009).

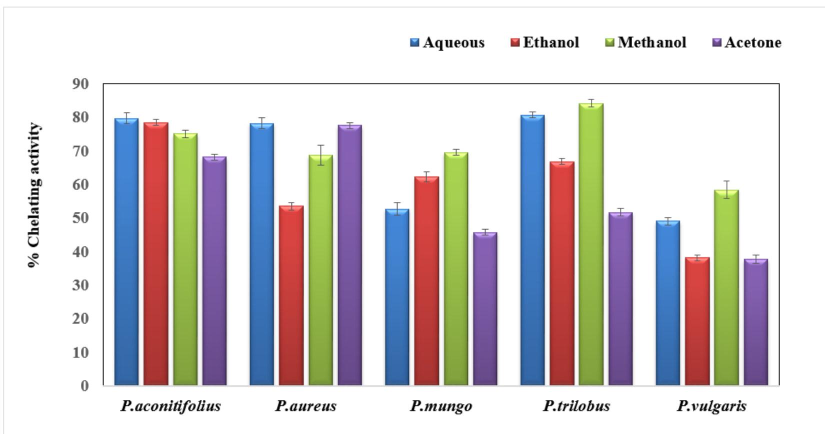

Figure 4. Ferrous ion chelating activity of various *Phaseolus* species

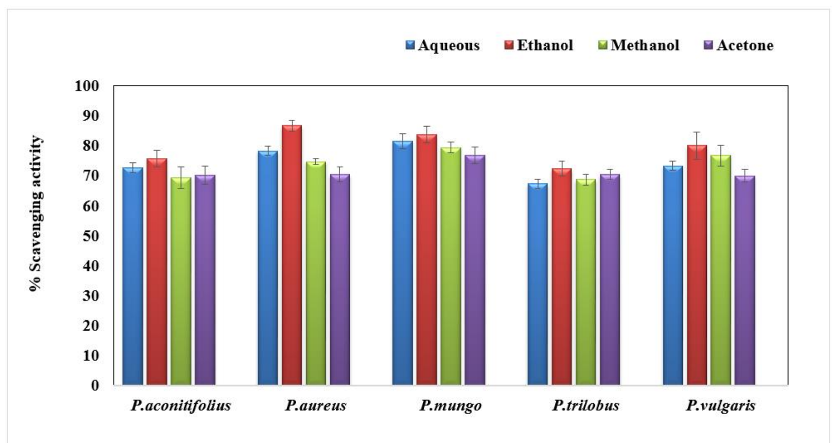

Figure 5. Hydrogen peroxide radical scavenging activity of various *Phaseolus* species

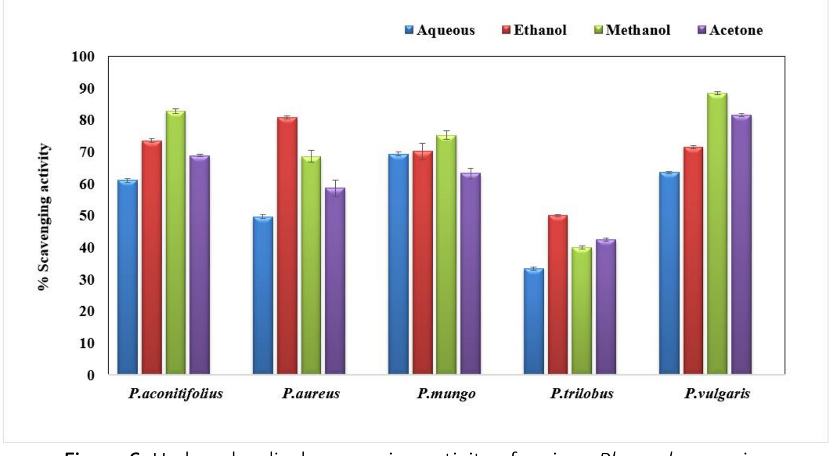

Figure 6. Hydroxyl radical scavenging activity of various *Phaseolus* species

3.4. Phosphomolybdenum reducing power

The reducing ability of the extracts estimated by phosphomolybdenum method revealed that the ethanol extracts of *P. vulgaris* (8.00 mg AAE/g), *P. aureus* (5.96 mg AAE/g), and *P. aconitifolius* (2.89 mg AAE/g) exhibited higher phosphomolybdenum reduction, while, in *P. mungo* (4.00 mg AAE /g) and *P.trilobus* (2.13 mg AAE/g) the highest reduction power was observed in aqueous and methanol extracts respectively (Figure 3). Thus different solvent extracts of different species possessed varied reducing power. Several studies have specified that the electron donation capacity of the phytochemical constituents present in the plants is linked with the antioxidant capacity (Lee et al., 2015; Gupta et al., 2016). The chemical constituents with reducing power revealed that they could donate electrons and reduce the oxidized intermediates of the lipid peroxidation process in such a way that they can act as primary and secondary antioxidants (Reische et al., 2008).

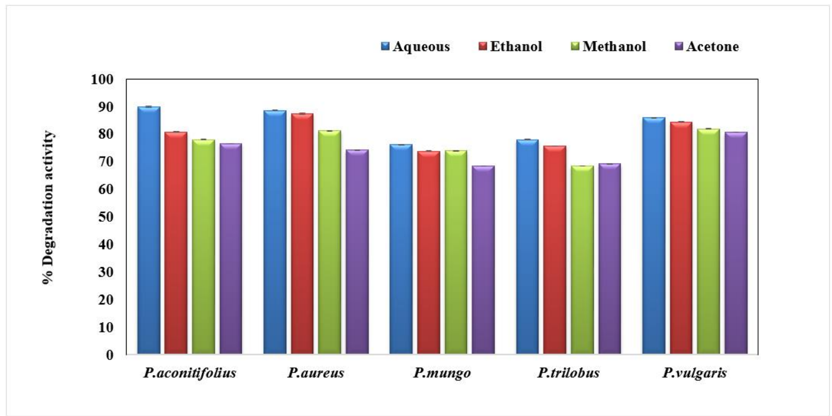

Figure 7. Deoxyribose degredation activity of various *Phaseolus* species

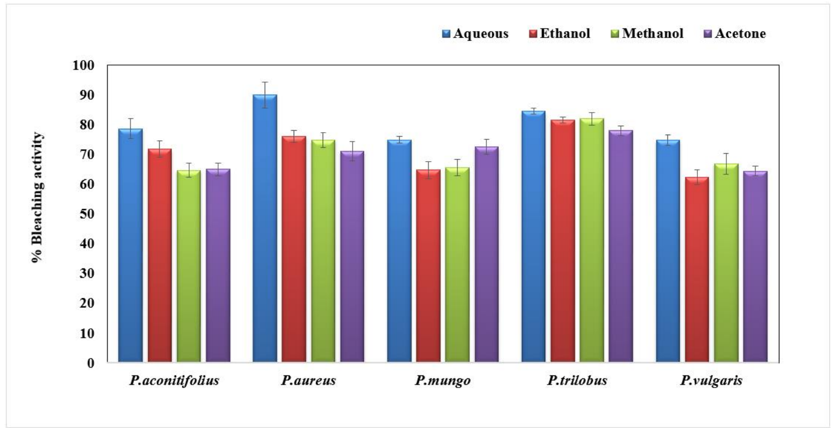

Figure 8. β-carotene bleaching activity of various *Phaseolus* species

3.5. Ferrous ion chelating activity

The assessment of *Phaseolus* species for chelating capacity revealed that the chelating effect of the extracts on ferrous ions ranged from 37.6% to 91.9% (Figure 4). The chelating capacity varied widely among the solvents; ethanol extracts of *P. vulgaris* chelated the least ferrous ions, whereas methanol extracts of *P. trilobus* chelated the most. Based on the chelating ability of the diverse species, the activity was observed as *P. trilobus* (84.1%) > *P. aconitifolius* (79.6%) > *P. aureus* (78.1%) > *P. mungo* (69.5%) > *P. vulgaris* (58.2%). Interestingly, the results revealed that the *Phaseolus* species exhibited potent chelating ability. The antioxidants prevailing in the plant extracts also form a coordination complex with the metal ions and thus, impeding the donation of electrons. This oxidation reaction gets detained, and no free radicals are formed. The degree to which phytochemicals can form metal ion complexes mainly depends on their chemical structures. Different phytochemicals have different chelating abilities (Arfan et al., 2012). The chelating compounds may also sterically hinder the formation of the metal hydroperoxide complexes (Reische et al., 2008).

3.6. Hydrogen peroxide radical scavenging activity

The capability of *Phaseolus* species to scavenge hydrogen peroxide displayed varied scavenging activity in the different species (Figure 5) and was found to be in the order *P. aureus* (86.6%) > *P. mungo* (83.6%) > *P. vulgaris* (80.0%) > *P. aconitifolius* (75.6%) > *P. trilobus* (72.3%). The activity varied among the solvent extracts of different species and among the solvents the extracts prepared in ethanol showed the extreme scavenging activity in all the species as compared to other solvent systems confirming that ethanol extraction makes it possible to extract better or even preserve the molecules accountable for the antioxidant capacity of the studied species. As Shah et al. (2014) described, different extracts yield differently, depending upon the nature of solvents. The current study results indicated that the extracts might contain constituents capable of inhibiting the hydrogen peroxide. The secondary metabolites present in plants may have the capability to counteract the effect of hydrogen peroxide generation and thus averting the adverse effects of excess hydrogen peroxide production in the human body (Halliwell et al., 2000).

3.7. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging ability of the extracts is attributed to the antioxidant capacity of the extracts; therefore, it has been determined in different species of *Phaseolus* (Figure 6). It was observed from the figure that the activity varied amongst the species and the solvents. The activity was in the order: *P. vulgaris* (88.5%) > *P. aureus* (80.8%) > *P. aconitifolius* (82.8%) > *P. mungo* (75.3%) > *P. trilobus* (50.0%). Ethanol extracts from the individual species showed comparatively more scavenging activity than other solvent extracts. The current study results specified that the different solvent extracts of *Phaseolus* species revealed different effects on the scavenging of hydroxyl radicals. Accordingly, the outcomes of the current study specified that the *Phaseolus* species were found to be more potent in quenching superoxide anion radical and hindering deoxyribose degradation induced by hydroxyl radical.

Table 2. ANOVA for antioxidant activity among different solvent extracts of five *Phaseolus* species determined by different antioxidant assays

Antioxidant assays	df	MS	*p*
FRAP	4	122.5	0.0008
DPPH	4	335.3	6.9100
MoO_2P reduction	4	19.73	8.5800
Fe^{2+} chelation	4	579.4	0.0095
H_2O_2 scavenging	4	72.59	0.0185
·OH scavenging	4	743.9	0.0010
Deoxyribose degradation	4	110.0	0.0124
β-Carotene bleaching	4	154.3	0.0147

3.8. Deoxyribose degradation activity

As evaluated by deoxyribose assay, the scavenging capacity of Phaseolus species showed diverse degradation ability amongst the species (Figure 7). The percent degradation in the species was perceived as *P. aconitifolius* (89.92%) > *P. aureus* (88.62%) > *P. vulgaris* (85.89%) > *P. trilobus* (78.04%) > *P. mungo* (76.17%). The activity also varied among the solvents in all the species and was highest in the ethanol extract of *P. aconitifolius*, suggesting that certain hydroxyl radicals are scavenged by the antioxidants present in the sample. The phytochemicals present in the sample can inhibit deoxyribose degradation by hydroxyl radicals (You et al., 2007).

Table 3. Comparison between phytochemical constituents and antioxidant activities as represented by correlation coefficient

	P. vulgaris		*P. aureus*		*P. mungo*		*P. trilobus*		*P. aconitifolius*	
	TPC	TFC	TPC	TFC	TPC	TFC	TPC	TFC	TPC	TFC
FRAP	0.894	0.257	0.830	0.017	0.926	0.711	0.831	0.980	0.353	0.493
DPPH	0.967	0.536	0.646	0.055	0.489	0.238	0.574	0.347	0.536	0.561
PMR	0.687	0.027	0.966	0.011	0.815	0.952	0.806	0.410	0.505	0.327
FC	0.663	0.722	0.136	0.555	0.861	0.489	0.520	0.711	0.438	0.435
HPS	0.940	0.344	0.598	0.159	0.805	0.454	0.382	0.378	0.925	0.741
HRS	0.802	0.465	0.011	0.669	0.446	0.145	0.573	0.542	0.406	0.572
DD	0.742	0.512	0.940	0.237	0.396	0.563	0.889	0.744	0.998	0.945
β-CB	0.886	0.667	0.496	0.230	0.975	0.620	0.286	0.717	0.946	0.787

TPC = Total phenolic content; TFC = Total flavonoid content; FRAP = Ferric reducing antioxidant power; DPPH = DPPH radical scavenging; PMR= Phosphomolybdenum reduction; FC=Ferrous ion chelation; HPS=Hydrogen peroxide scavenging; HRS=Hydroxyl radical scavenging; DD=Dexyribose degradation; β-CB= β-Carotene bleaching activity

3.9. β-carotene bleaching activity

The *Phaseolus* species analyzed for the β-carotene bleaching activity revealed that the ethanol extracts of all the species exhibited the maximum activity (Figure 8). The comparison among the species specified that *P. aureus* (89.9%) showed the highest bleaching activity as compared to *P. vulgaris* (74.7%), *P. mungo* (74.9%), *P. trilobus* (84.5%), and *P. aconitifolius* (78.6%). The β-carotene bleaching activity differs in different species of *Phaseolus*. Moreover, several antioxidant compounds in the plants can also impede the amount of β-carotene bleaching by deactivating the linoleate-free radical and other free radicals formed in the system (Siramon and Ohtani, 2007). The rate of β-carotene bleaching can be slowed in the presence of antioxidants. We speculate that the β-carotene bleaching activity of the *Phaseolus* species studied is likely due to the presence of phytocompounds.

3.10. Statistical analysis

The significant mean differences amongst the various solvent extracts of five *Phaseolus* species were assessed by ANOVA, and a significant discrepancy with $p < 0.05$ was considered significant (Table 2). Further, Pearson's correlation coefficient correlation was obtained to describe the relationship of phenolics and flavonoids with antioxidant activities (Table 3). Individually, both positive and negative correlations were observed between phytochemical constituents and antioxidant methods in different species. In *P. vulgaris*, a significant correlation was observed between TPC against FRAP, DPPH, H_2O_2 scavenging, β-carotene bleaching, and ·OH scavenging activity, while TFC showed a good correlation only against ferrous ion chelating activity. The correlation observed between TPC, FRAP and DPPH are in accordance with the data reported by other authors for *P. vulgaris* (Rodríguez Madrera et al., 2021). In *P. aureus*, the correlation of TPC with phosphomolybdenum and deoxyribose degradation activity was significant; however, the coefficient of correlation observed amongst the TFC, and antioxidant activity was poor. In *P. mungo*, the correlation of TPC and TFC with FRAP, phosphomolybdenum reducing, ferrous ion chelating, H_2O_2 scavenging, and β-carotene bleaching activity was observed to be extremely significant. Interestingly, in *P. trilobus,* the correlation of TPC and TFC displayed a significant correlation with FRAP, phosphomolybdenum reducing, ferrous ion chelating, deoxyribose degradation, and β-carotene bleaching activity but was not significantly correlated with other antioxidants. Further, *P. aconitifolius* also showed a significant relationship of TPC and TFC with H_2O_2 scavenging, β-carotene bleaching activity, and deoxyribose degradation. The difference in the correlation between phytoconstituents and the antioxidant

activities specifies the diversity of the group of phytochemical compounds in the different species and their varied responses to different antioxidant methods to assess the antioxidant capacity.

The observed distinct radical scavenging activities of the species can be ascribed to the diverse chemical nature of several phytochemical compounds that may react with different types of free radicals in unique ways (Chun et al., 2003). The positive correlations observed specified that the phenolics and flavonoids are the main contributors to the observed antioxidant activity in the studied *Phaseolus* species. Further, the negative correlation designated that it could be related to other antioxidant compounds present in the species. This difference in correlation might be due to the difference in the stoichiometry of the reactions amongst the antioxidant compounds present in the extracts and the various radicals, which may be considered a reason for the difference in their scavenging ability (Khan et al., 2012). The results are in accordance with others, who have revealed the positive association between the total phenolic content and the antioxidant activity (Sultana et al., 2007; Kim et al., 2008; Oliveira et al., 2009; Chen et al., 2015). The results strongly suggest that the antioxidant activities of different *Phaseolus* species could be attributed to the presence of phytochemical constituents. Many species of the genus *Phaseolus* have also been reported to possess antioxidant activities (Sreeramulu et al., 2009; Capistrán-Carabarin et al., 2019; Alcázar-Valle et al., 2020). Among the antinutritional composites, testified in beans, are oligosaccharides, trypsin inhibitory activity, phytic acids, and lectins. However, the latest studies have stated that trypsin inhibitors and lectins could deliberate health benefits. For example, trypsin inhibitors could opine protection against rotavirus, impede some types of carcinogenesis, and could be used as chemopreventive agents (De Mejia et al., 2003), and lectins may decrease lymphoma growth and could be used as diagnostic markers for tumors, as well as help in the prevention of obesity (Chávez-Mendoza and Sánchez, 2017). Therefore, it can be assumed that the studied *Phaseolus* species have a high biological potential for preventing diseases caused by the free radicals.

4. Conclusions

The current study revealed that the *Phaseolus* species examined possessed significant antioxidant activities. Individually, notable differences were detected among the species, with ranges of variability in terms of phenolics, flavonoids, and antioxidant activity. However, different antioxidant test models showed different levels of antioxidant activity. However, in most of the assays, ethanol and aqueous extracts exhibited the highest antioxidant potential, and, among the species, *P. aureus*, *P. trilobus*, and *P. vulgaris* possessed the highest antioxidant activity. Further, the correlation study revealed that the antioxidant capacity of these species might be due to the existence of phytochemical constituents. Since the species studied have the capability of scavenging the free radicals, thereby; they might serve as potent antioxidants. Thus, the research reported herein highlighted the possible antioxidant capacity of these underutilized beans and their intake, which may contribute considerable amounts of antioxidants to the diet. Further studies are needed to isolate, identify, characterize and explicate the structure of the bioactive compounds.

Acknowledgments

The authors would like to acknowledge the Department of Botany, Bioinformatics, and Biotechnology, Karnataka State Akkamahadevi Women's University, Vijayapura, for laboratory facilities provided to carry out this study.

Conflict of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Firdose R. Kolar: Conception, Design of study, Data analysis, Interpretation, Drafting manuscript, Final approval and accountability
Vinutadivya Nirmanik: Data acquisition
Annapurna Kagawad: Data acquisition
Laxmi Angadi: Data acquisition
Babu R. Lamani: Critical revision of the manuscript

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Supplementary File

None.

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