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IJPBP encourages contributions that explore new frontiers in plant-based pharmaceutical sciences and that demonstrate the global relevance and applicability of their findings. Submissions highlighting cross-disciplinary approaches or international collaborations are particularly welcome.

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Table of Contents

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RESEARCH ARTICLE

OPEN ACCESS

Myanmar traditional medicine formulations and their antioxidant, antiglycation and alpha-glucosidase inhibitory activities: Potentials for antidiabetes complications

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ABSTRACT

Myanmar Traditional Medicine (MTM) has been used since ancient times to treat life-threatening diseases like diabetes. In this study, various in vitro assays were used to prove that four MTM formulations were effective at treating diabetes. Antioxidant activities were determined using in vitro DPPH, nitric oxide (NO), and superoxide (SO) radical scavenging assays. The Folin-Ciocalteu method was used to quantify the total phenolic content, while the BSA-fluorescent antiglycation and α-glucosidase inhibitory assays were utilized to determine the antidiabetic activity of MTMs. Among the tested samples, MTM3 showed the best activities for almost all the biological assays tested in this experiment with the % inhibition of 82.89 ± 1.64 for NO and 65.02 ± 2.82 for SO radical scavenging activity, 92.12 ± 1.18 for α-glucosidase inhibitory activity and IC_{50} of 180.29 ± 1.6 µg/ml for the antiglycation activity. It also possessed the highest total phenolic content of 149.41 ± 3.7 mg GAE/g of extract/l among the tested samples. Therefore, the findings suggested that MTM could help diabetic patients improve their quality of life through antioxidant activity against several free radicals and their antiglycation and α-glucosidase inhibitory characteristics.

1. Introduction

Myanmar is a country rich in natural resources, with over 7.000 species of medicinal plants growing throughout the country and 70% of Myanmar's population relies on traditional medicine (Wai, 2015). Traditional medicine has been used to treat various diseases since ancient times and there is no doubt about its effectiveness, but it is still a traditional knowledge-based practice and needs to be promoted as evidence-based medicine through scientific validation. Although Western medicines and technology have become more influenced by the urban population, Myanmar Traditional Medicine (MTM) is still popular among rural people (Wai, 2015).

MTM covers all the fundamental traditional disciplines, as well as different treatises on traditional medicine and a variety of ways for prescribing a wide range of remarkably strong, efficacious, and low-toxicity traditional drugs (Mamatha, 2017; Ministry of Health, 2006). Ethnobotanical research, on the other hand, has become increasingly popular not only at the

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national level but also at the worldwide level, and it has revealed that a large gap exists between the scientific validation of ethnomedicine and the applications to which it is put (Rafe, 2017). MTM formulations have also received quite a little attention for evidence-based validation.

Diabetes mellitus is a prevalent chronic and metabolic disorder defined by elevated glucose levels resulting from either a lack of insulin or insufficient insulin production. The condition is characterized by long-term problems affecting the ocular, renal, cardiovascular, and neurological systems. This particular ailment is additionally linked to symptoms including excessive urination, exhaustion, loss of weight, impaired wound healing, impaired vision, and elevated amounts of glucose in the urine (Moradi et al., 2018). Diabetes is associated with an increase in oxidative stress, and there is substantial experimental and clinical evidence that reactive oxygen species (ROS) generation is elevated in diabetes (Matough et al., 2012). Non-enzymatic glycation is the primary factor responsible for spontaneous protein damage, resulting in a range of issues attributed to the development of non-reversible Advanced glycation end products (AGEs) and the induction of oxidative stress (Perera et al., 2013). Plant samples exhibiting both antioxidant and antiglycation capabilities possess enhanced potential for the treatment of a variety of biological illnesses, such as diabetes (Moe et al., 2018).

As globalization and east-west interactions intensify, the rising prevalence of type 2 diabetes in Asia has significant public health and socioeconomic consequences (Lee et al., 1998). This research attempted to validate the commercially available antidiabetic MTM on the aspects of antioxidant, antiglycation, and α-glucosidase inhibitory activities using in vitro assays and compared their effectiveness based on their formulations.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade chemicals such as 1,1-diphenyl- 2-picrylhydrazyl (DPPH), ascorbic acid, gallic acid, sodium nitroprusside, sulphanilic acid, N-(1-naphthayl) ethylenediamine dihydrochloride, glacial acetic acid, Folin-Ciocalteu's reagent, sodium carbonate, ethylenediamine tetra-acetic acid (EDTA), nitro blue tetrazolium (NBT), riboflavin, acarbose, α-glucosidase, *p*-nitrophenyl-α-D-glucopyranoside (PNPG), fructose, sodium azide, and bovine serum albumin (BSA) were purchased from Sigma Chemicals Co. (St. Louis, USA), or TCI Development Co. Ltd (Shanghai, China).

2.2. MTM formulations

Commercially available MTMs were purchased from Kyaukse district, Mandalay region in Myanmar, and brand names were given the codes MTM1, MTM2, MTM3, and MTM4. The formulations of purchased MTMs are shown in Table 1.

2.3. Antioxidant activity assays

2.3.1. In vitro DPPH free radical scavenging assay

The free radical-scavenging activity of the MTM formulations was determined with the DPPH free radical-scavenging assay as described by Lee et al. (1998). In a 96-well microplate, the reaction mixture containing 5 µl of the test sample (0.5 mg/ml in DMSO) and 95 µl of DPPH (300 mol/l in methanol) was deposited. The optical density (OD) was measured using a SPECTROstar Nano microplate reader (BMG LABTECH, Germany) at a wavelength of 515 nm after the microplate had been kept in the dark at 37 °C for 30 minutes. As the blank, DMSO was utilized, while ascorbic acid served as the standard. The inhibition rate (%) was calculated through comparison to the blank.

2.3.2. In vitro nitric oxide radical scavenging assay

Nitric oxide (NO) radical scavenging assay was performed as described by Hertog et al. (1993). 10 µl of the test sample (0.5 mg/ml) in DMSO, 20 µl of phosphate buffer saline (0.1 mol/l, pH 7.4), and 70 µl of sodium nitroprusside (10 mmol/l) were mixed, and incubated at 25 °C for 90–100 minutes to facilitate the formation of nitrite ions. After incubation, 50 µl of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was added and permitted to stand for 5 minutes for diazotization to be completed. Then 50 µl of 0.1% w/v N-(1-naphthyl) ethylenediamine dihydrochloride was added, agitated, and left to stand for 20 minutes. Under the diffused light, a pink chromophore was created. Using a SPECTROstar Nano microplate reader, the reduction of the pink-colored chromophore was measured at 540 nm relative to the corresponding blank solution. Ascorbic acid served as the standard for comparison.

2.3.3. In vitro superoxide radical scavenging assay

The free radical scavenging activity of the MTM formulations was also determined with superoxide (SO) radical scavenging assay modified from the protocol described by Patel Rajesh and Patel Natvar (2011). The reaction mixture containing 10 µl of the test sample (0.5 mg/ml) in DMSO, 160 µl of potassium phosphate buffer (0.067 mol/l, pH 7.4), 15 µl of EDTA (4.5 mmol/l), 10 µl of NBT (1 mg/ml), and 5 µl of riboflavin (0.2 mg/ml) was incubated under fluorescence light for 5 minutes. Using a SPECTROstar Nano microplate reader, the absorbance of the solution was determined at 560 nm relative to the blank solution. Gallic acid was used as the reference standard.

2.4. Antidiabetic activity assays

2.4.1. In vitro α-glucosidase inhibitory activity assay

The α-glucosidase inhibitory activity assay was performed as described by Abu-Zaiton (2010) to detect the antihyperglycemic potentials of the extracts. In a 96-well microplate, 10 µl of the test sample (0.4 mg/ml in DMSO), 20 µl of -glucosidase (0.5 unit/ml), and 120 µl of 0.1 M phosphate buffer (pH 6.9) were added together and incubated at 37 °C for 15 minutes. Then, 20 µl of 5 mM *p*-nitrophenyl-D-glucopyranoside in 0.1 M phosphate buffer (pH 6.9) was added to initiate the enzymatic reaction, followed by 15 minutes of incubation at 37 °C. The reaction was then neutralized by adding 80 µl of 0.2 M sodium carbonate solution, and absorbance was measured at a wavelength of 405 nm using a SPECTROstar Nano microplate reader. The reaction system without plant extracts was used as a control, while the reaction system without α-glucosidase was used as a blank to correct for background absorbance. Acarbose was utilized as the reference standard.

All of the aforementioned analyses were conducted at least in triplicate for each sample and positive control, and the inhibition rate was calculated using the following formula given below:

$$\text{Inhibition rate (\%)} = \left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

2.4.2. In vitro antiglycation activity assay

In vitro, antiglycation activity of MTM formulations was determined by measuring the ability of the MTM to inhibit the formation of advanced glycation end products (AGE) as described by Yarizade et al. (2017). In a 96-well black fluorescence plate, the reaction mixture (200 µl), containing 10 µl BSA (10 mg/ml), 70 µl of sodium phosphate buffer (0.1 M, pH 7.4), 100 µl of fructose (500 mM), sodium azide (0.1 mM) and 20 µl test sample (various concentrations in DMSO), was incubated at 37 °C for 7 days in the dark. After incubation, AGE formation was measured at the fluorescence intensity of excitation (370 nm) and emission (440 nm) by using an Agilent Cary Eclipse Fluorescence spectrophotometer (G9800, US). Rutin was used as the standard. The reaction mixture without fructose was used as the negative control and that without extracts as the positive control. Inhibition rate (%) was calculated by using the following formula given below:

$$\text{Inhibition rate (\%)} = \left(1 - \frac{\text{Fluorescence intensity of sample}}{\text{Fluorescence intensity of control}}\right) \times 100$$

Table 1. Formulation of MTMs

Name	Ingredients	Formulation (mg/g of MTM)
MTM1	*Swertia angustifolia*	17.19
	Tinospora cordifolia	245.70
	Trigonella foenum-graecum	245.70
	Quercus intecforia	245.70
	Capsicum frutescens	245.70
MTM2	*Trigonella foenum-graecum*	139.46
	Curcuma longa	101.24
	Centella asiatica	354.34
	Andrographis paniculata	253.09
	Piper nigrum	101.24
	Tinospora cordifolia	50.62
MTM3	*Ferula asafoetida*	142.86
	Curcuma longa	142.86
	Cinnamomum zeylanicum	142.86
	Aloe barbadensis	142.86
	Croton roxburghianus	142.86
	Tinospora cordifolia	142.86
	Andrographis paniculata	142.86
MTM4	*Gnetum gnemon*	230.78
	Odoriferous medicinal salt	115.39
	Rock salt	115.39
	Salamoniac	115.39
	Glycyrrhiza glabra	115.39
	Five types of stones (salt + saltpetre + natron + charcoal ash + limestone)	48.08
	Five types of seeds from *Foeniculum vulgare*, *Hyoscyamus niger*, *Apium gravedens*, *Carum carui*, *Anethum graveolens*	48.08
	Curcuma longa	38.46
	Rauvolfia serpentina	38.46
	Terminalia citrina	19.23
	Ray sting	19.23
	Myristica fragrans	19.23
	Caesalpinia crista	19.23
	Piper longum	19.23
	Trigonella foenum-graecum	19.23
	Caryophyllus aromaticus	9.61
	Zingiber officinale	9.61

2.5. Total phenolic content measurement

The total content of phenolic compounds present in each MTM formulation was measured with the method described by Waterhouse (2002) with slight modifications. Briefly, 2 µl of the test samples (1 mg/ml in 70% methanol) or gallic acid at varying concentrations (0, 0.5, 1, 1.5, 2.0, 2.5, and 5 g/ml in 70% methanol) were dispensed into each well of the 96-well microplate. 148 µl of distilled water and 20 µl of the 1 N Folin-Ciocalteu's reagent were added. The reaction mixtures were thoroughly combined and incubated at room temperature for 5 minutes. The reaction was then neutralized by adding 30 µl of sodium carbonate at a concentration of 0.2 g/ml. After incubating the samples at 40 °C for 30 minutes, the optical density (OD) was measured using a SPECTROstar Nano microplate reader at a wavelength of 765 nm. Three replicate assays were performed on each sample. Total phenolic content (TPC) was computed as gallic acid equivalent (GAE) using the gallic acid standard curve equation ($y = 0.0007x$, $R^2 = 0.9985$).

2.6. Statistical analysis

All data were expressed as mean ± standard error of the mean of at least triplicate measurements. One-way analysis of variance (ANOVA) and Dunnett's or Tukey's multiple comparison tests were performed to compare the difference between the MTM activity and standard control or between each other. In each analysis, $p \leq 0.05$ was considered statistically significant. For the gallic acid standard curve, the optical density (OD) of the gallic acid was plotted against the concentrations of 0, 50, 100, 150, 250, and 500 mg/l. The linear regression points were used to determine the GAEs of the phenolic concentration in tested samples. Statistical analyses were performed using Microsoft Office 2010 and GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The IC_{50} was defined as the concentration of MTM required to inhibit 50 % of radicals or enzyme activity under assay conditions.

3. Results and discussion

Four commercial anti-diabetic MTMs were tested for their biological activities by using in vitro assays and their activities were compared

based on their formulations. In vitro DPPH radical scavenging assay, nitric oxide (NO) radical scavenging assay, and superoxide (SO) radical scavenging assay were used to determine the antioxidant activities. Folin-Ciocalteu's method was used to determine the total phenolic content and BSA-fluorescent antiglycation assay and α-glucosidase inhibitory activity were used to evaluate the antidiabetic activity of selected MTM.

Among the four tested samples for six biological activities evaluations, MTM3 showed the best activities for almost all the biological assays tested in this experiment with the % inhibition of 82.89 ± 1.64 for NO and 65.02 ± 2.82 for SO radical scavenging activity, 92.12 ± 1.18 for α-glucosidase inhibitory activity and IC_{50} of 180.29 ± 1.6 µg/ml for the antiglycation activity (Figure 1, 2, 3, 4 and Table 2). It also possessed the highest total phenolic content of 149.41 ± 3.7 mg GAE/g of extract/l among the tested samples (Figure 5).

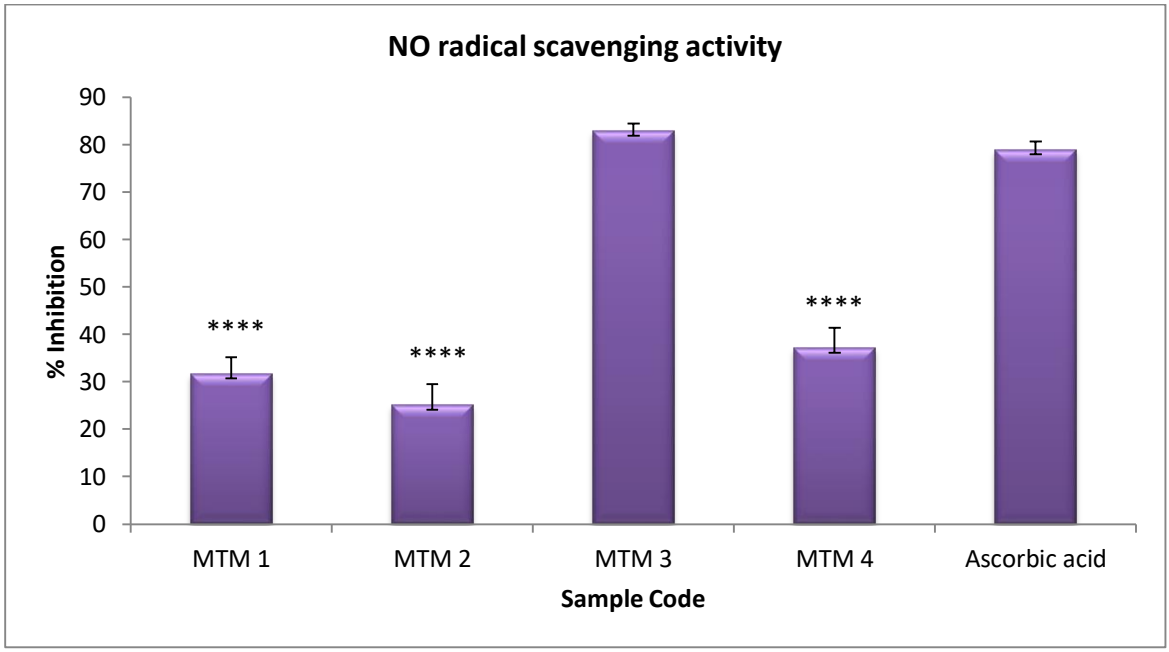

Figure 1. Comparison of antioxidant activities of Myanmar traditional medicine formulations through NO radical scavenging assay
Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group. NO: Nitric oxide

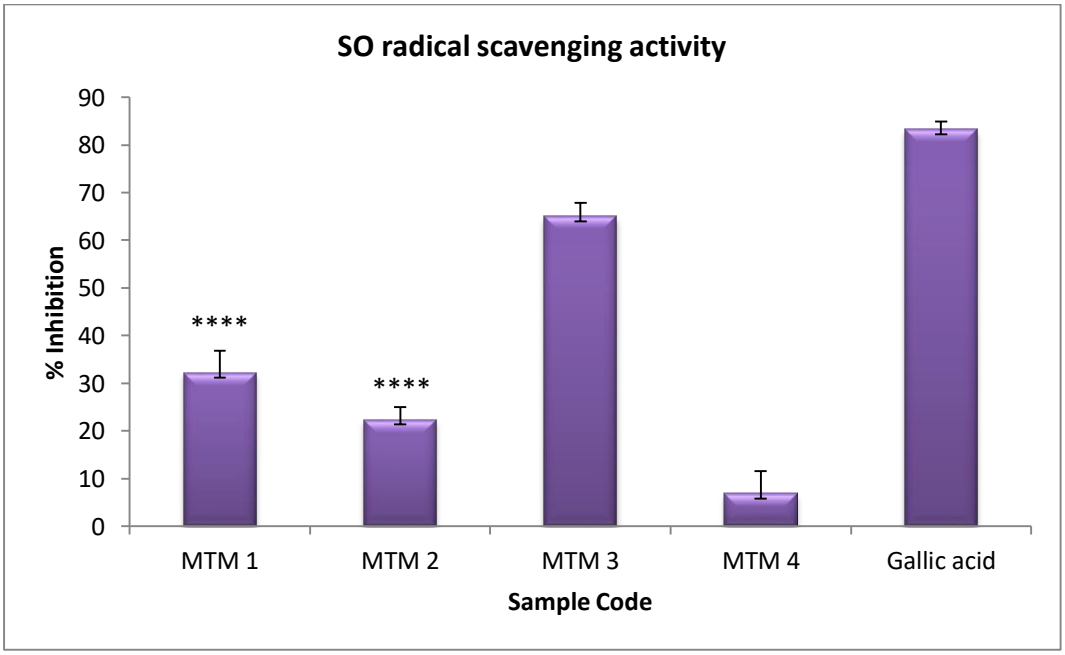

Figure 2. Comparison of antioxidant activities of Myanmar traditional medicine formulations through SO radical scavenging assay
Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group. SO: Superoxide

Figure 3. Comparison of antidiabetic activities of Myanmar traditional medicine formulations through α-glucosidase inhibitory activity assay
Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group.

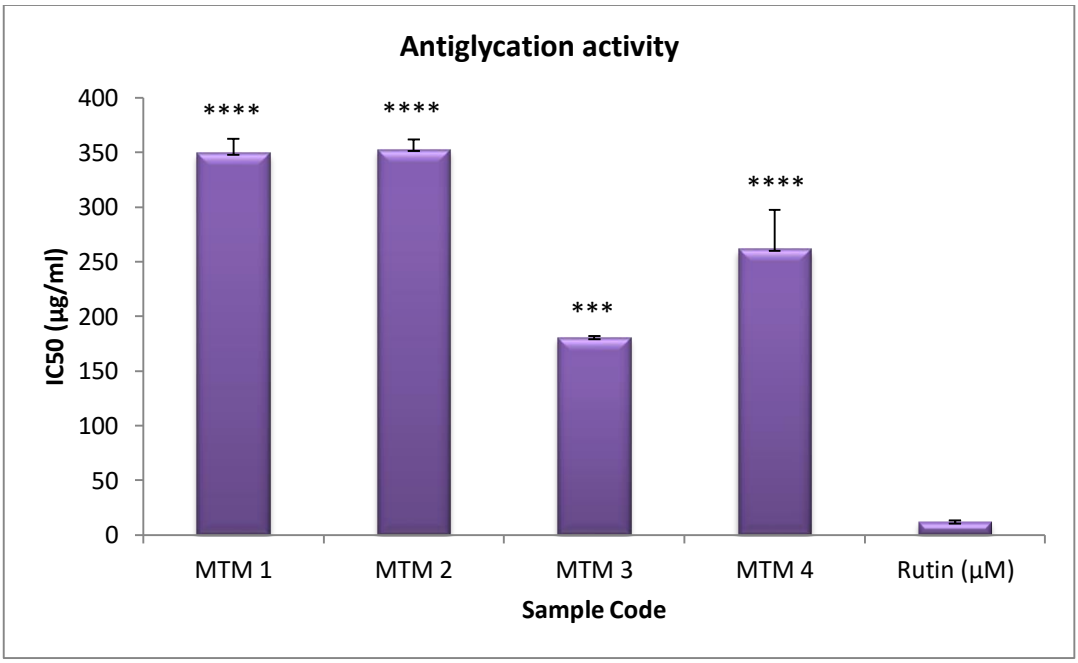

Figure 4. Comparison of antidiabetic activities of Myanmar traditional medicine formulations through antiglycation assay
Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group.

For the DPPH radical scavenging assay, MTM1 showed the best activity with a % inhibition of 53.66 ± 4.19 (Figure 6). It was also active in α-glucosidase inhibitory assay with a % inhibition of 62.39 ± 4.54 while positive control, acarbose, showed a % inhibition of 60.54 ± 5.57. MTM2 was not active for all the currently tested bioassays.

MTM4 was moderately active in antiglycation assay with IC_{50} of 260.88 ± 36.36 µg/ml and active in α-glucosidase inhibitory assay with % inhibition of 61.39 ± 0.96.

MTM3 was composed of seven traditional medicinal plants including *Ferula asafoetida*, *Curcuma longa*, *Cinnamomum zeylanicum*, *Aloe barbadensis*, *Croton roxburghianus*, *Tinospora cordifolia*, and *Andrographis paniculata*. Several research teams have studied *F. asafoetida*'s antidiabetic potential. *F. asafoetida* extract was examined for potential antidiabetic action against alloxan-induced diabetes in rats and associated hormones. *F. asafoetida* extract lowered blood glucose levels and increased serum insulin (Abu-Zaiton, 2010). Another study team determined the in vitro antidiabetic potential of *F. assafoetida* via DPP-IV and α-glucosidase inhibitory activities and concluded that *F. assafoetida* could be a

source for active ingredients as α-glucosidase and DPP-IV inhibitors to treat type 2 diabetes (Yarizade et al., 2017). It was also shown that ethanolic *F. assafoetida* oleo-gum-resin extract can regulate hyperglycemia and diabetes complications in streptozotocin (STZ)-induced diabetic rats (Latifi et al., 2019). The rest six medicinal plants used in this formulation were also known for their antidiabetic activities and several studies have been carried out to support their antidiabetic activity through in vitro and in vivo assays (Den Hartogh et al., 2019; Krishnakumar et al., 2014; Sharafeldin & Rizvi, 2015). *A. barbadensis* was also investigated for its antidiabetic activity through clinical trials (Yongchaiyudha et al., 1996). Therefore, formulation employing potent medicinal plants made this MTM3 effective to serve as an antidiabetic drug.

Table 2. In vitro biological activities and total phenolic content of Myanmar traditional medicine formulations

Name	DPPH (%)	NO (%)	SO (%)	Antiglycon (IC_{50})	α-Glucosidase inhibitory (%)	Total phenolic content (mg GAE/g extract)
MTM1	53.66 ± 4.19**	31.72 ± 3.48****	32.18 ± 4.70****	348.98 ± 13.65****	62.39 ± 4.54	31.78 ± 2.25[a]
MTM2	44.05 ± 0.87***	25.16 ± 4.37****	22.37 ± 2.61****	352.17 ± 9.98****	0.49 ± 0.06****	27.16 ± 1.69[a]
MTM3	48.57 ± 8.05**	82.90 ± 1.65	65.02 ± 2.82*	180.30 ± 1.63***	92.12 ± 1.18***	149.41 ± 3.64[b]
MTM4	36.37 ± 7.02***	37.09 ± 4.27****	6.82 ± 4.74****	260.88 ± 36.36****	61.39 ± 0.96	32.53 ± 1.59[a]
Ascorbic acid	84.78 ± 0.47	78.96 ± 1.71	-	-	-	-
Gallic acid	-	-	83.24 ± 1.73	-	-	-
Rutin (μM)	-	-	-	11.64 ± 1.86	-	-
Acarbose	-	-	-	-	60.54 ± 5.57	-

Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group.
DPPH: 2,2-diphenyl-1- picrylhydrazyl. For total phenolic content assay, different letters (a to b) indicate the difference ($p < .05$) in each group.

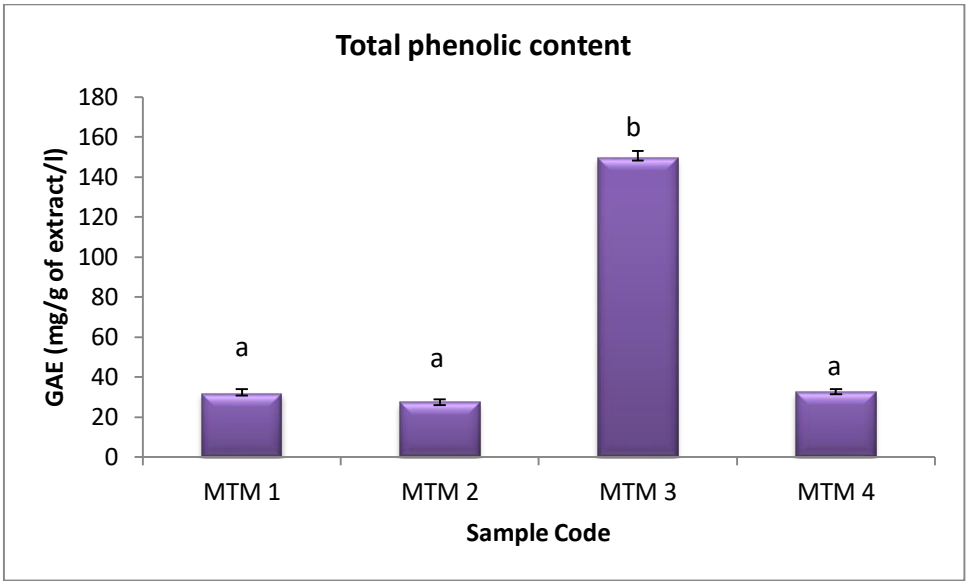

Figure 5. Comparison of TPC of Myanmar traditional medicine formulations determined by the Folin-Ciocalteu's assay and calculated as GAE in mg/g extract based on dry weight
Results were the mean of triplicates ± standard error of the mean. Different letters (a–b) indicated significant differences ($p < 0.05$) in each group.
GAE: gallic acid equivalent, TPC: total phenolic content

Another antidiabetic drug; MTM1 was comprised of five traditional medicinal plants including *Swertia angustifolia*, *T. cordifolia*, *Trigonella foecgraetun*, *Quercus intecforia*, and *Capsicum frutescens*. MTM2 was composed of six medicinal plants such as *T. foecgraetum*, *C. longa*, *Centella asiatica*, *A. paniculata*, *Piper nigrum*, and *T. cordifolia*. MTM4 is made up of 17 ingredients of which 12 are ingredients derived from medicinal plants and the rest are minerals, salts, and animal products which are normally used in MTM preparations.

It was observed that *T. cordifolia* was used in 3 formulations of MTM1, MTM2, and MTM3. *T. cordifolia* is a plant that is frequently used in Ayurvedic medicine for the control of diabetes. Studies conducted in the past have demonstrated that *T. cordifolia*, which is abundant in nutraceuticals, is an effective anti-diabetic plant material. It was shown that *T. cordifolia* aqueous extract dramatically lowered blood glucose and increased plasma insulin levels in alloxan-induced mildly diabetic rats, thereby demonstrating an insulinotropic activity (Joladarashi et al., 2014; Noor & Ashcroft, 1998; Noor et al., 1989).

T. foenum-graecum was included in 3 MTM formulations of MTM1, MTM2, and MTM4. The previous research revealed that a seed powder solution made from *T. foenum-graecum* has a substantial role to play in reducing dyslipidemia in individuals who have just been diagnosed with type II diabetes (Geberemeskel et al., 2019). *C. longa* was present in the formulation of MTM2 and MTM3. A comprehensive spectrum of physiological and pharmacological properties, including antioxidant, anti-inflammatory, anticancer, neuroprotection, and anti-diabetic actions was found in the bioactive constituents isolated from *C. longa* (Den Hartogh et al., 2019; Fazel Nabavi et al., 2015). MTM2 and MTM3 also used *A. paniculata* in the formulations. Previous research reported that *A. paniculata* or its most active constituent andrographolide demonstrated hypoglycemic and hypolipidemic effects in rats fed a high-fat, high-fructose diet (Nugroho et al., 2012).

There are several in vitro antidiabetic activity assays that could be used to study the antidiabetic activity of medicinal plants such as α-amylase inhibitory assay, dipeptidyl peptidase IV (DPP-IV) inhibition assay, PPARγ and GLUT-4 assay, PTP1B assay, glucose uptake assay, glucose adsorption assay, reporter gene assay, insulin secretion assay, calcium measurement assay, ATP measurement assay, and cAMP assay (Vhora et al., 2020). Therefore, it is imperative to carefully choose a suitable in vitro assay to assess the antidiabetic activity of the medicinal plants. A comprehensive examination of the multiple parameters involved in the pathway for glucose and hormone metabolism is essential. In the current research, two commonly used in vitro antidiabetic assays: BSA-fluorescent antiglycation assay and α-glucosidase inhibitory assay were used to determine the antidiabetic activity of MTM. MTM1, MTM3, and MTM4 which showed their antidiabetic activities through these two bioassays. However, MTM2 was not active for all the currently tested bioassays. Therefore, it is recommended that the aforementioned in vitro antidiabetic assays be employed to evaluate the comprehensive effects of the tested MTM to acquire a thorough understanding of their actions against diabetes mellitus.

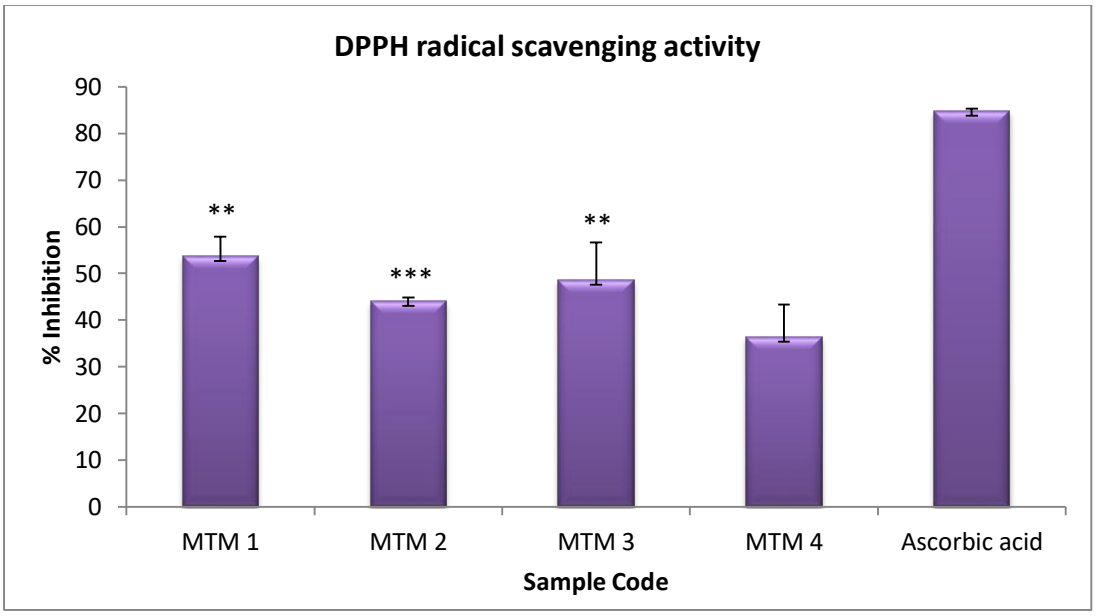

Figure 6. Comparison of antioxidant activities of Myanmar traditional medicine formulations through DPPH radical scavenging assay
Values are the mean of at least three replicates of experiments ± standard error of the mean.
*$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$, sample vs standard in each group. DPPH: 2,2-diphenyl-1- picrylhydrazyl

4. Conclusions

In conclusion, MTM were extensively utilized to treat type 2 diabetes mellitus and were developed using traditional knowledge. Although their actions were very different from one another, recent evidence-based validation research demonstrated that these MTMs possessed antioxidant, antiglycation, and α-glucosidase inhibitory characteristics in a range of in vitro experiments. These formulations showed antioxidant activity against several free radicals in addition to their antiglycation and α-glucosidase inhibitory characteristics. Therefore, the antidiabetic activities of MTM could be confirmed by evidence-based validations of current research experiments as stated in their description as an antidiabetic drug, and diabetic patients could improve their quality of life by using these MTM formulations that were comprised of a potent combination of MTM plants.

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Conflict of interest

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

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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The Su Moe: Conceptualization, Investigation, Data curation, Writing - original draft, Supervision
Htet Htet Win: Laboratory investigation, Practical, Investigation, Result analysis
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Supplementary File

None.

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REVIEW

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Exploring the phytochemistry and pharmacology of *Mangifera indica* L. (Mango) leaves: A review

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ABSTRACT

Mangifera indica L. (Mango), a member of the Anacardiaceae family, is native to the tropical and subtropical regions of the world. Leaves of *M. indica* exhibit pharmacological potential as a panacea. The current study aimed to systematically review the phytochemistry and biological effects of *M. indica* leaves (MILs). Google Scholar, PubMed, Scopus, and Web of Science were used to review the relevant literature. MILs are reported to possess remarkable medicinal properties owing to a plethora of phytochemicals, namely, minerals, vitamins, flavonoids, phenolic acids, terpenes, benzophenones, tannins, saponins, and alkaloids. MILs have been investigated for numerous therapeutic effects, including anticancer, anti-diabetic, antioxidant, antiviral, antibacterial, antifungal, antidiarrheal, antiulcer, gastrointestinal, anti-obesity, cardio-protection, hypotensive, analgesic, and hepato-protection. In this review, phytochemistry, pharmacology as well as toxicology of MILs have been critically discussed. Considering their phytochemical profile and pharmacological benefits, MILs can be used for the development of valuable pharmaceutical products. However, more comprehensive clinical trials are needed to be conducted for further evaluation of its effectiveness.

1. Introduction

One of the basic goals of Millennium Development Goals (MDGs) is the search to fight diseases such as acquired immune deficiency syndrome (AIDS), cancer, and cardiovascular disorders. Plants have always been highly beneficial to mankind for treating implacable diseases from time immemorial. Medicinal herbs are enriched in secondary metabolites, with better safety profile and least or no side effects, which make them an outstanding source of drugs and therapeutics. Therefore, an interest is developing in the application of herbal extracts as therapeutic agents (Mishra et al., 2022; Naik et al., 2020).

Mangifera indica L. is a member of the Anacardiaceae family. It is commonly cultivated in the tropical or subtropical regions of the world. It is considered an important plant in South and Southeast Asia. Pakistan, India, Bangladesh, Thailand, China, Indonesia, Nigeria, Philippines, Mexico, and Nigeria are considered among its main producer countries. *M. indica* also known as the 'king of fruit', is an evergreen tree that has numerous biological properties besides its very popular fruit (Kumar et al., 2021; Parvez, 2016).

Leaves of *M. indica* show remarkable medicinal, biological, and metabolic characteristics. Generally, *M. indica* leaves (MILs) are treated as a waste mainly produced by the pruning of *M.*

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indica, but in reality, these are highly important comprising a huge variety of phytoactive substances including phenolic, crude protein, essential oils, dietary fiber, vitamins, and minerals. MILs contain minerals including calcium (Ca), iron (Fe), sodium (Na), potassium (K), phosphorus (P), zinc (Zn), nitrogen (N), magnesium (Mg), manganese (Mn), boron (B), sulfur (S), copper (Cu) and cadmium (Cd), and vitamins including A, B, E, and C. MILs have been extensively studied due to their biological potential owing to a lot of bioactive compounds, namely mangiferin, flavonoids, benzophenones, terpenes, phenolics, alkaloids, saponins, tannins and many other medicinal compounds. The MILs show various pharmacological activities such as antimicrobial, antidiarrheal, antioxidant, anti-diabetic, anticancer, antiobesity, gastrointestinal, cardio-protection, and hepato-protection **(Figure 1)** (Kumar et al., 2021; Pan et al., 2018).

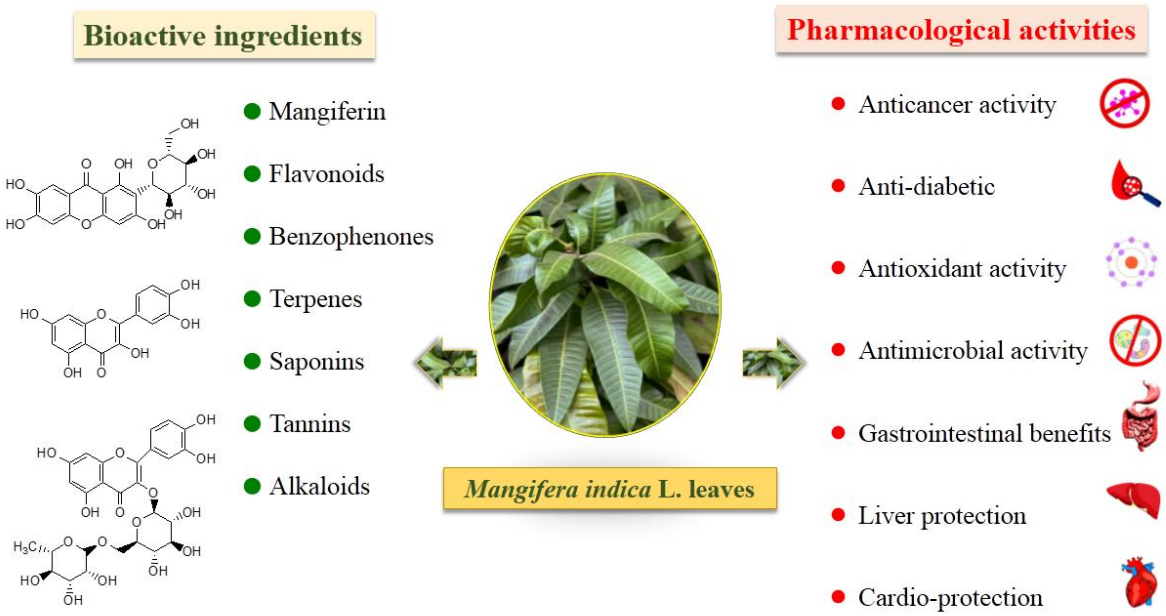

Figure 1. Phytochemistry and pharmacology of *M. indica* leaves

This review aims to discuss traditional uses, phytochemical profile as well as pharmacology of leaves of *M. indica*. Furthermore, the toxicology of MILs is briefly presented.

2. Methodology

The study was conducted by searching electronic databases including Google Scholar, Scopus, PubMed, and Web of Science for studies focused on the phytochemistry and pharmacological activities of MILs. All the articles published between 1990 and 2023 were examined.

3. Taxonomic classification

Kingdom: Plantae
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Rosidae
Order: Sapindales
Family: Anacardiaceae
Genus: Mangifera
Species: *Mangifera indica* L. (Yadav et al., 2018)

4. Botanical description

The leaves are lanceolate-elliptical, linear-oblong, spirally arranged on offshoots, located at both ends and release an aromatic odor on crushing. Leaf blades are mostly about 25 cm long and 8 cm wide, occasionally much larger. Leaves are reddish and thinly flaccid when first formed. The upper surface of leaves is dark green and shiny while the lower surface is glabrous light green (Parvez, 2016; Shah et al., 2010).

5. Traditional uses

In traditional medicine, dried leaves of *M. indica* were considered useful in the treatment of respiratory infections and diabetes (Zhang et al., 2019). *M. indica* was enlisted in TRAMIL (Program of Applied Research to Popular Medicine in the Caribbean) as an active agent in treating fever, ulcers, gastritis, and diarrhea (Robineau & Saejarto, 1996). MILs have also been listed in the Dictionary of Chinese Medicine for diabetes resistance and decrement of respiratory ailments (Shi et al., 2020). MILs are the main ingredients in some of the traditional Chinese medicine formulations such as Mango anticough tablets, and Yinhua mango granule (Xu et al., 2018). Moreover, aqueous extract of MILs has been utilized as traditional tea in certain Chinese districts, namely, Guangxi province (Zhang et al., 2013). In tropical Africa, *M. indica* is used medicinally as an astringent for toothache, skin diseases, internal hemorrhage, catarrh, and bronchitis. MILs tea are used for fever and diarrhea. The MILs are used as anti-diabetic agents in the folk medicine of Nigeria (Aderibigbe et al., 1999). The ash of burnt leaves is used to cure burns and scalds. The smoke produced from burning leaves is inhaled for relief of throat sickness (Parvez, 2016).

6. Phytochemistry of *M. indica* leaves

MILs exhibit huge medical value owing to their high concentration of bioactive chemical ingredients (Wu et al., 2020). Minerals, namely, K (589 mg), P (480 mg), Fe (343 mg), Mg (98 mg), Ca (368 mg), Na (28 mg), Zn (14 mg), Mn (3 mg), and N (2 mg) are found per 100 g dry weight in MILs (Ali et al., 2020). Various studies indicated presence of Fe (0.0062–0.034%), P (0.007–0.48%), N (0.003–2.6%), Mg (0.009–1.58%), Zn (0.0024–0.014%), Ca (0.003–4.41%), B (0.0016–0.0042%), S (0.37–0.88), copper (0.0021–0.0029%), Na (0.003–0.23%), cadmium (0.015%), and Mn (0.0028–0.003%) in total content of MILs extract (Kumar et al., 2021). Vitamin A (22.60 mg/100 g), B1 (0.04–0.48 mg/100 g), B2 (0.06–0.21 mg/100 g), B3 (0.38–2.20 mg/100 g), and C (13.20–53 mg/100 g) were also discovered (Okwu & Ezenagu, 2008; Princwill-Ogbonna et al., 2019; Rymbai et al., 2013).

The qualitative phytochemical study of MILs extract showed numerous medicinally significant secondary metabolites including flavonoids, tannins, saponins, and alkaloids **(Table 1)** (Ali et al., 2020). Chemical analysis of crude, ethyl acetate, and methanolic extracts of MILs utilizing ultrahigh pressure liquid chromatography identified various useful bioactive compounds which include nine flavanols, four xanthones, seven terpenoids, ten benzophenones, four derivatives of gallotannins, eleven phenols (Jhaumeer Laulloo et al., 2018). Another phytochemical study resulted in the identification and isolation of seventeen flavonoids and five benzophenones (Pan et al., 2018). In the leaves of the West African species of *M. indica*, quercitin (both free and glycosides), four anthocyanidins (3-monosides of paeonidin, delphinidin, and cyanidin), mangiferin, leucoanthocyanins, gallic and catechic tannins and kaempferol were reported (Okwu & Ezenagu, 2008).

Table 1. An overview of reported phytochemicals in leaves of *M. indica*

Type of extract	Chemical analysis	Phytochemicals	Reference
70% ethanol-water (3 × 50 l) extract	Positive-ion HR-ESI-TOF-MS	**Polyphenols:** Iriflophene, quercetin-3-*O*-β-D-xylopyranoside, quercetin-3-*O*-β-D-glucoside, quercetin-3-*O*-α-L-rhamnoside, quercetin-3-*O*-β-D-arabinoside, quercetine-3-*O*-β-D-galactoside, isovitexin, isoswertisin, vitexin, quercetin-4′-*O*-β-D-glucoside, luteolin-7-*O*-β-D-glucoside, quercetin, 3′,5′-dimethoxy-4′,5,7-trihydroxyflavone, mangiferin, 4′-*O*-*p*-hydroxybenzoyl mangiferin, amentoflavone, hypericin, and taxifolin	(Pan et al., 2018)
Ethanolic and methanolic extract	UPLC-MS/MS (positive mode)	**Xanthones:** Mangiferin, mangiferin-6′-*O*-gallate, mangiferin 3-methyl ether **Polyphenolic compounds:** Protocatechuic acid, gallic acid, derivative of gallic acid, methyl gallate, 2,5-di-tert-butyl phenol, tetrahydroxy sodium benzoate, ellagic acid, theogallin, derivative of theogallin with one OH missing **Flavonols:** Quercetin, rhamnetin, quercetin carboxylic acid, quercetin pentoside, quercetin 3-*O*-rhamnoside, epicatechin gallate hexamalonate, rhamnetin hexoside **Benzophenones:** Maclurin, iriflophenone glucoside derivative, iriflophenone 3-C-*β*-D-glucopyranoside, maclurin 3-C-(6′′-*O*-*p*-hydroxybenzoyl)*β*-D-glucoside, iriflophenone-di-*O*-galloyl-glucoside, iriflophenone tri-*O*-galloyl-glucoside **Terpenoids:** Lupeol, mangiferonic acid, manglanostenoic acid, cycloart-25-ene-3,24,27-triol, cycloartane-3,24,25-triol	(Jhaumeer Laulloo et al., 2018)
Ethanolic and methanolic extract	UPLC-MS/MS (negative mode)	**Phenolic compounds**: Sodium gallate **Xanthones:** Mangiferin, isomangiferin, mangiferin 3-methyl ether, mangiferin-6′-*O*-gallate **Flavanols:** Kaemferol, quercetin 3-*O*-glucoside **Benzophenones**: Maclurin 3-C-β-D-glucoside; 3-glucosyl-2,3',4,4',6-pentahydroxybenzophenone, maclurin 3-C-(6′-*O*-*p*-hydroxybenzoyl)*β*-D-glucoside, maclurin mono-*O*-galloyl-glucoside, maclurin di-*O*-galloyl-glucoside **Terpenoids:** Cycloartane-3,29-diol, 3*β*-form, 3,27-dihydroxycycloart-24-en-26-oic acid **Gallotannins:** Digalloyl glucoside, tri-*O*-galloyl glucoside, tetra-*O*-galloyl glucoside, penta-*O*-gallose-glucose **Other compounds:** Ferulic acid hexoside	(Jhaumeer Laulloo et al., 2018)
Essential oil obtained by hydro-distillation	GC–MS analysis	**Terpenes:** (-)-α-pinene, 1-terpineol, 3-methyl-camphenilol, α-humulene, α-elemene, 4-terpineol, camphor, α-gurjunene, α-guaiene, α-selinene, γ-selinene, (-)-α-panasinsen, palustrol, globulol, viridiflorol, α-eudesmol, octadecane, α-copaene, *cis*-guriune, α-cadinene, isocaryophyllen, elemol, guaiol, δ-cadinol, phytol isomer, α-terpinolene, *p*-cymene-8-ol, γ-cadinene, germacrene D, eremophilene	(Ouf et al., 2021)
Aqueous extract	High-resolution electrospray ionization mass spectrometry (HRESIMS) (positive mode)	**Benzophenone:** Acarbose, manindicin A, manindicins B, mangiferin, norathyriol	(Gu et al., 2019)
Aqueous extract	GC–MS analysis	Furfural, 2-furanometanol, *o*-catechol, hydroquinone, pyrogallol, oleic acid	(Martínez-Bernett et al., 2016)

MILs extract have flavonoids (1.054 ± 0.001 mg/g), tannins (0.977 ± 0.001 mg/g), alkaloids (0.300 ± 0.141 mg/g), and saponins (0.244 ± 0.001 mg/g) (Ali et al., 2020). Mangiferin has been reported as a major constituent (7.43%) (Pan et al., 2018). Flavonols including quercetin, quercetin 3-*O*-glucoside, quercetin carboxylic acid, quercetin pentoside, rhamnetin, quercetin 3-*O*-rhamnoside, epicatechin gallate hexamalonate, rhamnetin hexoside, and kaemferol were indicated in ultra performance liquid chromatography-MS/MS (UPLC-MS/MS) analysis (Jhaumeer Laulloo et al., 2018). Another spectrometry investigation indicated neomangiferin, kaempferol-3-*O*-rutinoside, and iso-quercitrin (Wu et al., 2020). Quercetin concentration of MILs was reported to range from 0.76-1.16 mg/g (Fitmawati et al., 2020).

Gallotannins such as digalloyl glucoside, tri-*O*-galloyl glucoside, tetra-*O*-galloyl glucoside, and penta-*O*-gallose-glucose were discovered in UPLC-MS/MS (negative mode) analysis of ethanolic and methanolic extract of MILs (Jhaumeer Laulloo et al., 2018). 4-Hydroxybenzoic, gallic, protocatechuic, coumaric, caffeic, and vanillic acids were found in MILs using mass spectrometry as the allelopathic bioactive compounds (Akhtar & Arshad, 2013). The gallic acid content ranged from 5.23 to 35.48 mg/g dry weight (Fitmawati et al., 2020). Additionally, cinnamic, and ferulic acids were also found (Kato-Noguchi & Kurniadie, 2020).

Benzophenones such as maclurin, iriflophenone 3-C-β-D-glucopyranoside, iriflophenone tri-*O*-galloyl-glucoside, maclurin 3-C-(6''-*O*-*p*-hydroxybenzoyl)β-D-glucoside, and iriflophenone-di-*O*-galloyl-glucoside were found in ethanolic and methanolic extract through UPLC-MS/MS (positive mode) analysis. Maclurin 3-C-β-D-glucoside, 3-Glucosyl-2,3',4,4',6-pentahydroxybenzophenone, maclurin 3-C-(6'-*O*-*p*-hydroxybenzoyl)β-D-glucoside, maclurin mono-*O*-galloyl-glucoside, and maclurin di-*O*-galloyl-glucoside were discovered in MeOH and EtOH extract though UPLC-MS/MS (negative mode) analysis (Jhaumeer Laulloo et al., 2018). Four derivatives of benzophenones such as acarbose, manindicins A and B, norathyriol, and mangiferin were discovered in an aqueous extract of MILs using high-resolution electrospray ionization mass spectrometry (HRESIMS) (Gu et al., 2019).

Essential oil of MILs contains monoterpenes (46.98 %), some trace amount of their analogs (10.67 %), sesquiterpenes (38.17 %), minor quantities of oxygenated hydrocarbons (4.19 %) and non-terpenoid hydrocarbons such as β-elemene, α-pinene, α-humulene, α-gurjunene, β-caryophyllene, β-selinene, and 3-carene (Džamic et al., 2010). 1-Terpineol, 3-methyl-camphenilol, α-elemene, 4-terpineol, camphor, α-guaiene, α-selinene, γ-selinene, viridiflorol, α-panasinsen, α-eudesmol, octadecane, α-copaene, *cis*-guriune, α-cadinene, palustrol, isocaryophyllen, elemol, guaiol, δ-cadinol, phytol, eremophilene, α-terpinolene, *p*-cymen-8-ol, γ-cadinene, and globulol were found in gas chromatography-mass spectrometry (GC-MS) analysis of MILs essential oil obtained by hydro-distillation (Ouf et al., 2021). Terpenoids such lupeol, mangiferonic acid, manglanostenoic acid, cycloartane-3, 24, 25-triol, and cycloart-25-ene-3, 24, 27-triol were found in UPLC-MS/MS (positive mode), and cycloartane-3, 29-diol and 3, 27-dihydroxycycloart-24-en-26-oic acid were observed in UPLC-MS/MS (negative mode) analysis of ethanolic and methanolic extract of MILs (Jhaumeer Laulloo et al., 2018).

Iriflophene, quercetin-3-*O*-β-D-xylopyranoside, quercetin-3-*O*-β-D-glucoside, quercetin-3-*O*-α-L-rhamnoside, quercetin-3-*O*-β-D-arabinoside, quercetine-3-*O*-β-D-galactoside, isovitexin, isoswertisin, vitexin, quercetin-4'-*O*-β-D-glucoside, luteolin-7-*O*-β-D-glucoside, quercetin, 3',5'-dimethoxy-4',5,7-trihydroxyflavone, mangiferin, 4'-*O*-*p*-hydroxybenzoylmangiferin, amentoflavone, hypericin, and taxifolin were found in 70% ethanol-water (3 × 50 l) using positive-ion HR-ESI-TOF-MS analysis (Pan et al., 2018). In a GC-MS analysis of aqueous extract, furfural, 2-furanometanol, *o*-catechol, hydroquinone, pyrogallol, and oleic acid were found (Martínez-Bernett et al., 2016). Chemical structures of some biologically active compounds present in MILs are depicted in Figure 2.

7. Pharmacological activities of *M. indica* leaves

Leaves of *M. indica* are considered a useful source of nutritive ingredients and cost-effective food supplements for the improvement of health as well as the treatment of mild and severe ailments. These show excellent medicinal, biological, and metabolic characteristics. The MILs extracts have been investigated because of their various medical effects including antioxidant, anticancer, antimicrobial, anti-diabetic, gastrointestinal, antidiarrheal, lipid-lowering, cardioprotective, and hepatoprotective effects.

7.1. Anticancer activity

Cancer is considered a highly dominant global disease after cardiovascular disorders. Therefore, it is necessary to propose a novel approach to control this worldwide problem. Polyphenols found in MILs, such as phenolic acids, gallotannins, mangiferin, and quercetin show chemo-preventive properties against several types of cancer owing to their potent antioxidant and anti-inflammatory effects (Jung et al., 2012). Mangiferin was found to diminish various tumors by hindering them from migration, invasion, and proliferation (Klein-Júnior et al., 2020).

An investigation was conducted to analyze the anti-tumoral activities of MILs extracts on MCF-10 and MCF-7 non-carcinogenic cells minimally and highly invading breast cancer cells. Leaves' extract exhibited protective activities against oxidation as well as cytotoxic actions on breast cancer and minimum harm to normal cells (Fernández-Ponce et al., 2017). The cytotoxic effect of different *M. indica* leaf extracts was assessed on lung cancer (A549) cells. The ethyl acetate extract exhibited the highest cytotoxicity against A549, followed by methanolic extract (Quizon et al., 2022). *M. indica* leaves extract was employed to prepare silver nano-rods. These nano-rods showed potential in vitro anticancer and antioxidant potential against cell lines of breast cancer and colorectal carcinoma (Anoop et al., 2018).

Ethanolic extract of *M. indica* leaves, at a concentration of 200 µg/ml, exhibited cytotoxicity against human cancer cell lines such as liver hepatoblastoma, bronchogenic carcinoma, ductal carcinoma, colon adenocarcinoma, and gastric carcinoma. The extract increased the percentage survival of normal cell lines, skin fibroblasts. At high doses, mangiferin increased the percentage of survival of lung and skin fibroblasts (Ganogpichayagrai et al., 2017). The bioactive components of *M. indica* leaves, extracted using methanol, showed high cytotoxicity on adenocarcinoma cell lines (Joona et al., 2013). The hexane-ethyl acetate extract of *M. indica* leaves showed cytotoxic activity on the L929 cell lines (Helen et al., 2013).

7.2. Anti-diabetic effect

Diabetes is known as a fetal disease that strongly disturbs human life. It is considered a danger for people regardless of their location geographically. It is defined by above-normal or high glucose level and it is partially because of damage due to the oxidation of β-cells of the pancreas, resulting in a cease in insulin secretion (Singab et al., 2014). An effective approach to treat diabetes mellitus (DM) is the inhibition of α-glucosidase and α-amylase enzymes, which results in the regulation of postprandial glucose absorption (Nair et al., 2013).

MILs have strong anti-diabetic potential owing to their hypoglycaemic compounds like flavonoids and benzophenones. In an investigation, four bioactive phytochemicals isolated from MILs extract, norathyriol, mangiferin, and manindicins A and B, were found to exhibit high anti-diabetic potential. Norathyriol had potent α-glucosidase inhibition with IC_{50} of 4.22 ± 0.19 µg/ml, which was four times more effective as compared to commercially used acarbose inhibitor (IC_{50}: 16.28 ± 1.22 µg/ml). Mangiferin exhibited lower α-glucosidase inhibition which can be enhanced by replacing the glucose group with hydrogen which may decrease the steric

hindrance during the mangiferin–enzyme interaction (Gu et al., 2019).

A study assessed MILs extract for its anti-diabetic effects using an in vitro model. The extract had pronounced inhibition towards α-amylase up to 51.4% ± 2.7 at 200 µg/ml of concentration. Furthermore, the extract showed a glucose adsorption capacity of 2.7 ± 0.19 mM glucose/g extract. Moreover, the extract significantly increased the uptake of glucose up to 143% ± 9.3 in LO-2 liver cells (Ngo et al., 2019). Anti-diabetic activity of mature as well as tender leaves of *M. indica* var. *totapuri* was conducted. Mature leaves extract (500 mg/kg) showed α-glucosidase inhibition with IC_{50} of 21.03 µg/ml, whereas, extract of tender leaves (500 mg/kg) showed α-amylase inhibition of IC_{50} 22.01 µg/ml (Bhuvaneshwari et al., 2014).

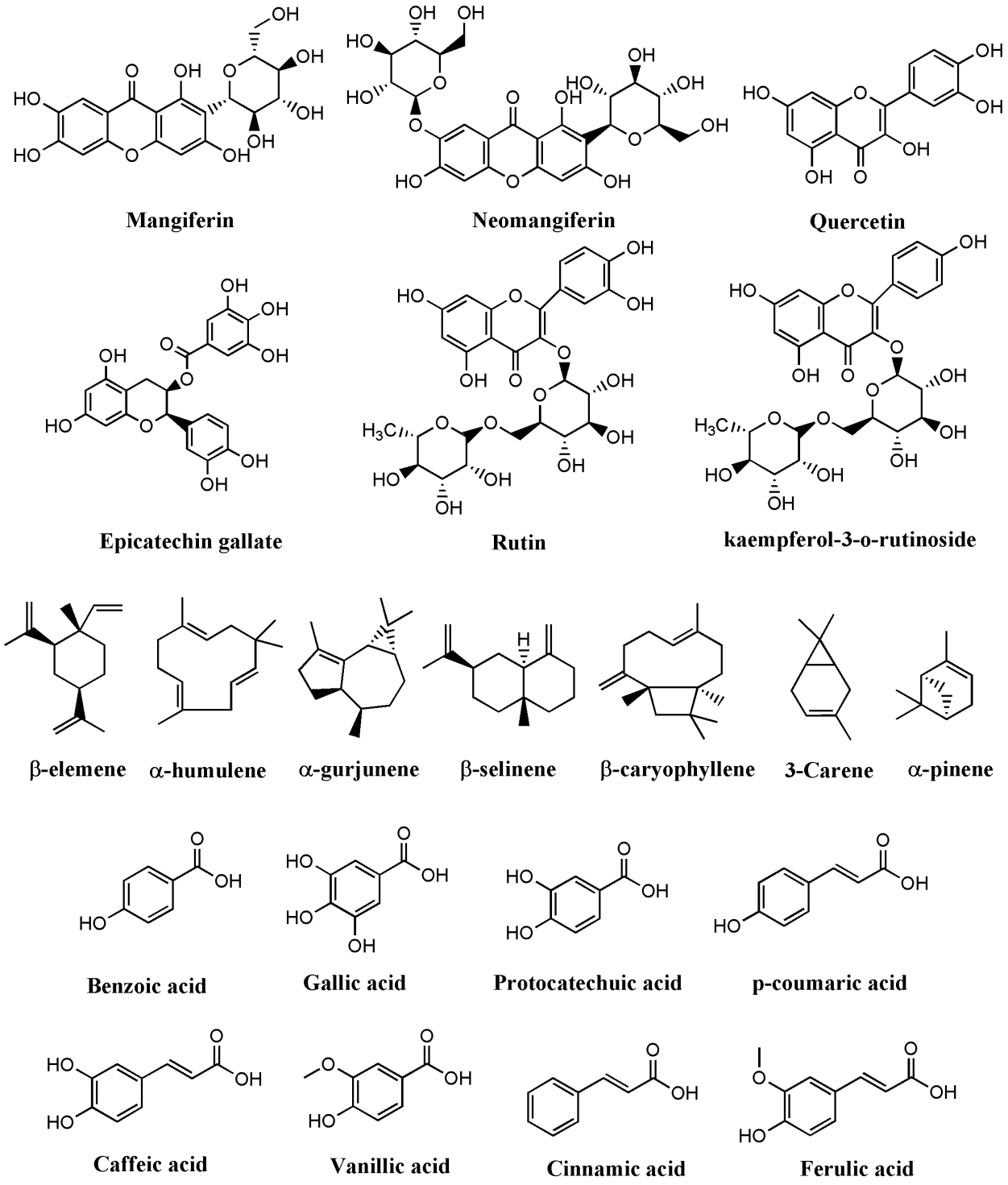

Figure 2. Chemical structures of important bioactive compounds in *M. indica* leaves

The anti-diabetic potential of ethanol extract of leaves of *M. indica* cv. *okrong* and its bioactive substance, mangiferin, was determined against yeast α-glucosidase and rat α-glucosidase employing *p*-nitro phenyl-α-D-glucopyranoside (1 mM) as substrate. Extract and mangiferin showed concentration-dependent inhibitory potential against yeast α-glucosidase with an IC_{50} of 0.0503 mg/ml and 0.5813

mg/ml, respectively, and against rat α-glucosidase with the IC_{50} of 1.4528 and 0.4333 mg/ml, respectively (Ganogpichayagrai et al., 2017). In another study, the antihyperglycaemic potency of *M. indica* leaves was studied in rats. The results indicated that aqueous MILs extract showed no significant impact on the blood glucose level in either STZ-induced hyperglycaemic or normoglycaemic rats. However, a reduction in elevation of blood glucose was observed in glucose-induced hyperglycaemic rats due to the aqueous extract (Aderibigbe et al., 1999).

7.3. Antioxidant effect

Free radicals produced during processes of metabolism cause several degenerative disorders including ischaemic disorders, AIDS, neurological disorders, and many others (Schraml & Grillari, 2012). Antioxidant compounds, on the other hand, also provide a strong antioxidant potential to reduce the harmful effects of free radicals. MILs extract showed significant inhibition of 63.3% ± 2.1 and 71.6% ± 4.3 in scavenging of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2-azinobis-3-ethyl benzothiazoline-6-sulfonic acid (ABTS) radicals, respectively. An inhibition of 66% ± 4.9 was observed in NO production from RAW264.7 cells without any sort of cytotoxicity (Ngo et al., 2019).

In another study, superoxide dismutase and DPPH assays demonstrated that MILs have a moderate antioxidant potential (Itoh et al., 2020). MILs methanolic extract also showed significant radical scavenging potential (Mohan et al., 2013). The antioxidant activity of leaves owing to the flavonoids and phenolics has been revealed in various investigations (Kumar et al., 2020). Flavonoids present in leaves have potential antioxidant features to protect cells from oxidative damage (Ali et al., 2020). Recently, mangiferin has been revealed to show antidepressant activity and regulation of the brain's biogenic amines. It decreases oxidative stress in the neurodegenerative diseases (Dutta et al., 2023). Polyphenols found in MILs such as phenolic acids, gallotannins, mangiferin, and quercetin show chemo-preventive properties against several types of cancer due to their antioxidant activity (Jung et al., 2012). Benzophenones as bioactive phytochemicals showed potential antioxidant and immunosuppressive activities (Gu et al., 2019). Tannins inhibit the formation and removal of reactive oxygen species, resulting in the reduction of scar tissue and improved wound healing (Ali et al., 2020).

The effectiveness of MILs was investigated in chitosan-incorporated films and the antioxidant potential of the MILs-incorporated chitosan films was enhanced in a dose-dependent aspect (Rambabu et al., 2019). The hydro-alcoholic extract of MILs fermented with either effective microorganisms or *Lactobacillus casei* exhibited high antioxidant potential. This examination displayed that fermented extract reduced the lipo-polysaccharide produced reactive oxygen species (Park et al., 2015). An advanced study demonstrated that MILs extract was highly appropriate as an antioxidant for the enhancement of biodiesel storage life (Neuana et al., 2021). In summary, several interesting reports have demonstrated that MILs are effective antioxidants with major applications in food and other industries (Kumar et al., 2021).

7.4. Antimicrobial activity

There is great interest in investigating the role of phytochemicals. Some medicinal plants with anti-microbial properties can destroy the effect of multidrug-resistant microbes, helping to cope with resistance to antimicrobial agents (Dzotam & Kuete, 2017). The major phytochemicals in MILs that are responsible for antimicrobial activity include alkaloids, phenolics, saponins, terpenes, glycosides, and tannins (Kumar et al., 2021). Its polyphenols can cease the growth of microbes (Ediriweera et al., 2017). Mangiferin exhibits various pathophysiological properties (Dutta et al., 2023). The essential oil from *M. indica* leaves also shows bacteriostatic activities as it contains various antimicrobial compounds like camphor (Ouf et al., 2021). Saponins have natural potential for the removal of microbes which enable them to be suitable for the treatment of yeast and fungal infections. These substances act as natural antibiotics which is helpful for the body to fight microbial invasion and infections. Plant-derived alkaloids are useful as fundamental therapeutic agents for their antispasmodic, analgesic, and antibacterial properties and they also protect against chronic diseases (Ali et al., 2020).

The antibacterial activity of MILs extract and its fabricated silver nanoparticles (AgNPs) was evaluated against three bacteria. The extract showed the inhibition of 12.5%, 24.9%, and 32.16%, respectively, and its AgNPs possessed the inhibition of 86.95%, 95.23%, and 99.99% against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, respectively (Hai et al., 2022). Antimicrobial analysis of essential oils of leaves extract of five Egyptian *M. indica* cultivars revealed notable antibacterial potential against *Staphylococcus* species, *E. coli*, *Bacillus subtilis*, *Aspergillus flavus*, and *P. aeruginosa* (Ouf et al., 2021). MILs extracts showed favorable anti-bacterial activities against *Enterobacter aerogenes* and *Mycobacterium tuberculosis* (Bharti, 2013).

The antibacterial potential of MILs extract against bacteria was assessed by using the disc diffusion method and poisoned food approach. Extract of MILs exhibited significant inhibition potential against a variety of gram-positive bacteria, including *B. cereus*, *Streptococcus agalactiae*, *B. subtilis*, *Lactobacillus bulgaricus*, *B. megaterium*, and two gram-negative bacteria including *Shigella sonnei* and *S. flexneri*. But *Proteus* spp. and *Salmonella typhi* showed resistance to the extract. This study showed that the leaf extract had significant antibacterial effects against gram-positive bacterial species and had no or weak potency against gram-negative bacterial species (Islam et al., 2010).

Ethanol, ether, and water extracts of MILs were studied for antibacterial activity utilizing the well diffusion approach. Ethanol extract exhibited inhibition diameters of 22 mm, 15 mm, and 19 mm, ether extract exhibited inhibition diameters of 23 mm, 5 mm, and 6 mm, and water extract possessed inhibition diameters of 7 mm, 5 mm, and 19 mm against *S. aureus*, *E. coli*, and *P. aeruginosa*. The ethanol extract was notably effective with a minimum inhibitory concentration (MIC) of 5481.0-43750.0 $\mu g.ml^{-1}$ (Bbosa et al., 2007a).

The bactericidal effect of MIL extracts against *Clostridium tetani*, which causes many deaths all over the world, was evaluated. Ether and ethanol extracts exhibited anti-bacterial potential with a MIC of 6.25 mg/ml and 12.5 mg/ml, respectively (Bbosa et al., 2007b). Furthermore, a chemical investigation of MILs extract to estimate antimicrobial potential showed the existence of five important flavonoid phytochemicals. These phytochemicals were found to be synthesized immediately after fungal attack and reduced the growth of targeted fungal species such as *Alternaria* and *Aspergillus* from 97% to 56% (Kanwal et al., 2010). Secondary metabolites with antibacterial properties have been found in MILs. Mangiferin, as an antibacterial agent, has shown effectiveness in preventing *S. aureus*, which has a high ability to trigger skin infections (Lubis et al., 2023).

Extract of MILs demonstrated antifungal potential against different species of fungi such as *A. niger* through disc diffusion method (Islam et al., 2010). In an investigation, flavonoids isolated from MILs showed significant antifungal activity against five fungal strains, such as *Penicillium citrii*, *A. alternata*, *A. fumigatus*, *A. niger*, and *Macrophomina phaseolina* (Kanwal et al., 2010). The antifungal assay of MILs extract and its fabricated silver nanoparticles (AgNPs) exhibited inhibitions of 28.55% and 99.99% against *Candida albicans* (Hai et al., 2022).

In vitro, mangiferin and isomangiferin can inhibit the replication of *Herpes simplex* virus type 1 (HSV-1) within cells (Zheng & Lu, 1990). The antiviral activity of mangiferin extracted from MILs was studied in vitro against *H. simplex* virus type 2 (HSV-2). Mangiferin was found to not directly inactivate HSV-2, but to inhibit the late stage of HSV-2 replication (Zhu et al., 1993). In another in vitro investigation, mangiferin was found to antagonize the cytopathic activity of human immunodeficiency virus (HIV) (Guha et al., 1996).

7.5. Gastrointestinal benefits

Diarrhea is known as an infectious disease and its main causes include drinking dirty water, consuming uncooked meat, poor hygiene and sanitation, and food intolerance (Mehesare et al., 2017). The key microorganisms that cause this ailment are various microbial agents such as *Vibrio cholera*, *C. albicans*, *E. coli*, *S. aureus*, and *S. flexneri* (Mokomane et al., 2018). The potential MILs extract was investigated on gram-negative gastrointestinal disorders caused by bacteria and the phytoconstituents of the extract were observed to have an excellent effect as an anti-diarrheal agent. Aqueous MILs extract were investigated against various pathogens such as *S. typhi*, *E. coli*, *S. sonnei*, and *V. cholera* at the dose level of 50, 100, 200, and 300 mg/ml. The antidiarrheal potential is increased by increasing the dose amount. So, the aqueous extract of MILs was confirmed to have the potential to treat diarrhea (De & Pal, 2011).

The antiulcer activity of the ethanol and petroleum ether extracts of MILs was assessed in vivo against aspirin-induced gastric ulcers. The ethanol extract (250 mg/kg) and petroleum ether extract (250 mg/kg) remarkably decreased the ulcer index (Neelima et al., 2012). Mangiferin of MILs can alleviate damaged gastrointestinal motility and reduce intestinal inflammation, consequently facilitating gastrointestinal transit. It also showed the anti-ulcerogenic activity (Severi et al., 2009). In another study, mangiferin was found to significantly suppress inflammatory mediators, cytokines, NO_3^-/NO_2^- levels, myeloperoxidase effect, and adhesion compounds in the ileum portion of the small intestine. Mangiferin may act as an excellent therapeutic agent for the treatment of human inflammatory bowel ailments (Swaroop et al., 2018).

7.6. Hepatoprotective activity

Oxidative stress can cause cell and tissue damage. Liver disorders like cirrhosis, necroinflammatory hepatitis, subclinical icteric hepatitis, and carcinoma have an association with oxidative stress and redox imbalance. Therefore, compounds or herbs with antioxidant potency and lipid peroxidation inhibition can exhibit hepato-protective potential (Pourahmad et al., 2010). MILs tea consists of mangiferin and other biologically active substances. Its hepato-protective effects were studied on rats with high-fat-induced obesity. Tea increased antioxidant potential, antioxidant enzymes, AdipoR2 and PPAR-α mRNA, and protein expressions. It also inhibited the SREBP1c and NF-κB p65 gene expressions in the liver. This tea also caused Cpt1 overexpression. There was a significant reduction in the accumulation of fat droplets and hepatic steatosis was also improved.

Consumption of MILs tea showed a strong hepatoprotective effect by decreasing oxidative stress and steatosis, and regulating lipid metabolism (Ramírez et al., 2018). The hepatoprotective effect of ethanol and methanol extract of MILs was investigated in mercuric chloride-triggered toxicity in Swiss albino mice. Mice treated with extracts showed the recovery of damaged hepatocytes. However, the effect of methanolic extract (50 mg/kg) was better than that of ethanolic extract, which may be due to its strong radical scavenging potential. The results showed that oral intake of MILs extract supplement significantly reduced liver toxicity in mice, probably due to its high antioxidant potential (Karuppanan et al., 2014).

7.7. Anti-obesity and cholesterol-lowering effects

A study evaluated the anti-obesity properties of MILs tea in obese male Wistar rats fed with a high-fat diet. The consumption of tea (24.7 ± 2.1 ml/day) exhibited anti-inflammatory and antioxidant benefits. Tea increased the interleukin-10 serum concentration and total antioxidant capacity, reduced accumulation of abdominal fat, increased lipoprotein lipase and PPAR-γ expression, and decreased FAS expression. Studies suggested that MILs tea had remarkable medicinal effects in the treatment of obesity and other related disorders through the regulation of the expression of transcriptional factors and enzymes associated with adipogenesis (Ramírez et al., 2018).

The hypocholesterol effect of methanol extract of MILs was studied through pancreatic cholesterol esterase inhibition analysis in vitro. This investigation showed that the methanol extract of MILs had remarkable hypo-cholesterol potential which was attributed to the presence of 3b-taraxerol (IC_{50}: 0.86 µg/ml) in the extract (Gururaja et al., 2015). In another investigation, the cholesterol-lowering potential of MILs extract was evaluated in vivo in female albino Wistar rats. Plasma triglycerides were significantly reduced using an oral dose of the extract (90 mg/kg) during days 21-42; this confirmed the cholesterol-lowering potential of MILs extract. (Gururaja et al., 2017).

7.8. Cardioprotective activity

A study was conducted to evaluate the activity of alcoholic extract of MILs on cardioprotection against doxorubicin (DOX)-induced cardiac stress. The results revealed remarkable protective effects of the alcoholic extract of MILs against oxidative stress. There was an increase in tissue antioxidant levels and a decrease in serum biomarker enzyme levels. Additionally, animals treated with MILs extract showed improvement in histological score, electrocardiographic parameters, lipid profile, and mortality. (Bhatt & Joshi, 2017).

Mangiferin (a bioactive component of MILs) reportedly protects against DOX-induced high mortality rates and electrocardiogram abnormalities. It also reduced biochemical markers of cardiac toxicity. (Arozal et al., 2014). A study reported that mangiferin ameliorates cardiac toxicity, reduces intracellular levels of reactive oxygen species (ROS) and downregulates related signaling cascades in STZ-induced diabetic models. Mangiferin protects cardiac and renal tissues from streptozotocin (STZ)-induced oxidative damage (Muruganandan et al., 2002).

Mangiferin is highly beneficial against isoproterenol-induced myocardial infarction in rats. Mangiferin pretreatment has been

shown to inhibit isoproterenol-induced effects on changes in mitochondrial infrastructure, functions of various enzymes involved in the TCA cycle, lipid peroxidation level, intracellular ATP level, and endogenous antioxidant mechanisms associated with cardiovascular diseases. The anti-inflammatory and antioxidative properties of mangiferin have been found to be the reason behind the protection caused by mangiferin (Prabhu et al., 2006). A study investigated the antihypertensive effect of ethanol extract of MILs using in vitro and in vivo assays. The dichloromethane fraction of the extract demonstrated hypotensive activity via ACE inhibition (99% ± 8), providing benefits in cardiac hypertrophy and baroreflex sensitivity (Ronchi et al., 2015).

7.9. Other health benefits

MILs have potential curative effects in respiratory disorders, especially whooping cough (respiratory tract infection). It is also useful in treating colds, bronchitis and asthma. Therefore, it is considered a powerful agent for respiratory disorders. The therapeutic properties of MILs make them an outstanding herbal mouthwash that acts as a pain reliever for gingivitis and other gum problems. Ashes of burnt leaves provide quick relief when applied to burns (Muralikrishna et al., 2014).

8. Toxicology of *M. indica* leaves

Various studies have reported beneficial activities of *M. indica* leaves extract against various diseases such as diabetes, cancer, neurodegenerative and cardiovascular disorders. These beneficial effects are confirmed by the presence of a wide range of bioactive substances. However, the harmful effects of leaf extract due to allergens have also been reported in a few studies. There are two ways in which *M. indica* allergy can be observed: immediate hypersensitivity reaction, including erythema, anaphylaxis, wheezing, angioedema, dyspnea, and urticaria, or late reaction, including periorbital edema and contact dermatitis. (Sareen & Shah, 2011). Transcriptome analysis of *M. indica* leaves and fruits reported the presence of sixty-six strong allergenic genes, mainly associated with the pollen allergen, NADPH-dependent flavin mononucleotide reductase, and the pathogenesis-related protein Bet v I family. A study reported that human subjects previously exposed to poison ivy/oak allergy had a chance of developing allergic contact dermatitis due to Mangifera indica upon initial exposure (Hershko et al., 2005). However, studies have generally suggested that Mangifera indica has some allergic reactions, but these side reactions are limited to the pollen allergen, latex, or previous exposure to urushiol (Goldstein, 2004). In a study, *M. indica* leaves extract showed toxicity in lung fibroblast (WI-38 VA-13 subline 2RA, ATCC CLS 300421) (Ganogpichayagrai et al., 2017).

9. Conclusions

According to numerous research reviews, M. indica leaves contain potent chemical compounds with distinct pharmacological activities. MILs have remarkable biological, medical and metabolic effects. MILs form bioactive compounds as well as minerals and vitamins. Its phytoactive compounds include flavonoids, benzophenones, terpenes, phenolic compounds, anthocyanidins, xanthones, saponins, tannins, and alkaloids. Reportedly, MILs have anticancer potential against lung cancer, breast cancer cell lines, colorectal carcinoma, adenocarcinoma, liver hepatoblastoma, bronchogenic carcinoma, ductal carcinoma, colon adenocarcinoma, and gastric carcinoma. MILs have strong antibacterial, antifungal, and antiviral properties. Chemical compounds of MILs are effective against HSV-1, HSV-2, and HIV. The leaves are very important as anti-obesity and lipid-lowering agents. MILs have potent hepato-protective, gastro-protective, cardio-protective and antihypertensive effects. They have been found to be very useful in treating numerous ailments such as diarrhoea, ulcers, diabetes and fatty liver disease. Thus, it was concluded that MILs contain potent phytochemical compounds with remarkable therapeutic benefits.

Phytochemical studies have shown that further investigation of *M. indica* leaves is needed to uncover other phytoactive agents and potential therapeutic effects. Clinical studies should also be conducted on MILs to further investigate their effectiveness.

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Conflict of interest

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

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In this study, no method requiring the permission of the “Ethics Committee” was used.

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Supplementary File

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RESEARCH ARTICLE

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A North Cameroonian cultivar of *Hibiscus sabdariffa* (Malvaceae) with calyces enriched in anthocyanins

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ABSTRACT

Folere (*Hibiscus sabdariffa* L.) is a traditional plant cultivated in the north of Cameroon near the city of Yagoua. Calyces are used to prepare beverages while the leaves are incorporated into meals. This Cameroonian roselle is characterized by a higher intensity of red color than that found in conspecific samples from Egypt, and it was of interest to us to evaluate its beneficial activities in comparison with European pharmacopoeia standards. The samples were first subjected to phylogenetic analyses. Five samples including samples from Egypt and a white flower variety were subsequently examined according to the European pharmacopeia criteria i.e. *(i)* taxonomic identification, *(ii)* sample preparation and characterization (desiccation, TLC, coloring power, presence of foreign matter), and *(iii)* determination of the acidity index. Anthocyanins and anthocyanidins were quantified in three extracts from methanolic maceration, infusion, and boiling in water. Taken together, the results confirmed that the Cameroonian variety indeed belongs to *H. sabdariffa* and meets European Pharmacopoeia standards. All extracts contained common anthocyanins (delphinidin-3-sambioside, delphinidin-3-glucoside, cyanidin-3-sambioside, cyanidin-3-glucoside), and delphinidin and cyanidin aglycones were also found in the organic extracts. As a result of anthocyanin quantification by HPLC in Cameroon samples, it was determined that they showed a high concentration of delphinidin type anthocyanins and a concentration 10 times higher than the Egyptian variety. This variety appears to be a very promising candidate for inclusion in industrial preparations, thanks to its high anthocyanin qualitative and quantitative properties, and also maintains economic interest for local populations.

1. Introduction

Located in tropical West Central Africa, Cameroon has a wide range of subclimates and is considered to be home to 90% of diverse African ecosystems (e.g. Sahel, Sudan, humid tropical forest, coastal and mountain ecoregions called Afro mountains) (Fokunang et al., 2011). To date, approximately 7850 plant species have been recorded, of which 160 are endemic (Onana, 2011). *Hibiscus sabdariffa* L., belonging to the family Malvaceae, was recorded by Fongnzossie et al. (2017) and Jiofack et al. (2010) in Cameroon. It is a worldwide species found in Asia (India, China, Malaysia, Indonesia, Vietnam, Taiwan), in the Middle East (Egypt, Saudi Arabia), as well on Africa (Sudan, Nigeria, Cameroon) but also in America (Mexico) and its vernacular name varies according to the localization (Maganha et al., 2010) even in a same country: for example its name is Folere in extreme north of Cameroon and in the Sudano Sahelian region (Jiofack et al., 2010), Mbanga in the east (Fongnzossie et al., 2017) and Oseille in Douala (Emmanuel & Di-

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dier, 2011). According to its large geographical distribution, genetic polymorphism is considered important (Da-Costa-Rocha et al., 2014). All parts of *H. sabdariffa* are used for their nutritional value and medicinal properties (as well as for their economic value in the cosmetic and textile industries) (Cisse et al., 2009). The protein content in fresh calyces is approximately 1.9% (m/m), in leaves 3.3% (m/m) and in seeds approximately 27.8% (m/m) (Cisse et al., 2009; Riaz & Chopra, 2018). Calyces are also rich in vitamins (niacin, riboflavin, ascorbic acid) and minerals (calcium, iron, magnesium) (Riaz & Chopra, 2018) and are frequently used as beverage in the preparation of aqueous extracts to obtain cold or hot drinks and as food coloring (Maganha et al., 2010; Riaz & Chopra, 2018). As a medicinal plant, infusions of calyces are prepared to treat gastric and hepatic disorders in India but also fever and hypercholesterolemia. In Malaysia, oil obtained from seeds **(Figure 1C)** is incorporated in soaps (Agrawal, 2020; Riaz & Chopra, 2018). In Northern Cameroon, the decoction of the leaves is reportedly used in dental care (Fongnzossie et al., 2017), but the leaves are also mixed with meat to prepare typical meals. The decoction of the fruits and leaves is also used in the treatment of amoebiasis, anemia and sexually transmitted diseases, as well as as a regular drink. Another commercial outlet is the use of fibers which can be useful to obtain jute, or woven into fishing nets and ropes (Da-Costa-Rocha et al., 2014). A great number of preclinical studies and clinical trials have been conducted with *H. sabdariffa* extracts or powder (Najafpour Boushehri et al., 2020). The most relevant effects are antihyperlipidemic, antihypertensive, and antidiabetic activities (Da-Costa-Rocha et al., 2014; Maganha et al., 2010; Ojulari et al., 2019; Riaz & Chopra, 2018). While some antilithiatic and diuretic results have been more contradictory in human studies (Laskar & Mazumder, 2020), other pharmacological activities such as antimicrobial, anti-inflammatory, anti-anemic, and anticarcinogenic need to be confirmed in human studies (Maganha et al., 2010; Ojulari et al., 2019; Riaz & Chopra, 2018). Therefore, with global demand increasing by up to 15.000 tonnes per year, increasing cultivation of this species seems clearly economically beneficial (Raghu et al., 2019).

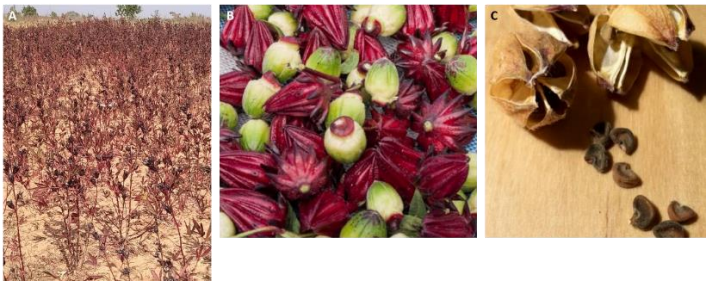

Figure 1. *H. sabdariffa*: cultivation of the red variety in Yagoua (Cameroon) (A), fruit and calyces (B), seeds from the fruits (C)

Various families of compounds, such as organic acids, phenolics, polysaccharides, and essential oils, have been isolated from *H. sabdariffa*, and the composition varies depending on the part of the plant and solvent extraction. For calyces, the organic acids give the species its sour taste and the most common acids are citric, malic, tartaric, hydroxycitric, succinic, and fumaric acids. Among polyphenols, flavonols (hibiscitrin, gossytrin, and their relative glycosides) are the major flavonoids while four anthocyanins have been reported (Da-Costa-Rocha et al., 2014). The major anthocyanins **(Figure 2)** are delphinidin-3-sambubioside (hibiscin) and cyanidin-3-sambubioside (gossypicyanin) and the others (delphinidin-3-glucoside, cyanidin-3-glucoside, cyanidin-3,5-diglucoside) are less present (Cisse et al., 2009; Da-Costa-Rocha et al., 2014). Some phenolic acids (protocatechuic acid, chlorogenic acid), and tannins (catechin, ellagic acid, epigallocatechin) have also been described in aqueous extracts as well as polysaccharides and volatile terpenoids (Da-Costa-Rocha et al., 2014).

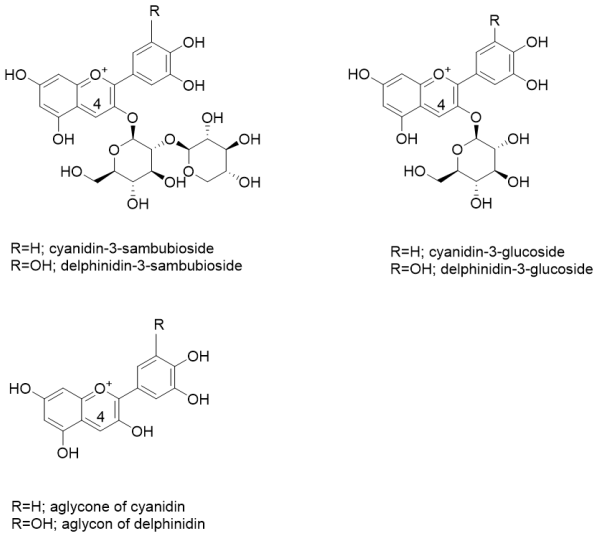

Figure 2. Anthocyanins reported in *H. sabdariffa*

In the extreme north of Cameroon near Yagoua, a cultivar of *H. sabdariffa* has been cultivated for a long time by women to ensure their subsistence. The calyces have a particularly intense red color; this is a sign of potential richness in anthocyanins. Given the great interest in the search for anthocyanins of economic importance (Veluru et al., 2022), the scope of this study was to describe this red

Cameroonian cultivar and to submit it to several assays of the European pharmacopeia (Karkade Monograph, 2017). Additionally, the dosage of anthocyanins was measured by comparing three different traditionally used extraction methods, respectively: decoction, infusion, and maceration. Phylogenetic analyses were also carried out independently. Three other varieties originating from Egypt and sold in pharmacies and a white variety from Cameroon were also compared with this traditional Cameroonian variety for interesting characteristics.

2. Materials and methods

2.1. Material

Thin layer chromatography (TLC) plates were 10 x 20 cm aluminum sheets coated with silica gel 60F254 (10-12 µm, Kieselgel 60 F254, MERCK 5554). Analytic grade solvents used for TLC, e.g. anhydrous formic acid (VWR Prolabo Chemicals, 13G220509), *n*-butanol (VWR Prolabo Chemicals, 16G084005), and sodium hydroxide were obtained from Sigma-Aldrich. Distilled water was obtained by using a 0.45µm filtration system (Vent Filter MPKO1, F7MA56578).

CAMAG system® including the Automatic TLC Sampler 4 (CAMAG, Switzerland) for depositing the samples on plates and the TLC Visualizer 2 (CAMAG, Switzerland) for photographing the plate before and after revelation were used in TLC analysis. All the system is controlled by the CAMAG® HPTLC software VisionCat.

Absorbances were recorded by using a spectrophotometer (Analytik Jena, Specord PC205) and the pH with a pH Meter (Fisher Scientific, Accumet AE150).

HPLC separation of the anthocyanins was accomplished on a Shimadzu Prominence chromatograph equipped with a quaternary pump LC-20AD 5P, an autosampler SIL-20A(HT), a photodiode array detection (SPD-M20A) with a degassing unity (DGU-20 A(5R). The gradient elution method was performed using a Phenomenex®, Kinetex C18 column (100 mm x 4.6 mm, 2.6 µm particle size, 100A), and the column oven CTO-20A was fixed at 30 °C. Mass spectrometric detection was performed using an Advion® expression cmS mass spectrometer (MS). The solvents used for HPLC analyses were of HPLC-grade (formic acid, acetonitrile).

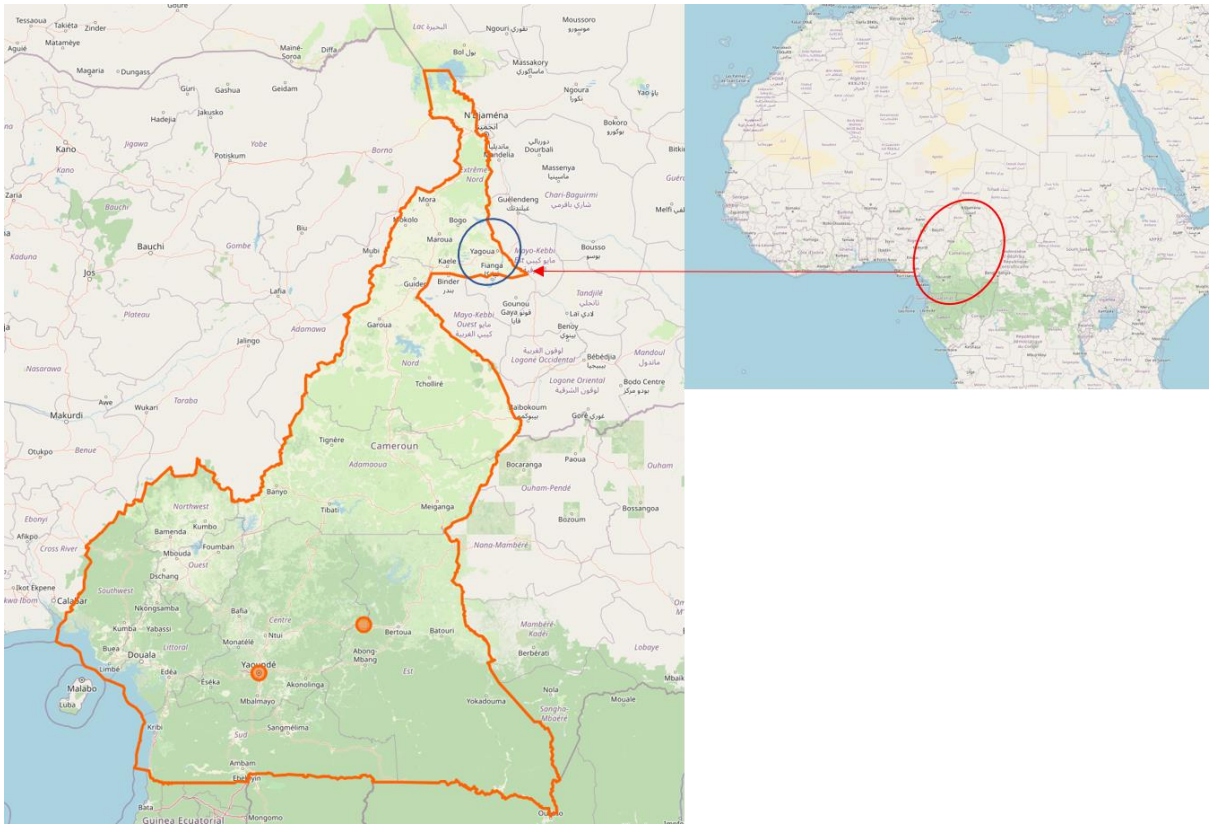

Figure 3. Localization (Yagoua district) of the red cultivar of sabdariffa in Cameroon.
(https://www.openstreetmap.org/#map=5/13.902/-2.900)

2.2. Description of the cultivation area and the crop

The red cultivar of *H. sabdariffa* is cultivated in the north of Cameroon in the region of Yagoua (capital of the department of Mayo-Danay), 115 km south-east of Maroua **(Figure 3)**. The cultivation fields are located around Yagoua city (10°20'34° N and 15°14'26° E, altitude 336 m) and are surrounded by Sahelian Savannah (Fongod et al., 2014). The Limani-Yagoua mega sand ridge itself has a warm and dry desert climate (Ngatcha et al., 2001). The calyces **(Figure 1A, B)** are collected twice a year: *(i)* a smaller crop during the rainy July-August season when cloudy and with rainfalls over 80 mm, and *(ii)* a large crop during the longer dry period (October-June) when sunny with temperatures exceeding 35 °C and with little rain (< 30 mm). The calyces are collected at maturity before fructification **(Figure 1C)** by women, with special attention to prohibiting children from working in the fields. Then calyces are dried in the shade away from the dust.

2.3. Plant material

Five samples of *Hibiscus* calyx (A-E) were selected: three samples originated from the north of Cameroon and two samples from Egypt but commercialized by different suppliers **(Table 1)**. Batch E was of officinal grade. The calyces from the north of Cameroon were

purchased from local producers in Yagoua after being dried traditionally under shade far away from the dust.

All dried-out samples were ground using a mortar and pestle and the resulting powder was sifted under a granulometry of 0.5 mm.

Table 1. Batches of *H. sabdariffa* and their acid percentage (mean of three assays, expressed in citric acid equivalent)

Code	Commercial batch number	Suppliers	Voucher number	Expressed in citric acid (%)
A	FDH90501	Cameroon (Yagoua)	REN_JB/A/21/01	13.27 ± 0.08
B	H1600239	Egypt (Cailleau)	REN_JB/A/21/02	17.66 ± 0.10
C	FDH803001	Cameroon (Yagoua)	REN_JB/A/21/03	15.90 ± 0.05
D	FDH803001	Cameroon (Yagoua)	REN_JB/A/21/04	13.93 ± 0.02
E	1974443	Pharmacist/Egypt	REN_JB/A/21/05	18.30 ± 0.01

2.4. Morphological analyses

Calyces from the five batches were examined macroscopically by using a Müller (HG550775) stereomicroscope (Gx6) and anatomical details of the ground powders were observed under an Olympus Cx41 light microscope. The identification and the microscopical examination of the specimens were done according to the European pharmacopeia protocols (Karkade Monograph, 2017).

2.5. Phylogenetic analyses

DNA was extracted from fresh seed samples according to the protocol of Werner et al. (2016), since DNA from the original plant material was degraded during and after the drying process. The nuclear ITS region and the chloroplast *rpl16* region were amplified and sequenced as explained by Werner et al. (2016).

The obtained sequences were then used to search via BLAST (Altschul et al., 1990) for the most similar sequences in GenBank. Sequences with more than 95% identity and ≥ 90% query coverage were selected. In the case of the ITS sequences, sequences of *Peltaea speciosa* (Kunth) Standl., *Taliparitihamabo* (Siebold & Zucc.) Fryxell, *Malvaviscus penduliflorus* DC., and *Pavonia spinifex* (L.) Cav. was added as the outgroups. Selected sequences were aligned with CLUSTALX (Larkin et al., 2007) using the default settings. Minor manual adjustments were made to correct evident errors using BioEdit (Hall, 1999), and the local alignment quality was checked with the TCS module of T-Coffee (Notredame et al., 2000).

Genetic distances were calculated by MEGAX counting the number of differences (Kumar et al. 2018). All ambiguous positions were removed for each sequence pairs (pairwise deletion option).

MrBayes v3.2 was used for the phylogenetic analyses (Ronquist et al., 2012). Trees were sampled across the substitution model space in the Bayesian MCMC analysis (Huelsenbeck et al., 2004) by using the option nst = mixed, removing the need for a priori model testing. Two runs with four chains (2 x 10^7 generations each) were run simultaneously. Chains were sampled every 10.000 generations and the respective trees were written into a tree file. Consensus trees and posterior probabilities of clades were calculated by combining the two runs and using the trees sampled after the chains converged. The inspection of the sump file created by MrBayes showed that (i) the chains had converged and that there was no tendency for the log-likelihood values to decrease or increase over time, (ii) the standard deviation of split frequencies was below 0.01 upon completion of the analyses, (iii) the potential scale reduction factor for each of the parameters was in the range of 0.999-1.001, and (iv) the effective sample size was above 500 for all parameters.

2.6. Determination of physicochemical properties

All assays were done according to the European Pharmacopeia protocols (Karkade Monograph, 2017).

2.6.1. Thin-layer chromatographic (TLC) analyses

The calyces (500 mg) were extracted with 5 ml of ethanol (60%) (Sigma-Aldrich, STBH9428) under stirring for 15 minutes at room temperature and then filtrated to obtain the TLC extracts.

Thin layer chromatography consisted of loading the extracts and the standard silica plates using the automatic TLC Sample IV. The volume for spotting was 5 µl with a band of 10 mm. Then, the plates were developed with anhydrous formic acid/water/*n*-butanol (10/12/40). All TLC plates were observed under visible light. The standard solution for TLC consisted of an equal mixture of quinaldine red (2.5 mg, Sigma Aldrich, MKCK2556) with sulfan blue (2.5 mg, Sigma Aldrich, MKCJ0273) dissolved in methanol (10 ml, Sigma Aldrich, STBJ4298).

2.6.2. Coloring intensities

Powder of each batch of *Hibiscus* calyces (500 mg) was extracted in hot water for 15 minutes and the temperature was stabilized at 30 °C. Then the volume of the filtrate was adjusted to 25 ml with distilled water to constitute the water solution. The absorbance of this solution was recorded at 520 nm. This assay was repeated three times. The threshold absorbance value required to comply with pharmacopeia criteria must be 0.350 for a whole plant and 0.250 for a fragmented plant.

2.6.3. Desiccation assay

The weight of calyces (1 g) of each batch number was determined before (m1) and after (m2) the incubation in an oven at 105 °C for 2 hours. The loss on drying was calculated according to the equation given below. This assay was done in triplicate. The threshold desiccation value required to comply with pharmacopeia criteria must be 11%.

$$Loss\ on\ drying\ (\%) = \frac{(m1 - m2)}{m1}\ x\ 100$$

2.6.4. Acid percentages

Powder of each batch of *Hibiscus* calyces (1 g) was mixed under agitation with 100 ml of water for 15 minutes. Then the filtrate (25 ml) was titrated by the addition of sodium hydroxide (0.1 M). The pH was recorded with a pH meter after each addition of NaOH (0.5 ml). The equivalent volume Veq (ml) is determined graphically, and the acid percentage is calculated according to the equation given below. The assays were repeated three times. The threshold acid

percentage value required to comply with pharmacopeia criteria must be 13.5% expressed in citric acid equivalent.

$$Acid\ (\%) = \frac{Veq\ x\ 6.4}{250}\ x\ 100$$

2.6.5. Foreign matters

Calyces of *Hibiscus* (100 – 500 mg) corresponding to a weight called m were examined for each batch under the stereomicroscope. Two types of foreign matter were identified and weighted: some fruit fragments (p1) and other diverse fragments (p2). The percentage of foreign matter was calculated according to the equation given below. The threshold for fruit fragments and diverse fragments values required to comply with pharmacopeia criteria must be respectively 2% maximum.

$$Foreign\ matters\ (\%) = \frac{weight\ (p1\ or\ p2)}{m}\ x\ 100$$

2.7. Determination of total anthocyanin content

2.7.1. Extraction

Protocols were followed according to ANSM guidelines for infusion, maceration, and preparation of herbal tincture (ANSM Monograph, 2013). Three extracts (organic, decocted, and infused) were realized to analyze their total anthocyanin contents. Extraction was done in triplicate for each sample. The organic extract (OE) consists of mixing 500 mg of *Hibiscus* powder with 40 ml of methanol and 0.1 ml HCl (1 M) for 30 minutes at room temperature. The decocted extract (DE) was obtained after boiling 500 mg of powder with 75 ml of water for 30 minutes. The infused extract (IE) was obtained after infusing 500 mg of powder in 25 ml of boiling water for 15 minutes. Then, all extracts were filtrated. They were evaporated, weighed, and stored in the dark at 4 °C under nitrogen.

An aliquot of each dried extract was dissolved either in water or in methanol (by the solvent of extraction) and filtrated (0.45 µm) to make an extract solution at 5 mg/ml. Then extract solutions from samples A and E were diluted to 1/3 and sample C to 1/5 before chromatographic analysis.

2.7.2. HPLC-DAD-MS analysis

An analytical method for identifying and measuring anthocyanins in *Hibiscus* was developed. The following gradient system was applied for separation: A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) T 0 min: 5% B, T 0-10 min: 5% B, T 10-40 min: 40% B, T 40- 47 min: 100% B, T 47-51 min: 100% B, T 51-53 min: 5% B, T53-60 min: 5% B. The injection volume was 20 µl and the flow rate was 0.8 ml/min. UV detection was performed at 530 nm. Mass spectrometric detection was performed using Advion® expression cmS mass spectrometer (MS) and all analyses were recorded in the negative ESI mode in a mass range of 100 to 1200 Da, applying the following parameters: detector gain 1200, ESI voltage 3.5 kV, capillary voltage 180 V, source voltage 20 V, source voltage dynamic 20 V, nebulizer gas pressure 60 psi, desolvation flow gas rate 4 l/min, capillary temperature 250 °C and gas temperature 20 °C. Data processing and evaluation for MS measurement were performed with the Data and Mass Express 2.2.29.2 software (Advion).

2.7.3. Validation of the anthocyanins quantification method

All validation parameters were determined following the International Conference on Harmonization (ICH) Guidelines (Branch, 2005) using HPLC analyses, and the following characteristics were evaluated: linearity, limits of detection (LOD) and quantification (LOQ), and repeatability inter-day and intra-day.

A stock standard solution of 3-*O*-glucoside cyanidin (C3OG) in methanol was prepared at 600 µg/ml, this was done in triplicate. Then, five working standard solutions were prepared by appropriate dilutions of each stock solution with methanol to generate concentrations ranging from 1.9 µg/ml to 30 µg/ml for the external standard calibration curve and the determination of the regression line. Three calibration curves for C3OG were built after injection in HPLC-DAD at 530 nm. Hence, the concentrations of anthocyanins were calculated, based on peak areas in the equivalent of 3-*O*-cyanidin glucoside at 530 nm and the calibration curve Peak Area C3OG = f (CC3OG). The limits of detection and quantification were determined from the y-intercept standard deviation and the slope of the calibration curve. For the calculation of the intra-day repeatability, a diluted solution of 3-*O*-cyanidin glucoside at 7.5 µg/ml was injected six times on the same day. These assays were repeated on three different days for inter-day repeatability. The coefficient of variation and standard deviation were then calculated. Coefficients of variation of less than 5% for intra-day and inter-day were accepted.

2.7.3. Statistical analysis

Statistical analyses were carried out for loss on drying and acid percentage. Data were analyzed using the one-way analysis of variance (ANOVA) and Tukey-test to compare the mean according to the single variable *Hibiscus* variety. For the results concerning the quantification of anthocyanins, data were analyzed using the two-way ANOVA and Tukey test to compare the significance between the varieties of *Hibiscus* for each extraction method.

3. Results and discussion

3.1. Botanical analyses

3.1.1 Morphological studies

Two Cameroonian red calyce samples were compared macroscopically and microscopically with a white one also from Cameroon but also with a sample from Egypt and another sample of officinal grade **(Table 1)**. All calyces were concrescent and urceolated in the lower part while the upper part showed five acuminated and recurved tips **(Figure 1SA, 1SB, supplementary file)**. Except for the white sample **(Figure 1SB, supplementary file)**, the others were of red and purple color. The calyculus consisted of 8-12 narrow leaflets **(Figure 1SC, supplementary file)** which were lighter in the lower part **(Figure 1SD, supplementary file)**. A median midrib slightly in relief **(Figure 1SD, supplementary file)** was observed on the tips as well as the excretory gland **(Figure 1SE, 1SF, supplementary file)**.

The powder of all red samples showed the presence of polygonal cells from the epidermis **(Figure 1SG, supplementary file)** with a thick wall **(Figure 1SH, supplementary file)** and some of them contained some prisms of calcium oxalate **(Figure 1SG, 1SL, supplementary file)** and anisocytic stomates **(Figure 1SG, supplementary file)**. Some unicellular flexuous trichomes **(Figure 1SI, supplementary file)** were observed as well as some glandular trichomes **(Figure 1SJ, supplementary file)**. Numerous spiral vessels were observed in the parenchyma **(Figure 1SJ, supplementary file)**. Some scarce smooth bent trichomes and parenchyma with cells containing mucilage-filled cavities **(Figure 1SK, 1SH, supplementary file)** and calcium

oxalate crystals **(Figure 1SL, supplementary file)** were observed in the powder, but no pollen grains were found. The preliminary observation under the microscope was important to characterize a species (Raghu et al., 2019) and in our case, all these data were in accordance with the description of *H. sabdariffa* reported in the European monograph (Karkade Monograph, 2017).

3.1.2 Phylogenetic analyses

The two samples showed no differences in the *rpl16* sequence (GenBank accession numbers ON157056 and ON157057, respectively) in comparison with one sequence of another *H. sabdariffa* sample from Sudan and a sequence record of *H. mechowii* Garcke. In comparison with another GenBank record of *H. sabdariffa*, one difference was observed. All other sequences included in the *rpl16* alignment were separated by at least 6 mutational steps. The Bayesian tree **(Figure 4)** supported the observations made based on the distance values. All *H. sabdariffa* samples are placed on a clade with good support (posterior probability 1) together with a specimen of *H. mechowii*.

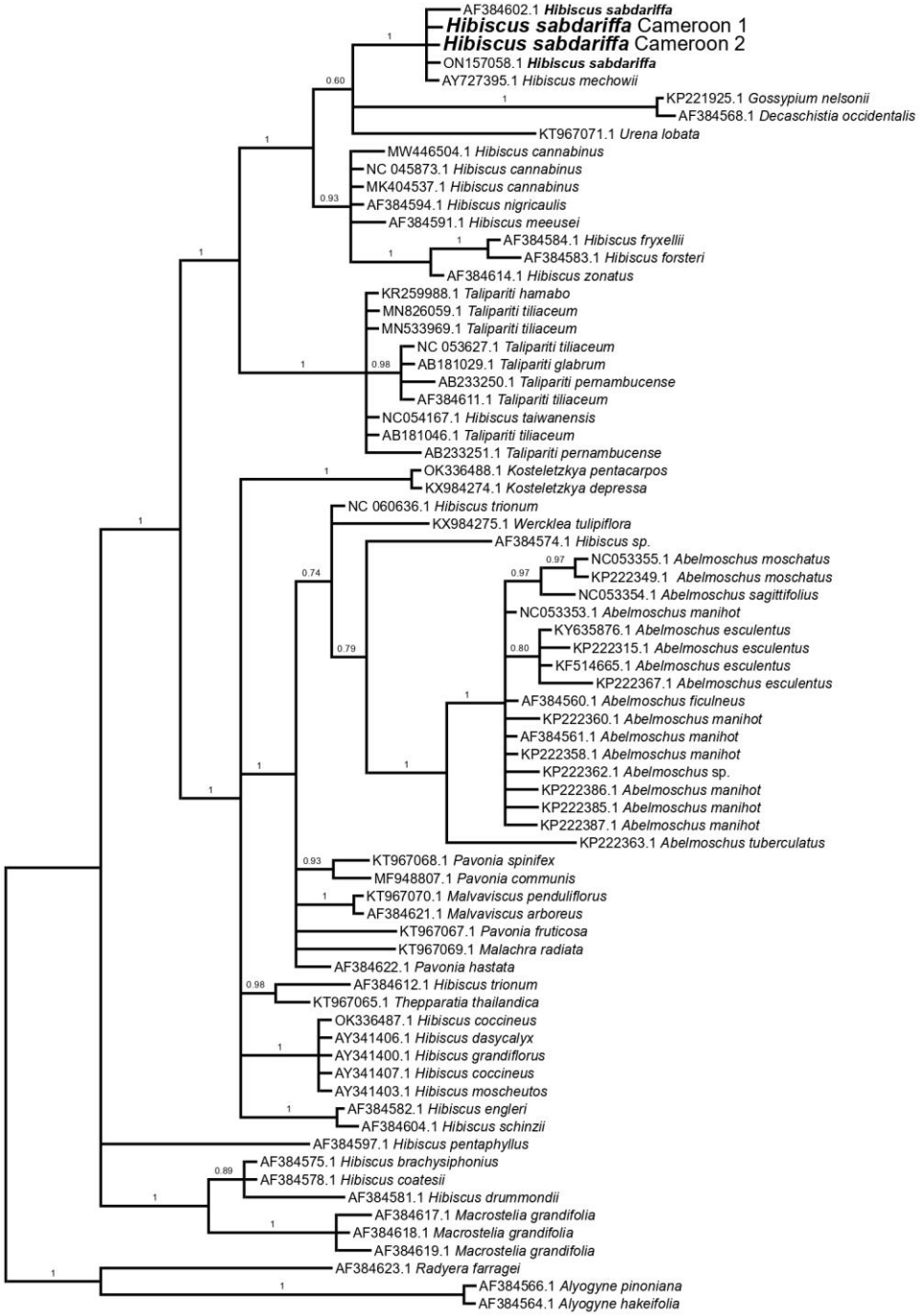

Figure 4. Baysian tree based on chloroplast *rpl16* sequences

GenBank accession numbers, species names, and support values for clades (posterior probability) are given. Sequences of *H. sabdariffa* are highlighted using boldface and the sequences from Cameroon by bigger font size. All samples of *H. sabdariffa* are situated on a clade with solid support together with one sequence of *H. mechowii*.

The ITS sequences for the two studied *H. sabdariffa* samples (GenBank accession numbers ON127554 and ON127555, respectively) were identical except for three ambiguous positions in the alignment, where sample 2 showed double peaks, indicating the

presence of different copies of the multicopy ITS region in the genome. In comparison with therpl16 sequences, a much more complicated pattern was observed. The available 14 *H. sabdariffa* sequences were separated into three groups. The more numerous group (11 samples) included specimens one and two from Cameroon, which were separated by 1-7 mutational steps from the other samples of this group. However, two identical samples of *H. sabdariffa* were separated by 21–25 differences but very close to *H. mechowii* (1 difference). The third group was represented by one individual only with a great distance from both other groups (25 and 35 differences).

The Bayesian tree (Figure 5) showed that the most numerous groups of *H. sabdariffa* are close to *H. asper* Hook.fil., *H. cannabinus* L., and *H. radiatus* Cav. The two-specimen group of *H. sabdariffa* was situated on a clade with a posterior probability of 1 together with the two sequences of *H. mechowii* and one sequence of *H. berberidifolius* A.Rich., while the isolated specimen was close to two *H. asper* sequences on a clade with solid support.

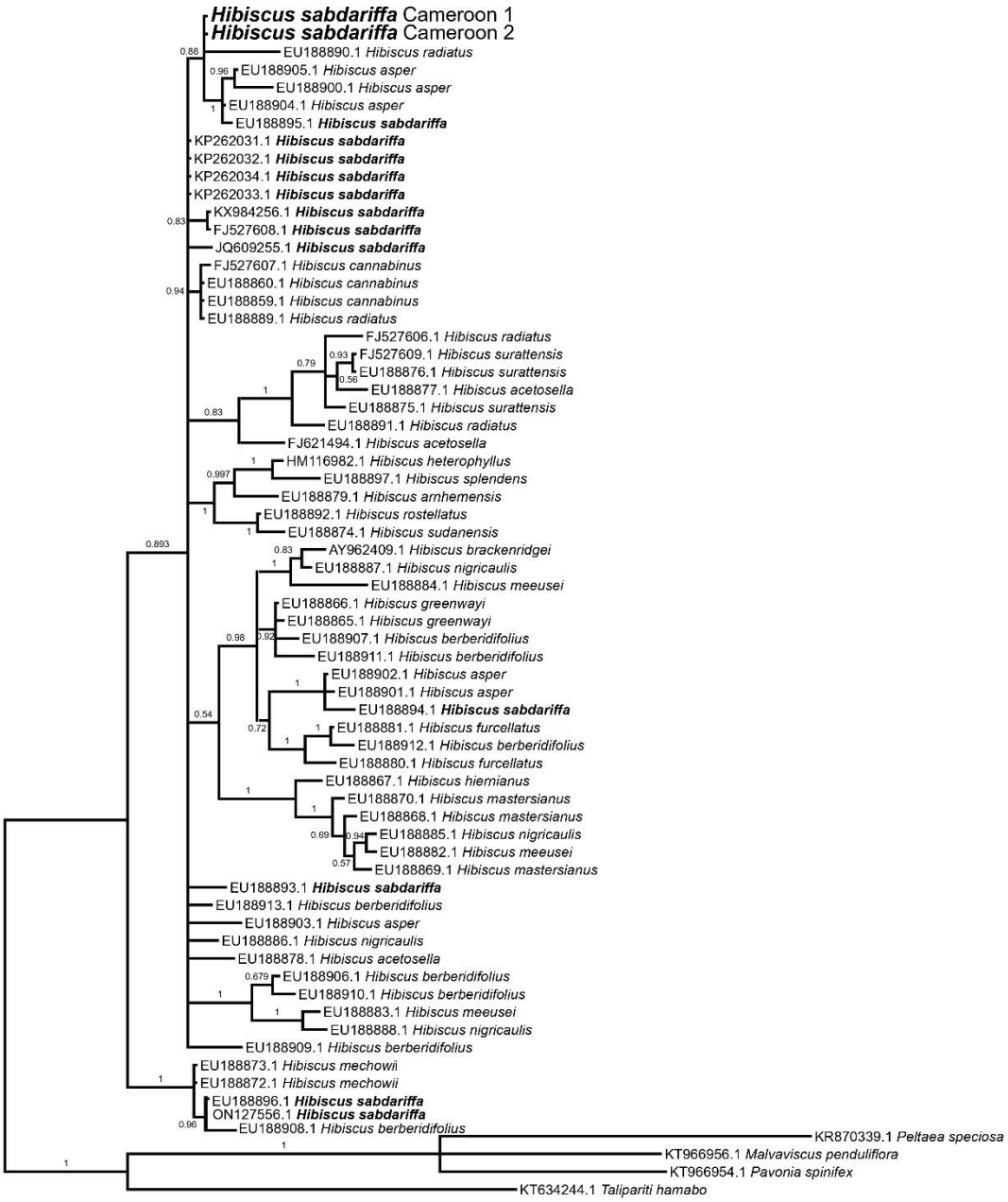

Figure 5. Baysian tree based on nuclear ITS sequences

GenBank accession numbers, species names, and support values for clades (posterior probabilities) are given. Sequences of *H. sabdariffa* are highlighted using boldface and the sequences from Cameroon by bigger font size. Most of the *H. sabdariffa* sequences from GenBank are close to the two samples from Cameroon, but several sequences are situated clearly apart. A similarly complex pattern can be observed for other species like *H. asper*.

Gene trees and species trees were not necessarily congruent; these results could be explained by incomplete lineage sorting, by which "ancestral polymorphisms persisted through several speciation events" (Maddison, 1997). Large effective population sizes and short phylogenetic branches (expressed in generations) favored incomplete lineage sorting (Pamilo & Nei, 1988). Another possible explanation for the incongruencies of gene trees and species trees was hybridization (Maddison, 1997).

In the case of *Hibiscus* section Furcaria, to which *H. sabdariffa* belongs, hybridization and alloploidy were well documented (Wilson, 2006) and the tetraploid *H. sabdariffa* (chromosome

number *n* = 36) was an allopolyploid (Wilson, 2006). This complex evolutionary pattern may explain the partial incongruence between morphological and sequence data, especially in the ITS tree that not only affected *H. sabdariffa*, but also species like *H. asper*, *H. berberidifolius*, *H. nigricaulis* Baker F., and *H radiatus*. In any case, the samples of *H. sabdariffa* from Cameroon were genetically very close to most of the *H. sabdariffa* sequences available at GenBank and taken together the available data support the correct identification of the samples with the present knowledge on the taxon.

3.2. Determination of physicochemical properties and analyses of foreign matters

Raw ethanol extracts of powder samples from each batch were deposited on a TLC plate. European Pharmacopeia monographs reported two bands (one blue and one purple) of high intensity and some other weaker bands were observable in visible light. As seen in **Figure 2S (supplementary file)**, *H. sabdariffa* extracts from batches A, B, C, and E also showed two bands with high intensity as well as weaker ones. The extract of *H. sabdariffa* from batch D exhibited no bands suggesting the absence or a very weak quantity of colored compounds. At this same concentration, batches A and C revealed two bands with higher intensity than batches B and E.

The coloring assay was based on the extraction of powder of *Hibiscus* with hot water. Then the absorbances of the five solutions were recorded at 520 nm **(Table 1S, supplementary file)**. Batches A, B, C, and E showed a saturation of the absorbances and agreed with the recommendation of the European Pharmacopeia (absorbance over 0.350 for powder). In contrast, the sample from batch D exhibited an absorbance under 0.350.

The loss on drying assay evaluated whether the sample was dried sufficiently; this is useful to prevent drug degradation. The loss on drying percentage for *H. sabdariffa* powders in batches A, B, and C was below 11%, while batches D and E were above this value recommended by the Pharmacopoeia **(Figure 3S, supplementary file)**. Statistical analysis indicated that loss on drying percentage varies significantly according to the variety studied. They are all different from each other (*p* value ranging from 0.0469 to < 0.0001), except for variety B, which is not different from either A or C **(Table 2S, supplementary file)**.

The acid percentage was determined for the five batches after the neutralization of the citric acid in the aqueous extracts with sodium hydroxide. The results are summarized in **Table 1** and show that batches B, C, D, and E exhibited an acid percentage of over 13.5%, which is recommended by the European guidelines. The percentage of batch A reached 13.27%, which is slightly lower. Statistical analysis indicated that there is also a significant difference between varieties, but this time they are all different from each other (*p* value ranging from 0.0067 to < 0.0001) **(Table 3S, supplementary file)**.

The analyses of foreign matters were realized in the five batches and two types of foreign matters were found in the samples: from the fruit and other sources **(Figure 4S, supplementary file)**. All samples respect the European Pharmacopeia (Karkade Monograph, 2017) criteria i.e. the samples must not contain more than 2% of fragments from the fruits (red funicles, grey or yellow fragments of the capsules or seeds) and less than 2% of others elements **(Figure 5S, supplementary file)**.

Three assays were performed on each of the five batches of *H. sabdariffa*. The white specimens of *H. sabdariffa* were used as negative control and the results showed that they did not meet the recommendations given by the European Pharmacopeia (for coloring and desiccation assays, for TLC analysis) in contrast to the red specimens from Cameroon and Egypt. Commercial samples stored in plastic bags and sold in the pharmacy did not respect the value of desiccation as condensation could be involved. Conservation for botanical drugs should be done in paper bags far from atmospheric humidity to guarantee better sample preservation and long-term quality of plants, akin to herbarium specimens (Fournier et al., 2011). The sour taste of karkade is specific to this drink and the relatively lower percentage of acidity for both samples retrieved for the red Cameroonian cultivar explained why it was less acidic (about 15% vs over 17%). Nevertheless, the values obtained here were in the range recommended by the pharmacopeias in contrast to some samples bought in a Turkish market (Özdogan et al., 2011). In the literature, the malic acid content in fresh calyces of *H. sabdariffa* was between 0.12 to 2.70% (m/m) (Cisse et al., 2009) and other authors reported a percentage of organic acids of about 15% whatever the acid (Da-Costa-Rocha et al., 2014).

3.3. Validation of the HPLC method for anthocyanins quantification

Three calibration curves for C3OG were built after injection in HPLC-DAD at 530 nm. Linearity was validated for the range of concentration (1.9–30 µg/ml). All results are available in **Table 4S (supplementary file)**. The method for the anthocyanins quantification in C3OG equivalent was validated for all parameters.

3.4. Extraction of H. sabdariffa, identification of major anthocyanins and their quantification in cyanidin 3-O-glucoside (C3OG) equivalent

Three types of extractions were done for the five batches of *H. sabdariffa*: (i) a maceration with the organic solvent methanol, (ii) a decoction, and (iii) an infusion process, the latter two used water. The choice of the solvents was an important step because it conditioned the chemical composition of the extract as reported by Laskar and Mazumder (2020), and thus the biological activity of the extract. Vasudeva and Sharma (2008) reported for example that methanol extracts of the calyx increased the hepatic enzymes (ASAT, ALAT), while aqueous extracts remained non-toxic even at high doses. In the same manner, water or ethanolic extracts from flowers showed different antioxidant activities (Da-Costa-Rocha et al., 2014). The higher yields for aqueous extractions could be explained by the extraction not only of anthocyanins and glycosylated flavonols but also by the extraction of mucopolysaccharides, pectin, and catechins the plant contains (Da-Costa-Rocha et al., 2014).

All extracts were analyzed by High-Performance Liquid Chromatography-Diode-Array Detector (HPLC-DAD-MS) and the analysis was focused on the anthocyanins easily detected at 530 nm as seen in **Figure 6**. Four peaks were detected for the methanolic macerated extracts A, C, and E named peak 1 (Rt = 16.6 min), peak 2 (Rt = 18.1 min), peak 3 (Rt = 20.9 min), peak 4 (Rt = 22.9 min), while two peaks (corresponding to peaks 1 and 2) were extracted with water whatever the method (infusion or decoction). The mass detection allowed us to determine two major ions for peak 1 with *m/z* $[M+H]^+$ = 597 and 465, peak 2 corresponded to two ions with *m/z* 581 and 449, and peaks 3 and 4 corresponded to respectively *m/z* 303 and 287. One could be noticed that the retention time for the anthocyanins differed along with the solvent and this was demonstrated by injecting the standard 3-*O*-cyanidin glucoside. No anthocyanins were detected in extract D.

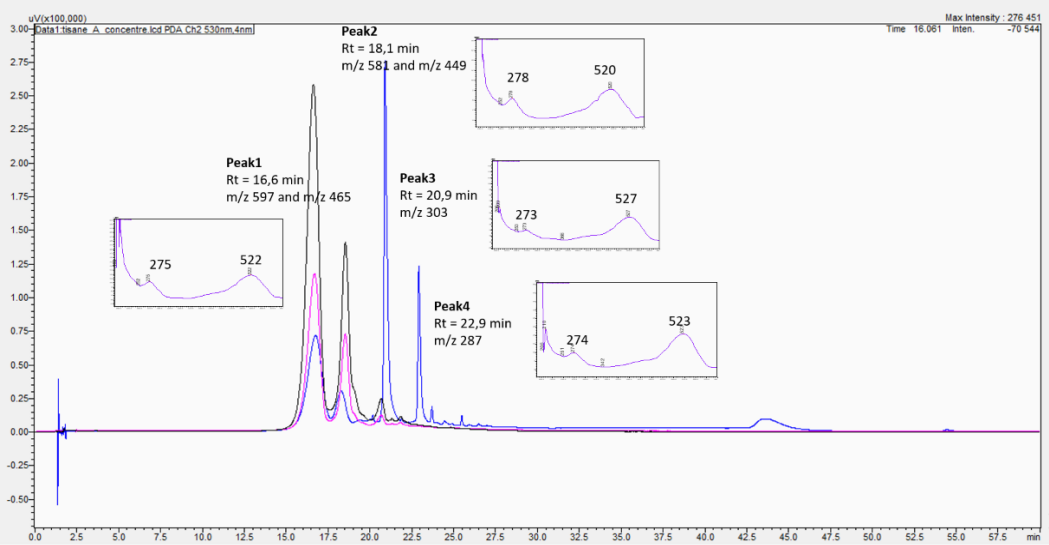

Figure 6. Chromatographic profiles of the three extracts of *H. sabdariffa* for batch A (in blue: maceration, in pink: decoction, in black: infusion)

The chromatographic data of each peak are the retention time (Rt), the mass spectra of the main fragment (*m/z*) and the UV spectra (in nm)

The mass detection allowed us to determine that peak 1 corresponded to delphinidin-3-sambubioside and delphinidin-3-glucoside, that peak 2 was cyanidin-3-sambubioside and cyanidin-3-glucoside. Peaks 3 and 4 were identified as the aglycones of delphinidin and cyanidin respectively (Schütz et al., 2006). These identifications agreed with the polarity of the solvents of extraction: the water extractions extracted the heterosides, while the organic extractions extracted the more apolar compounds, i.e. aglycones. Thanks to the mass spectrometry, delphinidin glycosides and cyanidin glycosides could be distinguished. Thus, the Cameroonian cultivar contained the major anthocyanins found in *H. sabdariffa*.

Quantification by High-Performance Liquid Chromatography–Diode Array Detector (HPLC-DAD) was calculated thanks to a calibration curve realized using the standard cyanidin-3-*O*-glucoside (C3OG). Each area under the peaks 1-4 of anthocyanins was determined and reported on the calibration curve to give a concentration in C3OG equivalent. As seen in Figure 7, the higher concentrations of anthocyanins were obtained for batch C > batch A > batch B and E whatever the mode of extraction. The extracted content was between 1.5 and 3.25 % m/m eq C3OG DM in extracts A and C and 0.16 and 0.55 % in extracts B and E (Table 2). Statistical analysis of the anthocyanin content indicated significant variations between Hibiscus varieties 2 by 2 for each given extraction method ($p < 0.0001$), except for varieties B and E for which $p < 0.05$ for decoctions and macerations, and which are not even significantly different for infusions ($p = 0.9556$). The anthocyanins content for the Cameroonian extracts was significantly higher whatever the mode of extraction (Table 5S, supplementary file).

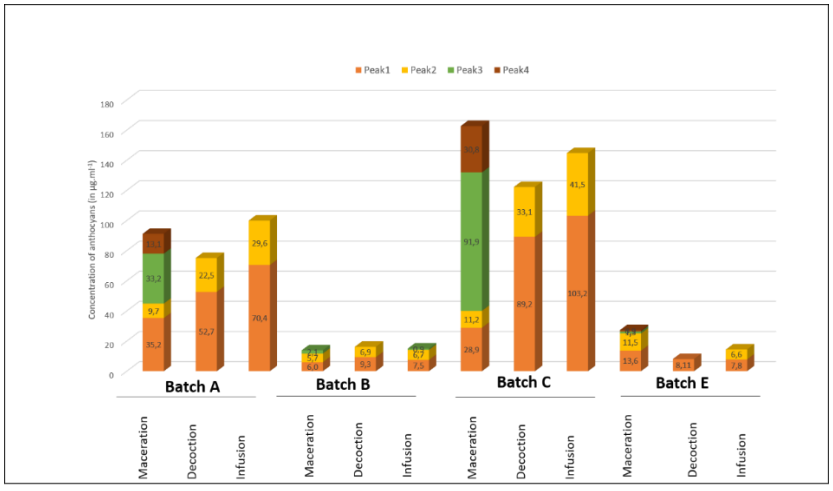

Figure 7. Concentration of the four anthocyanins in the three extracts of *H. sabdariffa* for the batches A, B, C, E (in µg/ml)

Peak 1: Delphinidin glycosides, Peak 2: Cyanidin glycosides, Peak 3: Aglycon of delphinidin, Peak 4: Aglycon of cyanidin

Table 2. Anthocyanin content in the five batches extract of *H. sabdariffa* along with the mode of extraction (mean of three assays, expressed in m/m eq C3OG (%)

	Maceration	Decoction	Infusion
Batch	Anthocyanin content (% m/m) ± δ	Anthocyanin content (% m/m) ± δ	Anthocyanin content (% m/m) ± δ
A	1.82 ± 0.06	1.50 ± 0.16	2.00 ± 0.08
B	0.35 ± 0.06	0.36 ± 0.06	0.32 ± 0.04
C	3.25 ± 0.08	2.45 ± 0.09	2.90 ± 0.05
E	0.55 ± 0.02	0.16 ± 0.01	0.29 ± 0.01

These results confirmed that the solvent of extraction was of great influence on the composition of the extracts: an alcoholic extraction allowed the extraction of more diversified anthocyanins with a great quantity of aglycons and fewer glycosides even if the yield was about the same whatever the solvent. For all batches, a slight decrease in anthocyanin content was observed for the extracts coming from decoction compared to infusion. It could come from the method of preparation because it is well-known that anthocyanins were unstable and were readily degraded after thermal or after long storage. Infusion was therefore recommended to obtain a better content of anthocyanins.

The anthocyanin quantification exhibited that the red cultivar from Cameroon (batches A and C) contained about ten-fold more content of anthocyanins than the Egyptian batches and overpassed the usual values found in the literature: 0.62% (m/m) (Riaz & Chopra, 2018) or about 0.15% (m/m) (Cisse et al., 2009). These extracts were about three-fold higher in delphinidin glycosides compared to cyanidin derivatives in agreement with the literature (Opletal et al., 2017; Riaz & Chopra, 2018). It was already described that upon drought stress, the plants could accumulate secondary metabolites and this high percentage of anthocyanins could be correlated to the place where the Folere is cultivated in semi-aridic places (Besharati et al., 2022). Jiofack et al. (2010) reported *H. sabdarrifa* cultivated in the Sudano-Sahelian region and it could be interesting to compare them.

As a result, *H. sabdariffa*, and especially this Cameroon cultivar is a huge source of anthocyanins, and greater than those fixed by the pharmacopeia for *Vitis vinifera* (0.2%) even if the advantage for the latter plant is the better stability of anthocyanins with the compounds having a hydroxyl group on C-4 position.

4. Conclusions

This *Hibiscus* cultivar cropped in Cameroon was taxonomically verified, chemically analyzed and scientifically characterized for its useful properties for the first time. Molecular analyses confirmed its identification as *H. sabdariffa* and the sequences were deposited at GenBank. All samples respected the identification tests, quantification of acidity, and several assays including desiccation, coloring, and foreign matters of the European Pharmacopeia. Moreover, the traditional extract realized as an infusion showed a very high content of delphinidin and cyanidin glycosides. This enriched source represents a great interest for companies because less herbal material is needed to realize standardized preparation for pharmacological purposes, cosmetic properties, and for coloring power. Until recently, the Arab Republic of Egypt was the only certified source country for commercial hibiscus flowers and in 2011 several producers and traders from Burkina Faso achieved certification through the FairTrade Labelling Organizations International FLO-CERT GmbH (Da-Costa-Rocha et al., 2014). We propose that Cameroon should also participate in this impulse. A political strategy supports the development of the local industry for producing drugs from medicinal plants (Fokunang et al., 2011) and this cultivar could also participate in local development communities.

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Conflict of interest

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

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Supplementary File

The supplementary file accompanying this article is available at https://ijpbp.com/index.php/ijpbp/libraryFiles/downloadPublic/19.

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RESEARCH ARTICLE

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Unveiling the antimalarial properties of *Terminalia ivorensis* (A. Chev) stem bark aqueous extract: In vivo efficacy testing and in silico predictions

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ABSTRACT

Due to the spreading resistance to antimalarial drugs, new therapeutics are urgently needed, preferably with novel modes of action. Extracts from *Terminalia ivorensis* have previously been shown to possess activity in vitro against multidrug-resistant and drug-sensitive strains of *Plasmodium falciparum*. However, to the best of our knowledge, no scientific study has been published describing the antimalarial potential of these extracts through in vivo efficacy testing. This study aimed to determine the safety and antimalarial efficacy of the *T. ivorensis* stem bark aqueous extract (TiH_2O) in a mouse model using the OECD 423 protocol and the suppressive and curative murine malaria models, and to predict in silico the pharmacokinetic properties and drug-likeness of two major phytochemical constituents. The in vivo antimalarial efficacy was assessed using the *P. berghei* NK65-infected mice. The TiH_2O treatment impact on biochemical parameters was measured using established standard procedures. The pharmacokinetics prediction was achieved through the pkCSM predictor and Swiss ADME. The TiH_2O extract was nontoxic in BALB/c mice at a lethal dose of 50 (LD_{50}) > 2000 mg/kg. The TiH_2O extract displayed strong antimalarial efficacy with 100% parasitemia suppression at 200 mg/kg b.w. after 4 days of treatment while its oral administration at 400 mg/kg b.w. in the curative model significantly decreased *P. berghei* parasitemia by 94.07% with a median efficacy dose (ED_{50}) of 96.80 mg/kg. The administration of TiH_2O extract restored the histological parameters disrupted by *P. berghei*, and the transaminase (ALT and AST) activity, creatinine, and bilirubin levels significantly decreased compared to the negative control mice. In silico explorations showed that the main constituents leucodelphidin (leucodelphinidin) and ellagic acid of the TiH_2O extract have drug-like properties, thus indicating that *T. ivorensis* might constitute a promising source of antimalarial chemical entities with good pharmacokinetics and drug-like properties. The results obtained further corroborated the preliminary in vitro antiplasmodial studies of *T. ivorensis* stem bark aqueous extract. The metabolome of TiH_2O extract should be further profiled in the prospects of characterizing novel natural product scaffolds to support antimalarial drug discovery.

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1. Introduction

Malaria, a life-threatening disease caused by *Plasmodium* parasites, continues to pose a significant global health burden (Sumbe & Barkade, 2023). The most severe form and treatment failure as well as the highest prevalence of malaria are attributed to *P. falciparum* and *P. vivax* (Sumbe & Barkade, 2023; World Malaria Report, 2023). The World Health Organization estimated an increase in malaria cases in 2022 with 249 million cases compared to 244 million cases in 2021 with Africa accounting for over 94% of all malaria cases and 95% (580 000) of deaths (World Malaria Report, 2023). The majority of malaria-related deaths in Africa (~ 80%) occur in children under the age of five, and the deadliest *P. falciparum* elicits the heaviest burden (Sumbe & Barkade, 2023). *Plasmodium* parasites spread to humans through mosquito bites and quickly enter the bloodstream, penetrate hepatocytes, and reproduce asexually, creating thousands of merozoites (Luth et al., 2018). In *P. vivax* and *P. ovale*, sporozoites may mature into "hypnozoites", a latent form that can revive weeks or months later and cause an infection recurrence. The liver exoerythrocytic cycle replicates asexually and thrives in the intraerythrocytic stage. The disease's symptomatic phase is brought on by merozoites that are discharged after leaving the lysed host red blood cells. After several cycles of replication, a small subset of parasites diverts from asexual replication and instead produce sexual progeny that differentiates into male and female sexual forms, called gametocytes that can subsequently be transmitted into the mosquito to perpetuate the infection cycle (Luth et al., 2018; Sumbe & Barkade, 2023). *P. falciparum* is not only the most deadly and common malaria parasite but also the hardest to eradicate, as it perpetually finds a means to evade every drug in the pharmaceutical arsenal (Rocamora & Winzeler, 2020).

Despite achievements with artemisinin-based combination treatments (ACTs), eradication efforts still face numerous obstacles, particularly in the African region (World Malaria Report, 2022) due to the recent emergence of artemisinin-resistant parasites (Asua et al., 2021; Mihreteab et al., 2023; Uwimana et al., 2020; Uwimana et al., 2021). This trend in the African context is of higher concern. In that regard, novel drugs are critically needed to help control malaria. The plant origin of famous antimalarial drugs such as quinine isolated from the bark of *Cinchona* trees and artemisinin isolated from *Artemisia annua* sustains the hope that continuous consideration of biodiversity could unveil novel starting points for new drug development against malaria. In this line, the investigation of natural products to identify novel pharmacophores with acceptable profiles as starting points for antimalarial drug discovery is a credible ongoing approach.

Terminalia ivorensis A. Chev (Combretaceae), also known as idigbo, black afara, shingle wood, brimstone, and black bark, is extensively used in traditional medicines around the world to treat a variety of illnesses, including malaria and yellow fever (Eloff et al., 2008). Of note, previous studies have indicated the potency of *T. ivorensis* against asexual blood stages of drug-sensitive and drug-resistant malaria parasites (Annan et al., 2012; Appiah-Opong et al., 2022; Komlaga et al., 2016). We have recently reported the antiplasmodial potency of this plant in vitro (Tali et al., 2022). However, the exploration of these natural product extracts using in vivo malaria models has not been reported yet.

The present study aimed to decipher the safety and efficacy properties of the aqueous extract from *T. ivorensis* (TiH_2O) and to predict the pharmacokinetic properties and drug-likeness of two of its active phytochemicals.

2. Materials and methods

2.1. Ethical approval

Animal experiments were performed following the protocol approved by the Joint Institutional Review Board, University of Yaounde 1 (No. BTC-JIRB2023-091), and the results were reported following the ARRIVE guidelines (S1 Checklist).

2.2. Plant materials

T. ivorensis (commonly referred to as “black afara” in Cameroon) was identified at Carrefour MEEC-Nkolbisson, Yaounde-Cameroon by Mr. Victor Nana, a taxonomist from the National Herbarium of Cameroon, Yaounde. Sample from the stem bark was collected around 6.30 am local time and a voucher specimen was deposited at the Cameroon National Herbarium under reference number 48878/HNC.

2.3. Extraction of plant material

The stem bark of *T. ivorensis* was ground into fine powders (Ø~0.5 µm) using a miller (Hammer Mill, Leabon 9FQ, Zhengzhou, PRC) and dried under shade at room temperature (25–29 °C) for two weeks (Tali et al., 2022). 1.5 kg of powder was macerated in 4.5 l of distilled water for three consecutive days. The macerate was then filtered using Whatman No 1 filter paper, and the residue was macerated afresh for two consecutive days and treated similarly. The extract was lyophilized using a Virtis Wizard 2.0 Freeze Dryer Lyophilizer (Model: XLS-70) (Tali et al., 2022). The extraction yields were determined relative to the initial weight of the powder, and the dried crude extract (greenish-brown color) was used for in vivo safety and efficacy studies.

2.4. Determination of the oral acute toxicity of TiH2O extract

The potential acute toxicity of TiH_2O extract was evaluated in mice as previously described (OECD, 2002). Three groups of three mice each were used for this study. Group one was orally administered by gavage as previously described (Anh Thu Pham et al., 2011) with a single dose (2000 mg/kg b.w.) of TiH_2O extract while group two (control group) was treated with 20 ml/kg distilled water. Group three was considered as the normal group and received no extract or water. The animals were observed for 30 minutes, 4 hours, and thereafter daily for 14 days to record any signs of toxicity.

2.5. Determination of bioactivity parameters in the murine experimental model

2.5.1. Amplification and maintenance of P. berghei NK65 in mice

A suspension of *P. berghei*-infected erythrocytes in PBS (1:1 v/v) was injected into the peritoneal cavity of healthy BALB/c mice. Parasite proliferation was monitored microscopically using Giemsa-stained slides, and when the parasitemia reached 10-20%, mice were sedated with a KX cocktail (ketamine/xylazine 120/16 mg/kg) (Sloan et al., 2011). For parasite amplification and maintenance, blood was collected by jugular puncture into EDTA tubes and diluted in PBS before inoculation to another group of healthy mice.

2.5.2. Determination of the antimalarial activity of TiH_2O

2.5.2.1. Assessment of the suppressive activity of TiH_2O

Parasitemia suppression by TiH_2O was assessed according to the method described by Knight and Peters (1980) using early *P. berghei*-infected mice. Chloroquine was used as a positive control (Knight & Peters, 1980). Treatment was administered by oral gavage to mimic the traditional route of administration. Three treatment groups of six mice each including one test group receiving TiH_2O, one positive control group receiving chloroquine (CQ), and one negative control group receiving distilled water were included. On day 1 of post-infection (D0), whole blood was drawn from the donor mouse by jugular puncture into an EDTA tube, and a suspension of *P. berghei*-parasitized erythrocytes in PBS was prepared at 1×10^6 RBCs/ml. Then, the experimental mice were infected intraperitoneally with 200 µl of the so-called suspension. Two hours after infestation, mice were treated orally with 100 mg/kg b.w. of TiH_2O. Positive and negative control mice received 10 mg/kg b.w. chloroquine and 25 ml/kg b.w. distilled water, respectively. All animal groups were thereafter monitored similarly for 4 consecutive days (D0–D3) between 8 a.m. and 10 a.m. with daily recording of parasitemia and survival rates. To evaluate the ability of TiH_2O to prevent weight loss due to infection, weight differences between days post-infestation were calculated relative to D0. Every 48 hours, Giemsa-stained thin smears were prepared from tail blood from the 5th to the 15th day (D4–D14) and examined microscopically at x100 magnification to determine the percent parasitemia relative to the total red blood cell (RBC) counts.

The average percentage of chemo-suppression was calculated for the treated groups as given below:

$$Chemosupression\ (\%) = \frac{A - B}{B} x\ 100$$

where A and B represent the average percent parasitemia in the negative control group and the test group respectively. Survival rate was monitored twice daily and was determined over 14 days (D0–D13) and compared between groups.

2.5.2.2. Assessment of the curative effect of TiH_2O

The assessment of the therapeutic activity of TiH_2O was conducted as previously described (Knight & Peters, 1980). On day 1, 30 healthy mice were intraperitoneally injected with 200 µl of *P. berghei* NK65-infected RBCs. After illness induction three days later corroborated by established parasitemia (3 to 4%), five groups of six mice each receiving daily oral doses of 100, 200, and 400 mg/kg/b.w. of TiH_2O (groups 1, 2, and 3, respectively); 10 mg/kg/b.w. of CQ (group 4); and 10 ml/kg distilled water (group 5) were considered. Giemsa-stained thin blood smears were examined daily to monitor parasitemia changes in response to drug exposure. Treatments were repeated for five consecutive days and the data were normalized to percent control activity using Microsoft Excel software, and median efficacy dose (ED_{50}) for TiH_2O was calculated using Prism 8.0 software (GraphPad) with data fitted by nonlinear regression to the variable slope sigmoidal dose–response formula given below:

$$y = \frac{100}{1 + 10^{logIC50 - x)H}} x\ 100$$

where H is the Hill coefficient or slope factor (Singh & Rosenthal, 2001).

2.5.2.3. Assessment of the changes in biochemical markers of mice blood

Upon completion of the curative test, mice were sedated with the KX cocktail, and their blood was collected in EDTA tubes by sectioning the carotid artery. The collected blood was centrifuged at 3000 rpm at 4 °C for 5 minutes, and the collected supernatant was stored at –20 °C for further analysis of biochemical markers. The LABKIT kit was used to quantify alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, creatinine, and total bilirubin levels.

2.6. Analysis of the histopathological changes

Cross-sectional samples (5 µm) of the liver, spleen, and kidney were dehydrated using ascending grades of alcohol, and 5-micron thick slides were stained with hematoxylin-eosin (HE) (Leica Biosystems) dye as recommended by the manufacturer and examined under a light microscope (at x40 magnification) and photographed using a microscope camera (Axioshop, Germany).

2.7. In silico prediction of ADMET properties of two antiplasmodial phytoconstituents of TiH_2O and putative targets

Our previous studies unveiled ellagic acid and leucodelphidin as the main pharmacophores incriminated in the activity of TiH_2O extract (Tali et al., 2022). Thus, ADMET and drug-likeness properties were predicted using the freely available Swiss ADME (Daina et al., 2017) and pkCSM predictor (Pires et al., 2015) tools. Chemical codes of ellagic acid and leucodelphidin were extracted from the chemical structure and used as input to the Swiss ADME (http://www.swissadme.ch/index.php) and pkCSM predictor (http://biosig.unimelb.edu.au/pkcsm/), to predict pharmacokinetic properties. In addition, plausible molecular targets were retrieved using Swiss target prediction tools (http://www.swisstargetprediction.ch/).

2.8. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Means were compared by analysis of variance (ANOVA), followed by Tukey's post-test using GraphPad Prism version 8.0 and values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. T. ivorensis aqueous extract (TiH_2O) is safe in mice

Oral dose of TiH_2O at 2000 mg/kg b.w. did not cause mortality or major behavioral changes among the experimental groups of animals, indicating its safety as per the criteria of the OECD (OECD, 2002). Additionally, no significant changes in the weight of the TiH_2O-treated mice were recorded (Figure 1).

3.2. TiH_2O displays antimalarial efficacy

3.2.1. TiH_2O halted disease installation in mice

The treatment with TiH_2O extract displayed significant chemo-suppressive activity within 4 days of post parasite inoculation. Like CQ, TiH_2O suppressed *P. berghei* growth by 100% relative to untreated mice (Table 1 and Figure 2A). However, at day 8 of post parasite inoculation, there was a parasitemia shift between the

TiH_2O (87.91%) and the CQ (100% suppression) treatments (Table 1 and Figure 2B).

On another hand, treatment of mice with TiH_2O resulted in a survival rate close to the chloroquine level up to day 8. Beyond day 9, all infected mice that received the vehicle died, whereas 5 out of the 6 mice (83.30%) were treated with TiH_2O and all CQ-treated mice survived until day 15 of the experiment (Figure 2C).

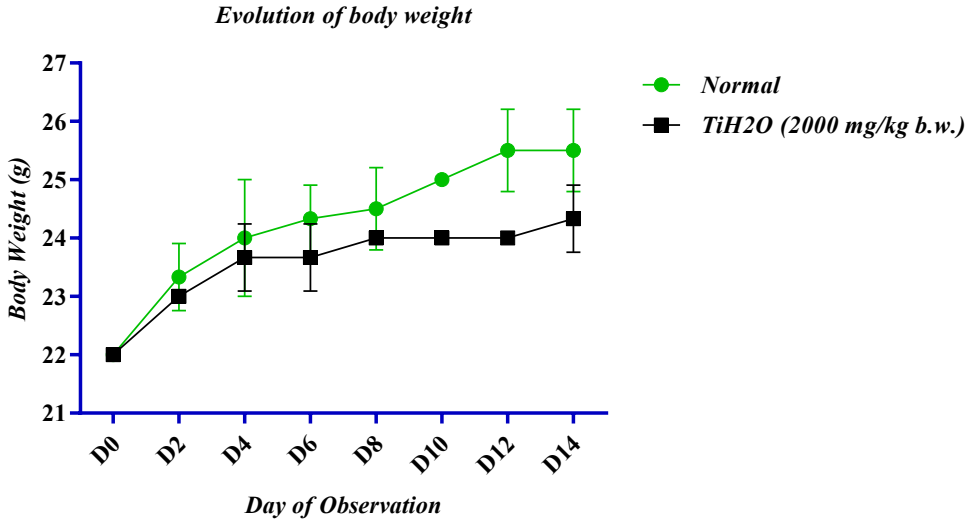

Figure 1. The body weight variation of mice under TiH_2O treatment.
Each point represents the mean ± SD; *n* = 3. TiH_2O: Aqueous extract of *T. ivorensis* stem bark. Administered dose: 2000 mg/kg b.w.

Table 1. Percentage of *P. berghei* suppression by TiH_2O extract

	Percent parasite suppression (%)	
	Day 4	Day 8
Chloroquine	100 ± 0.00	100 ± 0.00
TiH_2O	100 ± 0.00	87.91 ± 0.51***

Percentages of parasite suppression according to the treatment, calculated by comparison to TiH_2O-treated mice. ***$p < 0.0001$. TiH_2O: Aqueous extract of *T. ivorensis*.

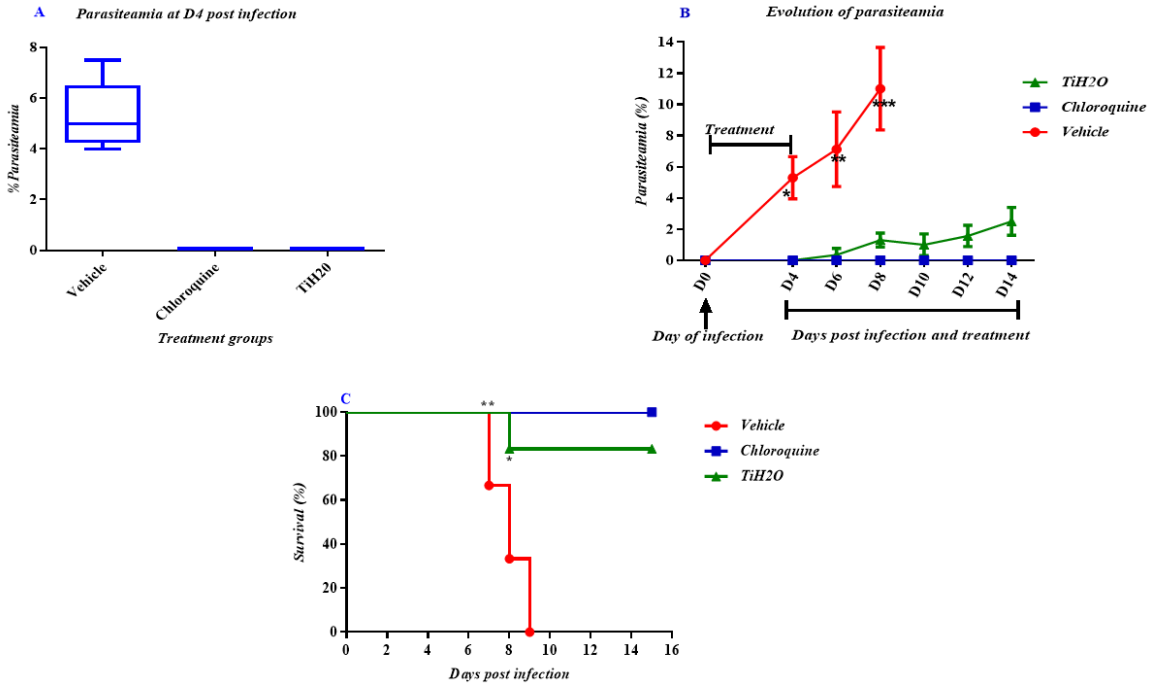

Figure 2. Antimalarial efficacy of TiH_2O in the *P. berghei* model
BALB/c mice were infected intraperitoneally with *P. berghei* NK65 (106 parasites/mice) and treated 2 h later with chloroquine (10 mg/kg), TiH_2O (100 mg/kg), or water (25 ml/kg) from day 0 to day 3. Parasite densities (parasitemia) were measured every 48 h from D4 to D14. (A) Effects of chloroquine; TiH_2O on the parasitemia of infected mice on day 4. (B) Evolution of parasitemia beyond day 4 post-infection (D4 to D14). Parasitemia was compared according to the treatment received (*$p < 0.05$; **$p < 0.005$ or ***$p < 0.001$). TiH_2O and CQ treatment were compared to vehicles. (C) Percentage of survival following infection in mice. Survival was measured daily and compared according to treatments received at D9 (**$p < 0.005$). TiH_2O and CQ treatment were compared to vehicle (**$p < 0.05$). TiH_2O: Aqueous extract of *T. ivorensis* stem bark

3.2.2. TiH_2O extract cures malaria symptoms in a murine model

The TiH_2O extract cured established malaria infection and restored key biochemical parameters and mouse histology. Compared to the negative control animals, TiH_2O administration at 200 and 400 mg/kg b.w. decreased parasitemia in a dose-dependent manner in *P. berghei*-infected mice from day 4 to day 8 (Figure 3). Compared to CQ at 10 mg/kg b.w., TiH_2O at doses of 100, 200, and 400 mg/kg b.w. inhibited parasite growth by only 88.8%, 97.4%, and 98.05%, respectively (corresponding to percent parasitemia of 11.2, 2.6, and 1.95%) within the same timeframe. Overall, the median efficacy dose (ED_{50}) of TiH_2O was 96.80 mg/kg b.w.

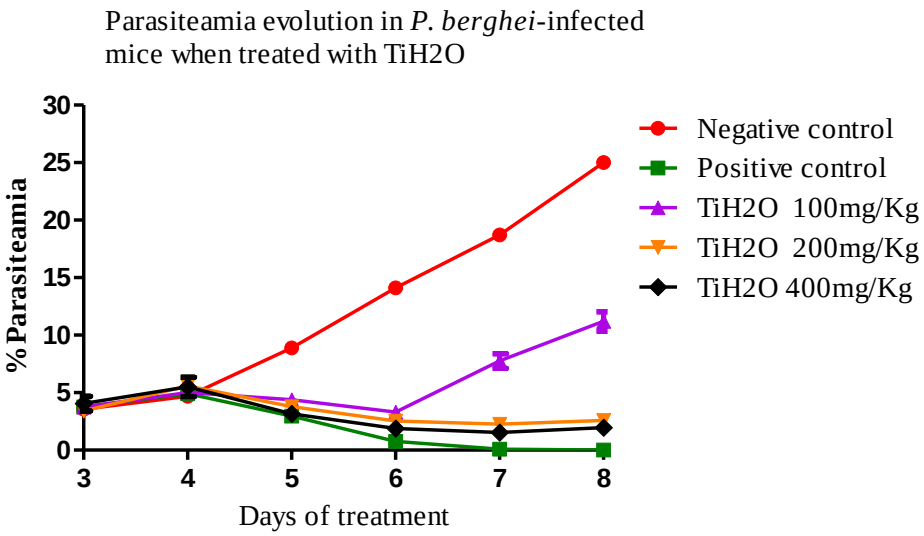

Figure 3. The curative effect of TiH_2O on *P. berghei*-infected mice
(A) The parasitemia levels were monitored from day 3 through day 8. TiH_2O: Aqueous extract of T. ivorensis stem bark

In summary, TiH_2O exhibited a comparable or superior suppressive and curative antimalarial effect at 200 and 400 mg/kg relative to CQ tested at 10 mg/kg. Additionally, in the daily post-drug treatment monitoring of animals, TiH_2O exhibited the ability to prolong mice survival, primarily attributable to its effectiveness in treating malaria infection by targeting and eliminating the malarial parasites, contributing to the prolonged survival of the mice. Additionally, the balance of the inflammatory response might contribute to the overall well-being of the animals by potentially impacting their survival. Also, if TiH_2O modulates the immune response to reduce excessive inflammation and promote a balanced immune reaction, it could positively influence the overall health of the mice. Indeed, ellagic acid and analogs are widely distributed in *Terminalia* spp., and their potential as anti-inflammatory and immunomodulatory agents is well documented (Abiodun et al., 2016; BenSaad et al., 2017; de Araujo et al., 2019; Deepika & Maurya, 2022). Besides, the observed recrudescence of parasites during daily observation of thin blood smears and post-drug exposure might be linked to latent *P. berghei* merozoites escaping the extract's effect by "hiding" in macrophages and neutrophils (Landau et al., 1999). In addition to our previous report (Tali et al., 2022), many other reports have highlighted the antimalarial efficacy of *Terminalia* species, all containing ellagic acid and derivatives among the main constituents, including Tali et al. (2022) who demonstrated that *T. mantaly* significantly cured murine malaria with ED_{50} of 69.50 mg/kg and LD_{50} above 2000 mg/kg. Similarly, Biruk et al. (2020) reported that an 80/20 methanol/water extract from *T. brownii* demonstrated good safety (LD_{50} > 2000 mg/kg) and significantly suppressed *P. berghei* ANKA proliferation (Biruk et al., 2020). Also, *T. albida* methanol extract was reported to drastically suppress *P. berghei* ANKA parasitemia by 100% (Camara et al., 2019). Moreover, these findings were corroborated by the previously reported activities of the ubiquitous ellagic acid (Njomnang Soh et al., 2012). Of note, there is no study in the literature that specifically describes the activity of leucodelphidin.

3.2.3. Treatment of mice with TiH_2O normalizes the biochemical parameters

The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) tests were conducted to depict liver dysfunctions and toxicological damages following plasmodial infection, and to ascertain treatment efficacy. The results showed a significant increase in ALT and AST activities in the negative control group of mice (infected and untreated), compared to the normal control (uninfected and receiving only vehicle) (Figure 4A and B). Oral administration of TiH_2O at 100, 200 and 400 mg/kg b.w. significantly decreased the levels of ALT and AST (***$p < 0.001$) in mice. Indeed, ALT and AST are enzymes found primarily in the liver, and their levels in the blood are often used as markers of liver function. Changes in these enzyme levels can be indicative of liver damage or dysfunction. In the context of *Plasmodium* infection (malaria) in mice, decreased levels of ALT and AST following drug administration could have therapeutic significance including 1) indication of therapeutic effect in terms of resolving or preventing liver damage associated with the infection; 2) an improvement in liver function, reflecting the drug's efficacy in addressing the underlying liver pathology associated with malaria; 3) a reduction in the parasite load in the liver; and 4) a broader improvement in the health status of the infected mice, indicating a positive response to the antimalarial treatment and corroborating the improved survival rate. Creatinine level significantly increased (**$p < 0.05$) (Figure 4C) in the negative control group compared to the normal control group. However, administration of CQ at 10 mg/kg/b.w. and TiH_2O at 100, 200 and 400 mg/kg b.w. elicited a consistent decrease (**$p <$ 0.05) in blood creatinine thereby depleting the deleterious creatinine elevation elicited by parasite growth. This decrease

further suggests that TiH_2O may be effective in mitigating the damage caused by malaria infection, particularly in the liver and kidneys. Similarly, the replication of *P. berghei* in infected mice correlated with a significant increase in bilirubin level compared to the normal group (Figure 4D). This effect was significantly normalized (**$p < 0.001$) by the effect of CQ at 10 mg/kg b.w. and TiH_2O at 200 and 400 mg/kg b.w. (Figure 4D). It should be noted that elevated bilirubin levels can be indicative of hemolysis and liver dysfunction. Therefore, a decrease in bilirubin levels following TiH_2O administration suggests potential therapeutic benefits.

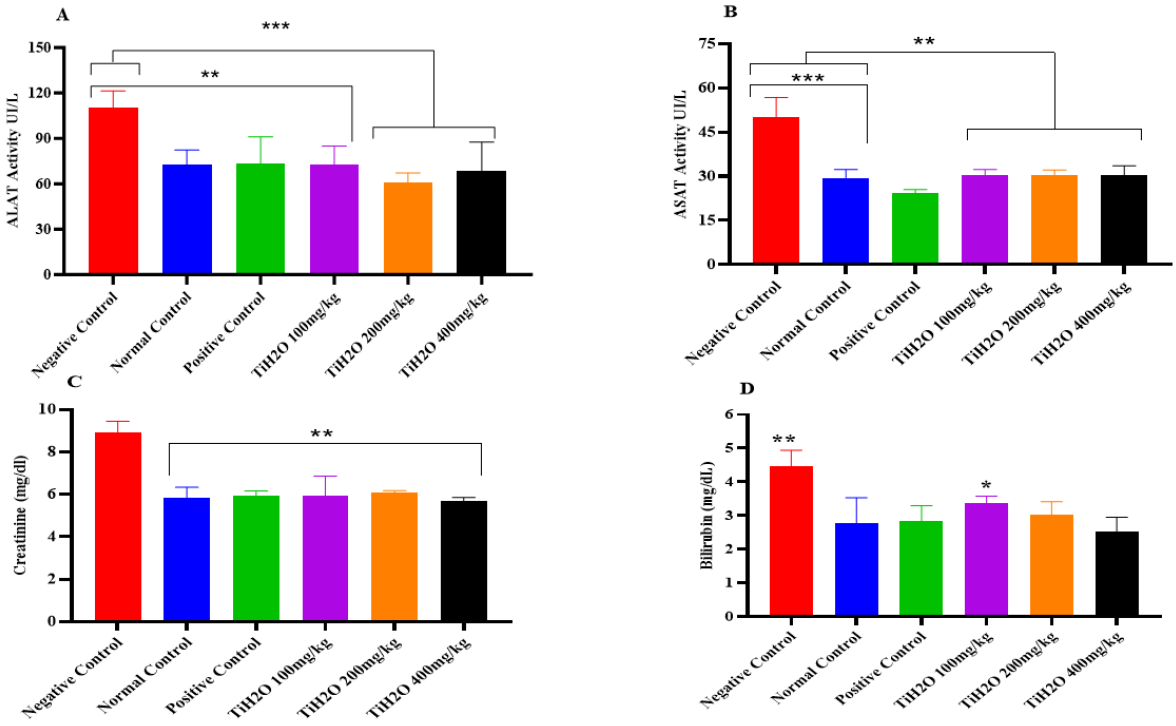

Figure 4. Effect of TiH_2O extract on ALT, AST, creatinine activity and bilirubin level

Each bar represents the mean ± SD, $n = 6$, A: Activity of ALT in *P. berghei*-infected mice and normal control groups, **$p < 0.05$; ***$p < 0.001$. B: Activity of AST in *P. berghei*-infected mice and normal control groups, **$p < 0.05$; ***$p < 0.001$. C: Activity of creatinine in *P. berghei*-infected mice and normal control groups, **$p < 0.05$. D: Level of bilirubin in *P. berghei*-infected mice and normal control groups, *$p < 0.05$; **$p < 0.001$

3.2.4. Histopathological profile of TiH_2O-treated versus untreated mice

The comparative effects of TiH_2O extract, CQ (positive control), and distilled water (negative control) on the microarchitecture of the examined organs indicated that parasite infection resulted in hepatic leukocyte infiltration, glomerulosclerosis in the kidneys, and disruption of the white and red pulp of the spleen in the infected, but untreated mice compared to the uninfected and untreated normal control (Figure 5). All these injuries may be caused by malarial anemia, cytoadherence phenomena, deposits of cell membrane debris, and malarial pigment. These factors lead to severe hypoxia, organ damage, increased lactemia, and shock (da Silva Junior et al., 2017). As a result of treatment with TiH_2O extract at doses of 200 and 400 mg/kg b.w. and CQ at 10 mg/kg, significant restoration of histological architecture of the liver, kidney, and spleen was elicited after 5 days of treatment.

3.3. Pharmacokinetics and drug-likeness profiles of leucodelphidin and ellagic acid

The data generated from the ADMET and drug-likeness prediction of leucodelphidin and ellagic acid (Figure 6) showed that both compounds satisfy the criteria of Lipinski's rule of five, indicating their drug-likeness. On another hand, leucodelphidin displayed poor absorption (51.01%) compared to ellagic acid (86.68%) (Table 2). Besides, the prediction suggested that ellagic acid and leucodelphidin cannot cross the blood-brain barrier (BBB) and do not inhibit hERG activity. Also, ellagic acid was predicted to inhibit CYP1A2 contrarily to leucodelphidin. The radar plot (Figure 7) indicates limited oral bioavailability for both compounds, probably due to highly unsaturated and polar features. In addition, the estimated plausible molecular targets for both compounds (Figure 8) indicated a preferential inhibition of kinases (60%) for ellagic acid followed by lyases (20%), while leucodelphidin was predicted to inhibit lyases (40%), followed by kinases (33.33%). Other less significant targets included the family of AG protein-coupled receptors (6.7%) and cytosolic proteins (6.7%) for ellagic acid, and proteases (13.3%), oxidoreductases (6.7%), and primary active transporters (6.7%) for leucodelphidin.

The predicted properties indicated that ellagic acid and leucodelphidin meet promising pharmacokinetics and drug-likeness properties, suggesting that both compounds possess characteristics that make them promising candidates as pharmaceutical compounds. Thus, further optimization might improve their ADME properties crucial for effectiveness in the body; their bioavailability to imply that enough of the compounds reaches the bloodstream and target tissues, thereby increasing the likelihood of a therapeutic effect; their metabolism such that by-products do not elicit toxicity. Besides, target prediction has unveiled plasmodial kinases and lyases as the main possible targets of ellagic acid and leucodelphidin. Notably, kinases are a class of molecular drug targets extensively investigated in multiple disease areas, including malaria as exemplified by the *P. falciparum* phosphatidylinositol 4-kinase type III beta (PfPI4KIIIβ) targeted by an inhibitor currently in

clinical development for malaria treatment (Paquet et al., 2017; Sinxadi et al., 2020). Kinases are critically involved in the development of *P. berghei* in mosquitoes (Tewari et al., 2010), and in the asexual blood stage in *P. falciparum* (Solyakov et al., 2011). Additional studies are therefore warranted to bring insights into the actual targets of both compounds.

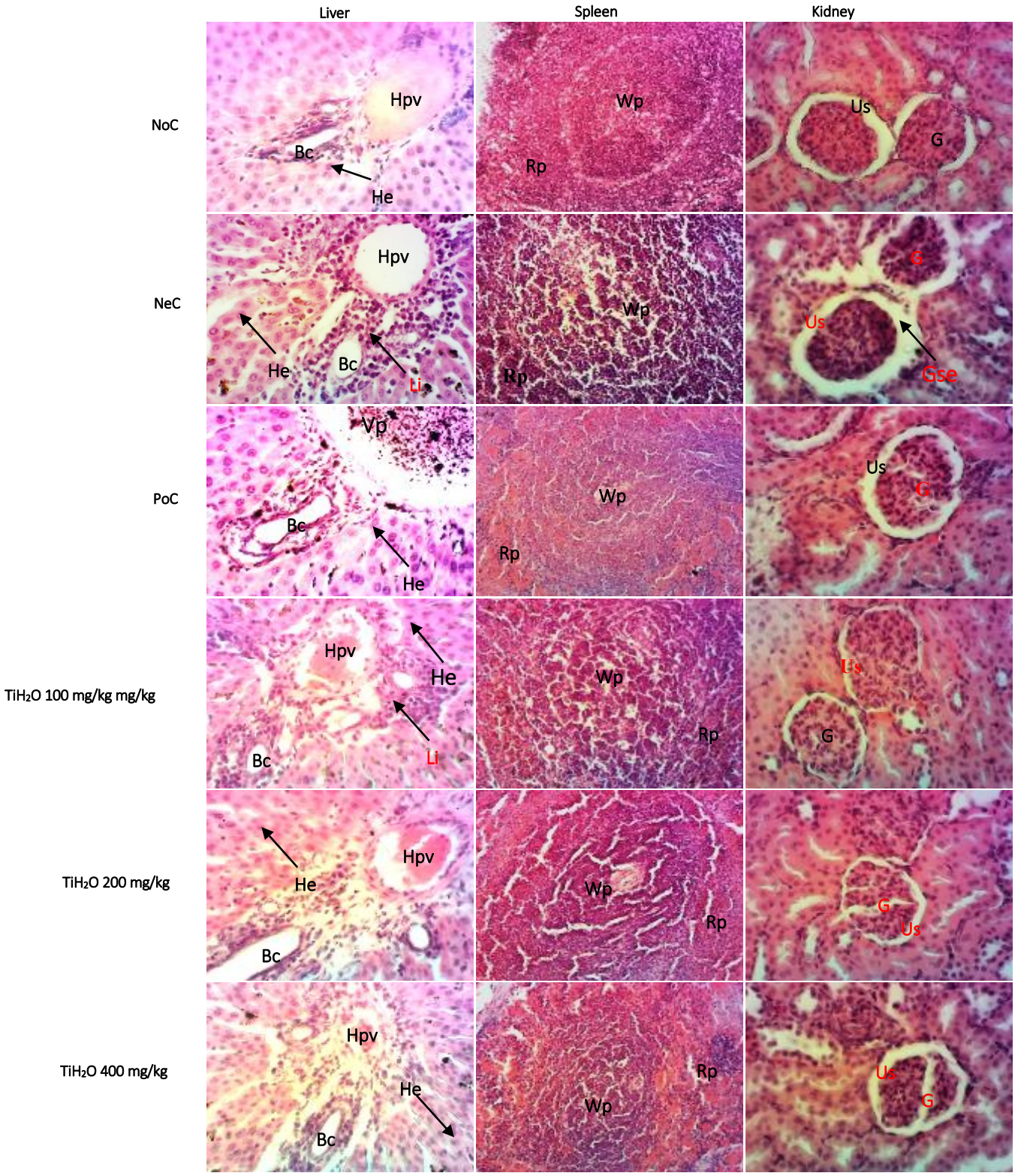

Figure 5. Micrography of liver, spleen, and kidney sections (HE x 400) of *P. berghei*-infected mice treated with TiH_2O (100, 200, 400 mg/kg), and CQ (10 mg/kg)

Hpv: Hepatic portal vein, He: Hepatocytes, Bc: Biliary canaliculus, Li: Leukocyte inflammation, Wp: White pulp, Rp: Red pulp, G: Glomerule, Us: Urinary space, Gse: Glomerulosclerosis, NoC: Normal control, NeC: Negative control, PoC: Positive control

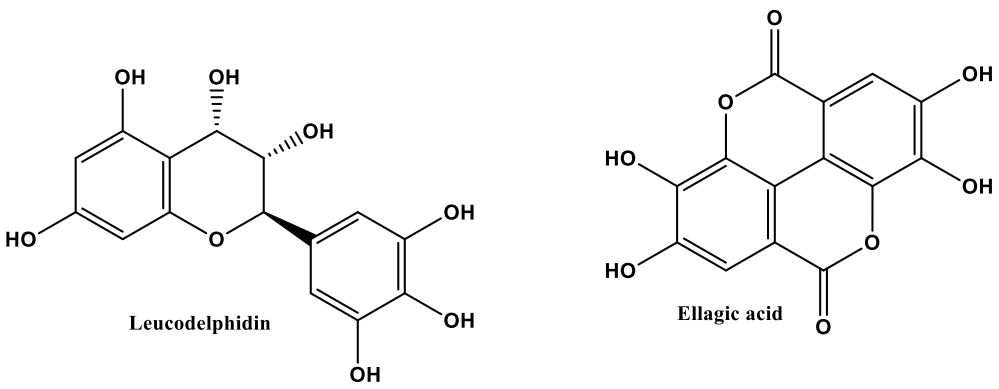

Figure 6. Pharmacophores reported as the main antiplasmodial ingredients of TiH_2O (Tali et al., 2022)

Table 2. Predicted pharmacokinetics and drug-likeness of leucodelphidin and ellagic acid

Property	Parameters	Predicted value		Unit/Classifications
		Leucodelphidin	Ellagic acid	
Drug-Likeness (MW < 500; HBD ≤ 5; HBA ≤ 10; logP ≤ 5)		Yes	Yes	
Absorption	Caco2 permeability	-2.916	-3.181	Numeric (log mol/l)
	Intestinal absorption (human)	-0.059	0.335	Numeric (log Papp in 10^{-6} cm/s)
	Skin Permeability	51.018	86.684	Numeric (% Absorbed)
	P-glycoprotein substrate	-2.735	-2.735	Numeric (log Kp)
	P-glycoprotein I inhibitor	Yes	Yes	Categorical (Yes/No)
	P-glycoprotein II inhibitor	No	No	Categorical (Yes/No)
Distribution	VDss (human)	1.721	0.375	Numeric (log l/kg)
	Fraction unbound (human)	0.486	0.083	Numeric (Fu)
	BBB permeability	-1.185	-1.272	Numeric (log BB)
	CNS permeability	-3.601	-3.533	Numeric (log PS)
Metabolism	CYP2D6 substrate	No	No	Categorical (Yes/No)
	CYP3A4 substrate	No	No	Categorical (Yes/No)
	CYP1A2 inhibitor	No	Yes	Categorical (Yes/No)
	CYP2C19 inhibitor	No	No	Categorical (Yes/No)
	CYP2C9 inhibitor	No	No	Categorical (Yes/No)
	CYP2D6 inhibitor	No	No	Categorical (Yes/No)
	CYP3A4 inhibitor	No	No	Categorical (Yes/No)
Excretion	Total clearance	0.216	0.537	Numeric (log ml/min/kg)
	Renal OCT2 substrate	No	No	Categorical (Yes/No)
Toxicity	Total clearance	0.216	0.537	Numeric (log ml/min/kg)
	Renal OCT2 substrate	No	No	Categorical (Yes/No)
	Total clearance	0.216	0.537	Numeric (log ml/min/kg)
	Renal OCT2 substrate	No	No	Categorical (Yes/No)
	Total clearance	0.216	0.537	Numeric (log ml/min/kg)
	Renal OCT2 substrate	No	No	Categorical (Yes/No)
	Total clearance	0.216	0.537	Numeric (log ml/min/kg)
	Renal OCT2 substrate	No	No	Categorical (Yes/No)
	Total clearance	0.216	0.537	Numeric (log ml/min/kg)
	Renal OCT2 substrate	No	No	Categorical (Yes/No)

hERG: Human ether ago-ago gene, CYP: Cytochrome P450

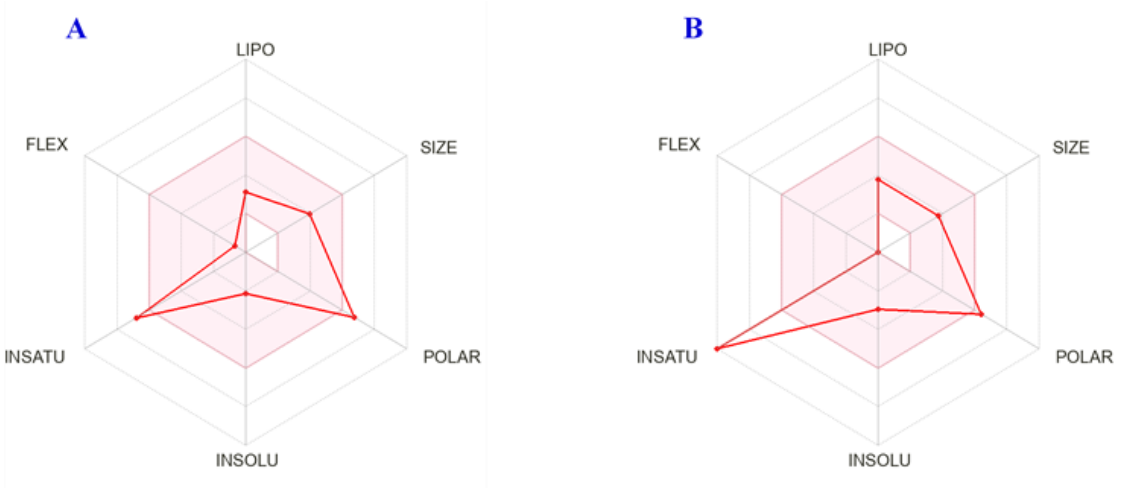

Figure 7. Bioavailability radar plots of (A) leucodelphidin and (B) ellagic acid
LIPO: Lipophilicity (between − 0.7 and + 5.0), SIZE: Molecular weight (between 150 and 500 g/mol), POLAR: Polarity (between 20 and 130 Å2), INSOLU: Solubility (≤ 6), INSATU: Saturation (fraction of carbons in the sp3 hybridization ≥ 0.25), FLEX: Flexibility (≤ 9 rotatable bonds)

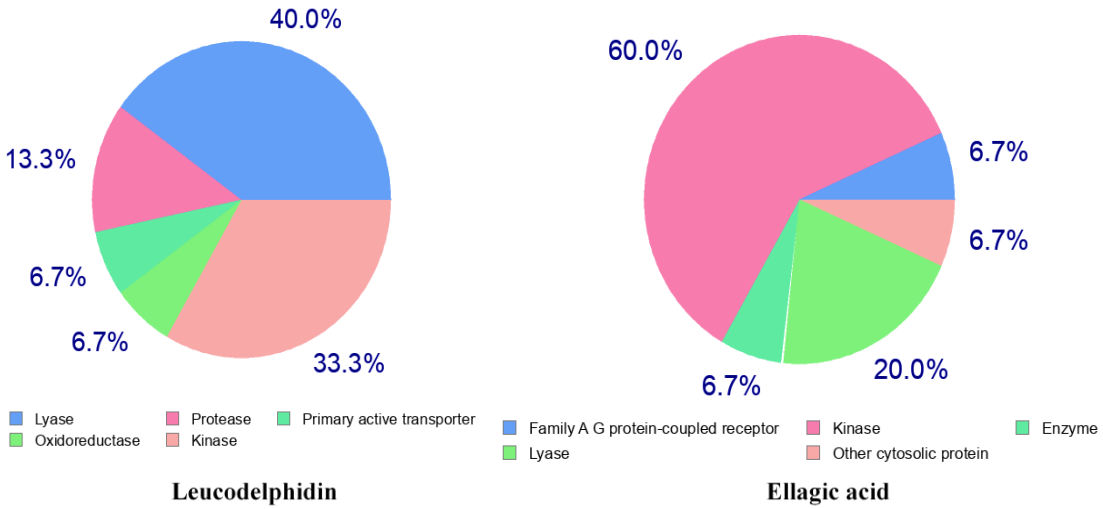

Figure 8. Pie chart of the top 50% of putative target prediction for ellagic acid and leucodelphidin

4. Conclusions

By amalgamating the previously reported in vitro antiplasmodial potency and the in vivo data reported herein, the outcome compellingly supports the conclusion that *T. ivorensis* could serve as a reliable source of new promising pharmacophores for antimalarial drug discovery. However, limitations include a lack of mechanistic insights to understand the actual mode of action of *T. ivorensis* metabolites against malaria parasites. Thus, in-depth exploration is warranted to comprehensively profile the metabolome and other biological parameters of this extract or their chemical isolates. In addition, this analysis adds to the continuous validation of *T. ivorensis* to treat malaria.

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Conflict of interest

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

Statement of ethics

Ethical approval for this study was obtained from the Joint Institutional Review Board for Animal & Human Bioethics (JIRB), University of Yaounde 1, Cameroon (No: BTC-JIRBI2023-091).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Supplementary File

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RESEARCH ARTICLE

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Organ-dependent variability in mineral composition, phytochemicals and antioxidant potentials in *Polygonum equisetiforme* parts

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ABSTRACT

Polygonum equisetiforme is a perennial herbaceous plant thriving in the arid regions of Tunisia and widely used in health care and self-medication. The objective of the current study was to investigate the distribution of minerals, phenolic compounds, and antioxidant potentials in various plant parts including the fruit, stem, leaf, and root. The mineral composition was determined using flame atomic absorption spectrometry. The phenolic content of the samples was investigated using colorimetric assays and identified and quantified using HPLC-ESI/MS. The study found that the different parts of *P. equisetiforme* contain significant amounts of essential minerals such as sodium, potassium, calcium, magnesium, copper, zinc, and iron. The leaf and root extracts had high amounts of polyphenols, flavonoids, and tannins. Through LC-ESI-MS analysis, eleven flavonoids and eight phenolic acids were characterized. The most abundant compounds were gallic acid, quinic acid, catechin (+), and hyperoside. The findings suggest that different parts of *P. equisetiforme* are valuable sources of essential minerals and phenolic compounds, which can have potential health benefits.

1. Introduction

Polygonum equisetiforme is a perennial herbaceous plant that grows in the Tunisian dry lands and has long been used in traditional medicine as a remedy for several ailments. *P. equisetiforme*, also known as "Gourdhab," is a common wild plant that grows in drought and salt-affected areas (Boughalleb et al., 2020; Mahmoudi et al., 2018). It exhibits high phenotypic plasticity, adapting to different conditions in terms of seed morphology, germination, size, and reserve. The plant's timing of flower production is also plastic, allowing for faster reproduction and complete life history. It produces a large number of flowers to survive numerous interacting stresses and allocates resources to support vegetative growth over reproductive growth (Bidak et al., 2007). The plant is well adapted to climatic conditions, with a creeping port that helps absorb the maximum amount of moisture and reduces contact with open air to minimize transpiration (Mahmoudi et al., 2020b). Our previous work on this plant has shown

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that seed lipids and proteins are widely affected by the geographical area under different bioclimatic zones, from semi-arid to Saharan regions (Mahmoudi et al., 2018). The plant produces more secondary metabolites under arid conditions (Mahmoudi et al., 2019). Recently, it was found to be a salt-tolerant species able to survive at salinities up to 300 mM, with a root system that allows the plant to thrive in the Saharan and arid environments (Boughalleb et al., 2020). Additionally, the plant establishes a widespread distribution across the flooded soils as well as the active sand dunes of the Tunisian desert. It is considered one of the most palatable plants with high nutritional value for domesticated animals feeding which provides potent economic benefits to rural communities (Mouldi, 2014). It has been reported that the aerial parts of the plants as well as the seeds are very rich in polyphenols (Mahmoudi et al., 2018; Mahmoudi et al., 2019). *P. equisetiforme* is widely used in folk medicine for treating colds, coughs, and sore throats (Khafagi & Dewedar, 2000). Additionally, it is employed as a flavoring for tea (Facciola, 1990). Previous chemical characterizations of *P. equisetiforme* have revealed that this plant is very rich in phenolic acids and flavonoids (Hussein et al., 2017), with significant antioxidant, hepatoprotective, antibacterial, and antifungal effects (El-Toumy et al., 2017; El-Toumy et al., 2019). The essential oil of the plants showed higher free radical scavenging activities against DPPH and ABTS radicals (Abd-ElGawad et al., 2023). Water-soluble polysaccharides isolated from *P. equisetiforme* extracts showed considerable antioxidant potential and anti-tumoral effects against colon and breast cancer cells (Ibrahim & El-Hela, 2012).

Despite its potential health benefits, little is known about the chemical composition and biological properties of its different organs. Therefore, the present report aims to evaluate the mineral composition, polyphenol contents, as well as antioxidant activities in the fruit, leaf, stem, and root of the Tunisian *P. equisetiforme*. In particular, we focused on the importance of phenolic compounds, which have been shown to play a crucial role in the biological activity of many plants, to provide insight into the potential health benefits of this plant.

2. Materials and methods

2.1. Chemicals and Reagents

All chemicals were of analytical-reagents grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Loba Chimie Ltd (Mumbai, India).

2.2. Plant collection

Seedlings of *P. equisetiforme* were collected in July 2016 at the EL-Fja, Medenine region, Tunisia (33°28′55.20″ N, 10°39′23.78″ E, at 95 m a.s.l.) and were identified by Dr. Raoudha Abdellaoui, (Laboratory of Rangeland Ecosystems and Valorization of Spontaneous Plants and Associated Microorganisms, Arid Regions Institute, Medenine, Tunisia) and voucher specimens of fruit (PEF1601), stem (PES1602), leaf (PEL1603), and root (PER1604) were deposited in the seed bank of the Arid Regions Institute. The fruit, leaf, stem, and root of the plant were cleaned, separated, shade-dried, ground to a fine powder, and stored for subsequent analysis.

2.3. Mineral analysis

Firstly, an oven drying step was done, at 60 °C, for the leaf, stem, fruit, and root of *P. equisetiforme* until a constant weight. After that, the minerals were subjected to an acidic extraction process utilizing hydrochloric acid and nitric acid (Boughalleb et al., 2021). The mineral composition in the different organs of the plant was determined using flame atomic absorption spectroscopy (Shimadzu AA-6800) as detailed in Maher et al. (2023).

2.4. Determination of phytochemical contents

2.4.1. Preparation of methanolic extracts

The fine powders of the different plant parts of *P. equisetiforme* were extracted by simple maceration in hydromethanolic solution 80% (v/v) in a light-protected flask (at 40 °C for 24 h), the macerate was then subjected to centrifugation at 4500 rpm for 15 min. Finally, the supernatant was filtered through a 0.2 µm syringe filter and stored at –20 °C for subsequent analysis (Mahmoudi et al., 2021b).

2.4.2. Total phenolic content

To evaluate the polyphenols in *P. equisetiforme* organs, the Folin-Ciocalteu method was adopted (Dewanto et al., 2002). A 125 µl of sample extract was added to 125 µl of Folin-Ciocalteu reagent and 1250 ml of Na_2CO_3 (7%). The resultant mixture was subsequently diluted with deionized water until the final volume reached 3 ml. The absorbance was read at 760 nm after an incubation away from the light of 90 min at room temperature. The polyphenol contents in the plant parts were expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE/g DW) (Maher et al., 2023).

2.4.3. Total flavonoid content

The amount of flavonoids in *P. equisetiforme* parts was evaluated according to (Dewanto et al., 2002; Helrich, 1990). In a 5 ml flask, 100 µl of plant part extract samples were mixed with 75 µl of 7% $NaNO_2$ and 150 µl of 10% aluminum chloride hexahydrate. After that, a volume of 500 µl of 1 M NaOH was added, and deionized water was added to the resulting mixture to obtain a final volume of 3 ml. Absorbance was read at 510 nm versus a blank. The concentration of flavonoids is expressed as quercetin equivalents per gram of dry weight (mg QE/g DW) using the standard curve (Maher et al., 2023).

2.4.4. Condensed tannin content

The level of tannins in the plant organs was measured based on the vanillin-sulfuric acid colorimetric reaction (Broadhurst & Jones, 1978). A volume of 4% vanillin solution (1.5 ml) and concentrated sulfuric acid (750 µl) were added to a tube containing 25 µL of plant extract. Absorbance was determined at 500 nm versus a blank after incubation at 15 min in the dark. The condensed tannin content is expressed as mg catechin (CE) equivalents (mg CE/g DW) by utilizing the standard curve prepared from authentic catechin.

2.4.5. Analysis by high-performance liquid chromatography coupled with electrospray ionization–mass spectrometry

The LC-MS system consists of two LC-20ADXR pumps for mobile phase delivery, SIL-20AXR autosampler for automated sample injection, SCL-10A system controller serves as the central control unit, CTO-20 AC column oven, electrospray ionization source for sample ionization, and DGU-20AS degasser enhance separation efficiency. The MS was operated in negative mode electrospray ionization (ESI). The experiment involves the use of two mobile phases, A and B, in linear gradient chromatography. Mobile phase A comprises of water with formic acid (0.1%), while mobile phase B

consists of water with formic acid (0.1%) and methanol with formic acid. The linear gradient involves a stepwise increase in the proportion of mobile phase B in the following sequence: from 10 to 20% (0-14 minutes), from 20 to 55% (14-27 minutes), from 55 to 100% (27-37 minutes), 100% (37-45 minutes), and a decrease to 10% (45-50 minutes). The characterization of the polyphenol compounds was conducted using a comparative approach, whereby the MS spectra as well as the retention times of the detected compounds were matched against those of reference standards and the concentrations of the identified compounds were expressed as µg/g DW (Mahmoudi et al., 2020a; Mahmoudi et al., 2021a).

2.5. Determination of antioxidant potential

2.5.1 Total antioxidant activity

The total antioxidant potential of *P. equisetiforme* parts was investigated according to the phosphomolybdenum assay (Prieto et al., 1999). In brief, 200 µl of samples were added to 2 ml of reagent solution composed of sulfuric acid (0.6 M), sodium phosphate (28 mM), and ammonium molybdate (4 mM). The mixture was incubated for 90 minutes at 95 °C and the absorbance was determined at 700 nm versus a blank. Results are expressed as equivalents of gallic acid (mg GAE/g DW) (Maher et al., 2023).

2.5.2. DPPH anti-radical activity

The antiradical potential effect of the different plant parts was evaluated against the DPPH free radical according to Sánchez-Moreno et al. (1998) as detailed in Maher et al. (2023).

2.5.3. Reducing power potential

The reducing power potential of the plant part extracts was evaluated using the procedure outlined by Oyaizu (1986). A volume (2.5 ml) of phosphate buffer (0.2 M, pH = 6.6) and potassium ferricyanide (1%) were mixed with 1 ml of plant extracts and incubated for 20 minutes at 50 °C, After the incubation step (20 min at 50 °C), 2.5 ml of TCA (10%) was added to the mixture. After that, the solution was centrifuged at 13.000 rpm for 10 min, and 2.5 ml of the supernatant was mixed with an equal volume of distilled water and 0.5 ml of $FeCl_3$ solution (0.1%). The DO was read at 700 nm. The reducing potential in the extracts was expressed as EC_{50} values (mg/ml) which correspond to the concentration at which ferrous ions were chelated by 50% (Maher et al., 2023).

2.6. Statistical analysis

The statistical analysis was carried out using IBM SPSS statistical software (version 20.0, IBM Corp., Armonk, NY, USA). The data were presented as means ± standard deviation (SD) from three replicates and subjected to analysis of variance (ANOVA). To compare the means, Duncan's post-hoc tests were performed. Linear regression analysis was employed to determine the EC_{50} values for the reducing power assays. The significance level was set at 5% to determine the differences between means.

3. Results and discussion

3.1. Variations in mineral composition

In the current study, the macro and microelement contents in the leaf, stem, fruit, and root of *P. equisetiforme* were quantified using flame atomic absorption spectrometry (FAAS). As shown in Table 1, the stem and fruit parts contained higher amounts of Na, Ca, and Mg compared to the leaf and root. While leaf and stem contained higher K levels (6.59 and 6.08 mg/kg), followed by fruit (4.87 mg/kg) and root (2.81 mg/kg). It was shown that Cu and Fe were higher in the root part with respective values of 0.22 and 0.65 mg/kg. The stem has the highest Zn content (0.44 mg/kg) compared to the leaf and root parts (0.36 and 0.22 mg/kg). However, the studied microelements were not detected in the fruit. The present findings corroborate our previous study on Tunisian *Polygonum* species, which highlighted significant differences in mineral composition among the leaves, stems, and roots of *P. maritimum* and *P. aviculare* across various plant organs. Among *P. maritimum* parts, the leaf had higher Na, K, Ca, and Mg levels with respective values of 14.2, 9.5, 12.5, and 5.8 mg/Kg. However, the root part possessed a higher concentration of Fe. Additionally, in *P. aviculare* parts, the stem showed higher mineral amounts compared to the other organs (Mahmoudi et al., 2021b). These contents were similar to the value that had been previously recorded in several Polygonaceae i.e. *Fagopyrum tataricum* (Huang et al., 2014) and *Persicaria tinctoria* (Park et al., 2016). Moreover, Corlett et al. (2002) recorded higher amounts of the mentioned minerals in the aerial parts of other *Polygonum* species including *P. odoratum* and *P. runcinatum*.

Table 1. Mineral element composition *P. equisetiforme* parts (mg/kg dry matter)

	Na	K	Ca	Mg	Cu	Zn	Fe
Fruit	8.62 ± 0.7^{a}	4.87 ± 1.47^{b}	7.69 ± 3.44^{b}	2.6 ± 0.58^{a}	-	-	-
Leaf	4.626 ± 0.3^{c}	6.599 ± 0.04^{a}	4.552 ± 0.04^{d}	1.22 ± 0.09^{d}	0.104 ± 0.06^{c}	0.364 ± 0.006^{b}	0.43 ± 0.02^{b}
Stems	8.72 ± 0.91^{a}	6.08 ± 0.06^{a}	8.37 ± 0.68^{a}	2.12 ± 0.1^{b}	0.18 ± 0.06^{b}	0.44 ± 0.11^{a}	0.28 ± 0.02^{c}
Root	7.76 ± 0.74^{b}	2.81 ± 0.05^{c}	7.0 ± 0.08^{c}	1.93 ± 0.12^{c}	0.22 ± 0.04^{a}	0.26 ± 0^{c}	10.65 ± 0.29^{a}

Data expressed as means ± standard deviation ($n = 3$). The different lower-case letters in the same row indicate significantly different values ($p < 0.05$)

Many research studies have established a significant correlation between the pharmacological properties of plants and their mineral composition. Additionally, several minerals are crucial components for various human physiological processes in the human body. Mineral elements play a vital role in various biological processes, including the formation of macromolecules (i.e. proteins, nucleic acids, and carbohydrates). The chemical characteristics of these elements significantly impact the structure as well as the function of macromolecules, which are essential for the proper functioning of living organisms (Kolasani et al., 2011).

For instance, the mineral element Mg is an essential cofactor for many enzymes that are involved in carbohydrate metabolism. Mg ions help stabilize the structure of enzymes, which enables them to function efficiently in the catalysis of biochemical reactions involved in the breakdown and synthesis of carbohydrates. Similarly, Ca ions are essential for the proper functioning of enzymes involved in blood clotting and muscle contraction. Mineral elements are also involved in the regulation of enzyme activity through the modulation of protein structure and function. Additionally, iron is indispensable as enzymes that participate in cellular respiration, which is the process by which cells convert glucose into energy (Kanjaksha, 2006). Furthermore, mineral elements are also involved in the formation and maintenance of the structure of carbohydrates, which are macromolecules that play crucial roles in energy storage, structural support, and cell recognition (Kolasani et

al., 2011). The findings of this study highlight the importance of *P. equisetiforme* parts as a potential source of essential minerals for human consumption and medicinal purposes.

3.2. Variations in phytochemical contents

Polyphenols are a large and diverse group of naturally existing organic compounds and abundantly present in numerous plant-derived food sources including fruits, vegetables, whole grains, seeds, and tea. They are characterized by several phenol groups and possess a wide range of chemical structures. The beneficial health effects of these components are well documented. Polyphenols are known to possess antioxidant, anti-inflammatory, and anticancer properties, and may help to protect against a range of chronic diseases. Common polyphenols include flavonoids, phenolic acids, stilbenes, and lignans, and their levels can vary widely depending on factors such as the type of plant, the growing conditions, and the processing and preparation methods used. The different plant extracts of *P. equisetiforme* are rich in polyphenols, flavonoids, and condensed tannins. The levels of these compounds vary among different parts of the plant. The highest concentration of polyphenols was found in the leaves (51.75 mg GAE/g DW), followed by the root with 48.6 mg GAE/g DW, stem with 40.15 mg/g GAE/g DW, and fruit with 14.55 mg GAE/g DW (Figure 1). Similarly, the highest concentration of flavonoids was found in the leaves with a content of 62.75 mg quercetin equivalents (QE) per gram of DW, followed by the root with 59.55 mg QE/g DW, stem with 55.95 mg mg QE/g DW, and fruit with 21.05 mg mg QE/g DW. In terms of condensed tannins, the root contains the highest levels at 15.1 mg/g, followed by the stem and leaf, with the fruit having the lowest levels. The amounts of polyphenols, flavonoids, and tannins in different populations of Tunisian *P. equisetiforme* from distinct bioclimatic zones, namely the Saharan, arid, and semi-arid areas, were found to be in the range of 31-113 mg GAE/ g DW, 29-130 mg QRE/g DW and 8-33 mg CTE/g DW, respectively (Mahmoudi et al., 2018). Compared to other Tunisian *Polygonum* species, the halophyte sea knotgrass (*P. maritimum*) exhibited higher TPC levels in its stem, leaf, and root parts. In contrast, knotgrass (*P. aviculare*) exhibited lower levels which ranged between 14.1 mg GAE/g DW (in leaves) and 31.90 mg GAE/g DW (in stems). Additionally, the stems of both species possessed higher TFC amounts compared to the other plant parts while the condensed tannins were higher in the roots (Mahmoudi et al., 2021b). Furthermore, these contents are consistent with those measured in other Mediterranean *Polygonum* sp. (El-Haci et al., 2013; Maria Joao Rodrigues et al., 2019a; Maria Joao Rodrigues et al., 2019b). These findings indicate that the leaves, roots, and stems of *P. equisetiforme* are valuable sources of polyphenols, flavonoids, and condensed tannins that may have potential health benefits for humans.

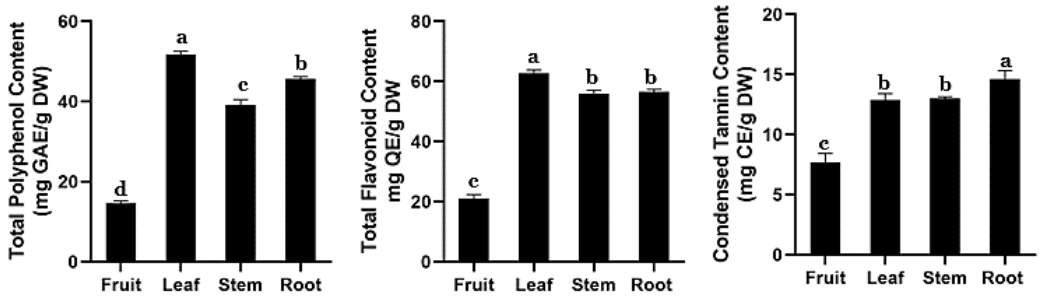

Figure 1. Total polyphenol, total flavonoid, and condensed tannin contents in different parts of *P. equisetiforme*
The different lower-case letters indicate significant ($p < 0.05$) differences. Data expressed as means ± SD ($n = 3$).

3.3 Variations of polyphenolic compounds

Polyphenols in the fruit, leaf, stem, and root of *P. equisetiforme* were identified through an LC-ESI/MS analysis as summarized in Table 2. Eight compounds were characterized and the most abundant component in all plant parts is quinic acid, with the highest concentration in the fruit (259.81 µg/g) and the lowest in the leaf (26.61 µg/g). Gallic acid is also present in all plant parts, with the highest concentration in the fruit (48.34 µg/g) and the lowest in the leaf (8.34 µg/g). Among the other phenolic acids, the concentrations vary depending on the plant part. For example, 4-*O*-caffeoylquinic acid is found only in the fruit, stems, and root, with the highest concentration in the stems (44.89 µg/g). Caffeic acid is not identified in the stems, but is present in the fruit, leaf, and root, with the highest concentration in the fruit (0.82 µg/g). Syringic acid is found in all plant parts, with the highest concentration in the fruit (2.28 µg/g) and the lowest in the root (1.13 µg/g). *trans*-Ferulic and *p*-coumaric acids are also present in all plant parts, with the highest concentrations in the fruit (6.14 µg/g and 1.85 µg/g, respectively) and the lowest in the root (2.8 µg/g and 2.48 µg/g, respectively). Overall, these results suggest that *P. equisetiforme* contains a diverse array of phenolic acids in its different plant parts and that the identified compounds exhibit high variation based on the plant parts. Furthermore, in this study, the presence and quantity of eleven flavonoid compounds were identified in the fruit, leaf, stems, and root of *P. equisetiforme* using LC-MS. Catechin (+) was found to be the most abundant flavonoid compound in all four parts of the plant, with the highest concentration in the stems (1706.35 µg/g) and lowest in the leaf (4.38 µg/g). Epicatechin and hyperoside were also present in all parts of the plant, with the highest concentrations found in the root and stems, respectively. Rutin was identified in all parts of the plant, but its concentration was highest in the fruit (0.82 µg/g). Quercetin was identified in the leaf with the highest amounts among all the plant parts, measuring 22.37 µg/g. However, detectable levels of quercetin were also seen in the root and stem, although at relatively lower concentrations. Both naringin and quercetin were present in all parts of the plant but in relatively lower amounts. Naringenin was identified in the fruit, stems, and roots but was not found in the leaf. Cirsiliol was present in all parts of the plant, with the highest concentration found in the root (24.63 µg/g). The compounds that were identified have been previously documented in both the seeds and the shoots of the plant. They are significantly affected by environmental fluctuations in different bioclimatic conditions across Tunisia (Mahmoudi et al., 2018; Mahmoudi et al., 2019). Furthermore, various phenolic acids and flavonoid compounds have been identified in other *Polygonum* species, with varying amounts found in different parts of the plant, including *P. maritimum*, *P. aviculare* (Mahmoudi et al., 2021a; Mahmoudi et al., 2021b) and *P. cognatum* (Gümüşçü et al., 2022).

The identified compounds have been documented to possess diverse biological activities. For instance, quinic acid and gallic acid are widely distributed in nature and are found in many fruits, vegetables, and plants. These compounds have been shown to have anti-inflammatory, antioxidant, antimicrobial, and antifungal effects (Cao & Prior, 1998). Furthermore, these compounds have been shown to exhibit high gastrointestinal and neuroprotective effects which may have potential benefits for the management of gastrointestinal disorders and improving cognitive function in animal models of neurodegenerative diseases (Jang et al., 2017; Song et al., 2019). Also, catechin has been shown to possess several biological activities, such as antioxidant, cardiovascular, and anti-cancer effects (Bernatoniene & Kopustinskiene, 2018; Vickers, 2017).

It is well known that phenolic content, as secondary metabolites of plants, is widely influenced by environmental factors such as salinity and aridity. Our previous study demonstrated that antioxidant activities and phytochemical contents, including phenolic acids and flavonoid compounds, in *P. equisetiforme* extracts increased with salinity. Specifically, an increase in phenolic acids, particularly quinic, gallic, and protocatechuic acid, was observed, followed by quercetin-3-*O*-galactoside, catechin, and epicatechin (Boughalleb et al., 2020). Additionally, these compounds were found to increase with aridity in the Tunisian Saharan climatic range (Mahmoudi et al., 2019).

These results provide valuable information on the distribution patterns of polyphenolic components in different *P. equisetiforme* parts, which could have important implications for the use of this plant in folk medicine and the development of new therapeutics.

3.4. Variations in the antioxidant potential

The results indicate that *P. equisetiforme* contains antioxidants in all its parts – fruit, leaf, stem, and root. The antioxidant activities were measured through three in vitro assays: total antioxidant capacity (TAC), DPPH, and reducing power assays. The TAC values were found to be in the range of 11.85-24.55 mg GAE/g DW, with the highest potential found in the stem **(Figure 2)**. The DPPH radical scavenging activities ranged between 11.58 to 20.6 mg TRE/g DW, with the highest value again found in the stem. The reducing power values, measured by EC_{50}, ranged from 40.6 to 58.5 µg/ml, with the highest reducing power found in the stem extract.

Table 2. Phenolic compounds identified by LC-ESI/MS in the different parts of *P. equisetiforme*

	RT	m/z	Fruit	Leaf	Stem	Root
Quinic acid	2.065	191	259.81 ± 2.14^{a}	26.61 ± 0.46^{d}	91.31 ± 0.16^{c}	191.63 ± 10.61^{b}
Gallic acid	4.056	169	48.34 ± 0.8^{a}	8.34 ± 0.16^{d}	17.06 ± 0.04^{c}	45.43 ± 0^{b}
Protocatechuic acid	7.072	153	7.36 ± 0.2^{a}	0.84 ± 0.02^{d}	4.57 ± 0.1^{c}	6.33 ± 0.22^{b}
Catechin (+)	11.439	289	28.95 ± 0.59^{c}	4.38 ± 0.16^{d}	44.3 ± 0.2^{b}	1706.35 ± 39.23^{a}
4-*O*-Caffeoylquinic acid	12.198	353	0.49 ± 0.01^{c}	nd	0.1 ± 0^{b}	44.89 ± 0.1^{a}
Caffeic acid	14.841	179	0.82 ± 0.01^{b}	0.65 ± 0.14^{c}	nd	2.56 ± 0.08^{a}
Syringic acid	16.506	197	2.28 ± 0.03^{a}	1.37 ± 0.03^{c}	1.87 ± 0.02^{b}	1.13 ± 0.03^{c}
Epicatechin	16.856	289	2.69 ± 0.03^{d}	3.92 ± 0.02^{c}	11.77 ± 0.04^{b}	42.69 ± 1.06^{a}
p-Coumaric acid	21.185	163	6.14 ± 0.2^{a}	5.59 ± 0.06^{b}	3.38 ± 0.02^{c}	2.8 ± 0.05^{d}
trans-Ferulic acid	23.451	193	1.85 ± 0.04^{b}	2.36 ± 0.05^{a}	1.29 ± 0.07^{c}	2.48 ± 0.04^{a}
Hyperoside	24.634	463	15.83 ± 0.65^{c}	41.63 ± 0.21^{a}	20.1 ± 0.13^{b}	4.44 ± 0.96^{d}
Rutin	24.52	609	0.82 ± 0.03^{b}	0.36 ± 0.02^{c}	0.21 ± 0.01^{c}	1.39 ± 0.15^{a}
Quercetrin	27.216	447	0.93 ± 0.02^{d}	22.37 ± 0.59^{a}	1.47 ± 0.02^{c}	11.09 ± 0.12^{b}
Naringin	26.516	579	0.05 ± 0^{d}	1.62 ± 0.01^{b}	0.31 ± 0^{c}	1.91 ± 0.02^{a}
Quercetin	32.67	301	3.68 ± 0.12^{b}	6.53 ± 0.4^{a}	3.27 ± 0.05^{b}	1.06 ± 0.02^{c}
Naringenin	34.37	271	0.33 ± 0.01^{b}	nd	0.23 ± 0.01^{b}	0.79 ± 0.05^{a}
Luteolin	36.95	285	nd	0.38 ± 0.13^{a}	nd	nd
Cirsiliol	35.811	329	2.53 ± 0.05^{c}	2.06 ± 0.11^{c}	4.27 ± 0.07^{b}	24.63 ± 0.84^{a}
Cirsilineol	38.952	343	nd	0.12 ± 0.02^{a}	0.09 ± 0.01^{b}	nd

Data expressed as means ± standard deviation (n = 3). nd: not detected. The different lower-case letters in the same row indicate significantly different values ($p <$ 0.05), nd: Not determined

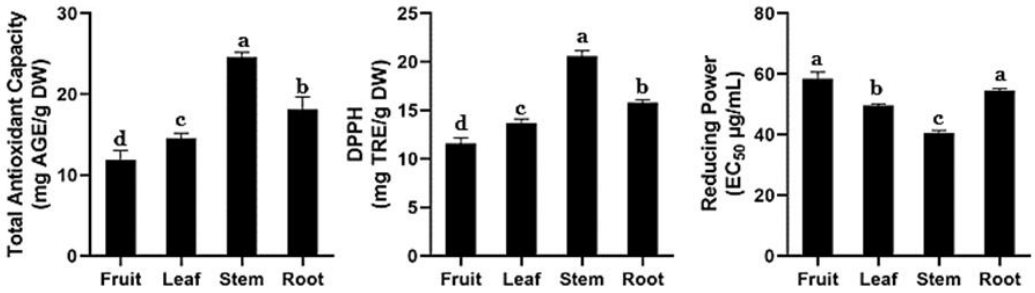

Figure 2. Total antioxidant capacity, DPPH radical scavenging activity, and reducing power in *P. equisetiforme* parts
The different lower-case letters indicate significant ($p <$ 0.05) differences. Data expressed as means ± SD (n = 3).

Our findings corroborate the results of many studies that have reported high antioxidant potential in several other species of *Polygonum* (El-Haci et al., 2013; Mahmoudi et al., 2018; Mahmoudi et al., 2019; Mahmoudi et al., 2021b; Maria João Rodrigues et al., 2016). In many studies investigating the antioxidant potential of plant extracts, polyphenols have been identified as the major contributors to the observed effects. This is likely a result of the remarkable ability of polyphenols to neutralize free radicals and chelate transition metals, both of which are important mechanisms of antioxidant activity (Yang et al., 2004). Overall, the study suggests that this plant has significant antioxidant activity in all of its parts, with the stem extract exhibiting the highest potential for antioxidant activity. These results may have implications for the use of *P. equisetiforme* as a source of natural bioactive compounds for

therapeutic or functional food applications, although further research is required to provide a more comprehensive understanding of the specific bioactive components responsible for these observed effects.

4. Conclusions

In conclusion, the analysis of mineral and polyphenolic contents of different parts of *P. equisetiforme* demonstrated that this plant has the potential to be a sustainable source of minerals and polyphenols. Specifically, stem and fruit parts were found to contain higher amounts of certain minerals such as Na, Ca, and Mg, while leaf and stem contained higher levels of K. Cu and Fe were found to be higher in the root part, while Zn was highest in the stem. Moreover, the phytochemicals varied among different parts of the plant with the highest concentration found in the leaves. These findings suggest that incorporating *P. equisetiforme* into sustainable agricultural practices in Tunisian arid regions may provide a promising way to promote both human health and environmental sustainability. Additionally, the antioxidant, anti-inflammatory, and anticancer properties of the plant's polyphenols may provide an important means to combat a range of chronic diseases.

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Conflict of interest

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

Statement of ethics

In this study, no method requiring the permission of the “Ethics Committee” was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Supplementary File

None.

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RESEARCH ARTICLE

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Toxicity study and ameliorative effects of the aqueous leaf extract of *Lecanoidiscus cupanioides* Planch (ex. Benth) on the stress-induced ulcer

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ABSTRACT

Lecaniodiscus cupanioides Planch (ex. Benth) is effective in treating burns, fevers, and inflammatory conditions. This study investigates the toxicity effects of the aqueous leaf extract (ALE) of *L. cupanioides* and its effect on stress-induced ulcers in animal models. The plant was collected and a 1:30 (g/ml) plant powder/solvent ratio was extracted using an ultrasonic bath at 50 °C for 45 min. Up-and-down procedure was used for acute toxicity. During subacute toxicity testing, a total of 20 mice were divided into four groups of five animals each. While group 1 served as control, groups 2, 3, and 4 received 250, 500, and 1000 mg/kg of the extract daily for 21 days. On day 22nd, animals were sacrificed and samples were collected for hematology, biochemical, and histological analyses. In the stress-induced ulcer activity, male albino mice were randomly separated into 5 groups of 5 animals, treated with the test drug, and then dissected after being stressed using the water immersion model. LD_{50} was > 5000 mg/kg, and in biochemical examination, there was a significant decrease in the ALP level at medium and high doses (p value < 0.05) and non-significant alterations in the values of urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), body weight and hematology parameters (p > 0.05). The histology results showed no evidence of liver and kidney toxicity. In conclusion, aqueous leaf extract of *L. cupanioides* was safe following a single dose (at 5000 mg/kg) and following repeated doses for 21 days (at 250, 500, and 1000 mg/kg). This study demonstrated that the extract had a significant effect on stress-induced ulcers at various dose levels when compared to the control (native, negative control, and omeprazole). The animals treated with 200mg/kg and 400mg/kg of the extract showed a significant increase in ulcer score, ulcer index, ulcer severity, and total acidity (p < 0.05). The ulcer protection ability of the aqueous plant extract was dose-dependent.

1. Introduction

Medicinal plants have been employed across the world to treat health problems and diseases. These plants are becoming more commonly used in health care delivery, particularly in resource-constrained situations. Approximately 80% of the global population relies on traditional medicine for basic health care (Ekor, 2014; Ugwah et al., 2013). Although the employment of these plants has demonstrated significant potential with considerable worldwide demand, there are still questions regarding not just their use but also their safety (Ifeoma & Oluwakanyinsola, 2013). It is generally known that using medicinal herbs without first assessing their efficiency and safety profile can lead to harmful consequences for several organs. The liver and kidneys are the primary targets since they are involved in the metabolism

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and excretion of toxic substances (Hodges & Minich, 2015). Before being used on humans, newly created medications must undergo extensive toxicity testing (Arome & Chinedu, 2013), safety testing of these chemicals, pharmaceuticals, food and food additives, cosmetics, and industrial items is essential (Erhirhie et al., 2018). Since safety remains a key concern with the use of medicinal plants, it is imperative to undertake toxicity studies on them to determine their risk profile.

Peptic ulcer disease involves both gastric and duodenal ulcers, which cause death over the years (Malfertheiner et al., 2009). According to studies, peptic ulcer remains one of the main pathologies in about 10% of the human population (Sonnenberg, 2013). About 80–90% of patients with duodenal ulcers have *H. pylori* infection, and the same goes for 70–90% of patients with gastric ulcers (Thomas et al., 2005).

Major causes of peptic ulcers include *H. pylori* bacteria, emotional stress, NSAIDs, alcohol abuse, and smoking. Associated signs and symptoms include abdominal discomfort and nausea, bloating and abdominal fullness, dark or black stools, water brash, hematemesis, melena, and rarely acute peritonitis, among others (Iqbal et al., 2012; Ramasubramaniaraja & Babu, 2011; Roy, 2016). The majority of peptic ulcer cases are due to *H. pylori* infection. Antiulcer drugs include antagonists of the histamine H_2 receptor antagonists (such as ranitidine), and inhibitors of proton pump (such as omeprazole), among others (Jain, 2016).

However, problems, such as nephrotoxicity, gynecomastia, thrombocytopenia, hepatotoxicity, and impotence (Chan & Leung, 2002; Sheen & Triadafilopoulos, 2011), which are associated with synthetic drugs have awakened the research on medicinal plants, which are natural and also described as an important source of modern medications (Albarri et al., 2017; AlMatar et al., 2018; Dharmani et al., 2005; Sharma et al., 2011).

Lecaniodiscus cupanioides Planch (ex Benth) is a tropical plant with a large geographic range in Asia and Africa. It is a member of the Sapindaceae family and is known in Nigeria by several names, including Ukpo (Igbo), Utantan (Edo), Kafi-nama-zaki (Hausa), and Akika (Yoruba). Traditionally, it is effective in treating burns, fevers, measles, abdominal swelling brought on by liver abscesses, and wounds and sores (Nafiu et al., 2013), as well as other ailments including cancer, measles, jaundice, diabetes, sexual dysfunction, typhoid, wounds, skin infections, and galactagogues (Ojo & Ndinteh, 2023).

Due to their higher cultural acceptance, lower side effects (Sam, 2019), lower costs, and lower availability (Alharbi et al., 2017), herbal and traditional medicine have gained ground as alternate remedies to several diseases.

As part of drug discovery, newly created medications must undergo extensive toxicity testing (Arome & Chinedu, 2013; Erhirhie et al., 2018). Though most medicinal plants are assumed to be safe, safety evaluation is not out of place (Olaniyan et al., 2016).

Interestingly, herbal remedies are becoming recognized for the management and treatment of peptic ulcers, as a viable alternative to synthetic pharmaceuticals that are sold commercially (Keshavarzi et al., 2014).

In this present study, *L. cupanioides* was investigated for its toxicity and anti-ulcer effects using the water immersion model in male albino mice.

2. Materials and methods

2.1. Plant material

The fresh healthy leaves of *L. cupanoides* were collected in July 2022 from Orba, which is situated in Nsukka, Enugu, Nigeria, and its geographical coordinates are 6° 51' 0" North, 7° 27' 0" East. Proper identification and authentication were done by a taxonomist, Mr. Felix Nwafor in the Department of Pharmacognosy and Environmental Science, University of Nigeria, Nsukka with voucher reference number of UNH/04/0330C. The fresh leaves weighing about 3 kg were cleaned and air/shade dried at room temperature. About 2 kg of dry sample was pulverized.

2.2. Preparation of the aqueous leaf extract

2.2.1. Ultrasonic-assisted extraction

Preliminary analysis to select the appropriate solvent for the extraction was done using ethyl acetate, dichloromethane, methanol, and water. These solvents were selected based on documented evidence of their ability to extract phenolic compounds. A 1:30 (g/ml) herbal powder/solvent mixture was extracted using an ultrasonic bath at 50 °C for 45 min. The extract was filtered and the filtrate was concentrated using a freeze dryer (Lyoquest – 55 plus, Germany). The total phenolic contents and yields of the extracts obtained were determined and the solvent with the highest ability to extract phenolic compounds (water) was selected for the next phase of extraction. The aqueous filtrates (extracts) were first frozen which resulted in the formation of ice crystals. The frozen filtrates in glass flasks (75 ml each) were connected through a manifold to the freeze dryer. The manifold allowed 8 samples to be freeze-dried at the same time. The manifold was opened to a headspace with a partial pressure of water vapor below the equilibrium vapor pressure of ice. This very low partial pressure was achieved using a condenser at -55°C – which caused the sublimation of the ice crystals (primary sublimation) and also the desorption of non-crystalline water present within the extract (secondary drying). The vacuum was maintained in the freeze-drying chamber using a vacuum pump to remove air from the chamber to increase the rate of transfer of water vapor from the extract to the condenser. The freeze-drying process continued uninterrupted till the samples were all dried which was indicated by an increase in the extract temperature to approach the value of the shelf temperature. Additional drying time (secondary drying) was allowed to ensure that non-crystalline water had been completely removed (Adami et al., 2020).

2.3. Phytochemical analysis procedure

The phytochemical analysis of the leaf extract and fractions was carried out using standard methods (Harborne, 1998; Sofowora, 1993; Trease, 2002).

2.4 Effect of solvent on total phenolic content of aqueous leaf extract

The effect of solvent on total phenolic content of aqueous leaf extract (ALE) of *L. cupanoides* was determined using the method described by Kim et al. (2003). One milliliter of the extracts (100 µg/ml) was mixed with 0.2 ml of Folin-Ciocalteu's phenol reagent. After 5 minutes, 1 ml of 7.6% Na_2CO_3 solution was added to the mixture followed by the addition of 2 ml of distilled water. The mixture (in duplicate) was incubated at 40 °C for 30 minutes, after which the absorbance was read at 760 nm using a UV-VIS spectrophotometer against a blank (containing every other

component of the mixture except the sample). The total phenolic content was estimated from the calibrated curve which was made by preparing gallic acid solution and expressed as milligrams of gallic acid equivalent (GAE) per gram of the extracts.

2.5. Ethical approval

The ethical review committee of the College of Pharmaceutical Sciences at Chukwuemeka Odumegwu Ojukwu University gave the animal study ethical clearance and issued an ethical approval number, PHACOOU/AREC/2023/019.

2.6. Source of experimental animals

2.6.1. Source of experimental animals for toxicity study

Adult mice (body weight:25-40 g) were procured in the Faculty of Pharmaceutical Sciences, Chukwuemeka Odumegwu Ojukwu University, Igbariam, Anambra state. These animals were used in the toxicity experiments and were housed in the animal house of the same institution, at approximately 12h/12h of light/dark cycle and at 25 °C. Vital grow pelleted feed, and free water were given ad libitum. Oral feeding was carried out using a stainless-steel gavage cannula coated at the tip to prevent lesions to the upper abdominal area.

2.6.2. Source of experimental animals for stress-induced ulcer

Male Swiss Albino mice (25 – 30 g) were used for this study. All the animals were obtained from the Animal House of the Department of Pharmacology, Enugu State University of Science and Technology, Enugu State, Nigeria. Animals were allowed to acclimatize for one week before the commencement of the study. Food and water were provided ad libitum. All animal experiments were conducted in compliance with the NIH guide for the care and use of laboratory animals [National Institute of Health (NIH) (2011) Pub No: 85-23].

2.7. Acute toxicity study

The acute toxicity test was carried out using the up-and-down procedure described by (Erhirhie et al., 2018). Healthy mice were divided into two main groups. The first group served as the untreated healthy control group of mice which received distilled water (10 ml/kg), while the other group received a single dose of 5000 mg/kg of the plant extract dissolved in 10 ml per body weight. After a single oral administration of the extract, mice were observed for the first 4 hours, 24 hours, and once daily for 7 days for signs of toxicity and mortality.

2.8. Sub-acute toxicity studies

A total of 20 mice were grouped into five categories of six animals each of both sexes.

Group 1 served as the untreated healthy control and received distilled water daily, whereas groups 2, 3, and 4 received a low dose (250 mg/kg body weight), a medium dose (500 mg/kg body weight), and a high dose (1000 mg/kg body weight) of *L. cupanioides* leaf extracts, respectively, daily for 21 days.

Group 1: Distilled water (control)
Group 2: 250 mg/kg of *L. cupanioides* leaf extract
Group 3: 500 mg/kg of *L. cupanioides* leaf extract
Group 4: 1000 mg/kg of *L. cupanioides* leaf extract

The mice were fasted in the evening of the 21st day and were euthanized on the 22nd day with an overdose of chloroform. Blood was collected from the inferior vena cava into plain tubes, and allowed to clot, then centrifuged at 3500 rpm for 10 minutes. The serum obtained was used for the estimation of biochemical parameters. The whole blood collected was placed in EDTA tubes for the determination of hematology parameters. The liver and kidney tissues were excised and placed in 10% formal saline for histological study (Erhirhie et al., 2023).

2.8.1. Serum biochemistry

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase activity (ALP), blood urea nitrogen (BUN), and creatinine ranges were determined using the commercial assay kits (Erhirhie et al., 2023).

2.9. Measurement of body weight

Mice body weights were taken before administration and every week to observe variation in body weight.

2.10. Hematology analysis

Assay of hematological parameters wa carried out by using Midray BC-2800 Auto Hematology Analyzer (Udoudoh et al., 2020). A volume of 18 µl of the sample was presented to the analyzer for full blood count.

2.11. Histopathological examination

Following the conclusion of the examination, the kidney and liver of sacrificed mice were removed to look for lesions associated with toxicity such as hemorrhage, reduced glomerulus, and central vein. The removed organs were promptly preserved in 10% formalin solution and prepared for histological analysis (Luna, 1968).

2.12. Experimental design for anti-ulcer study

2.12.1. Animal grouping and treatment

Male albino mice were used for the study. They were randomly divided into 5 groups of 5 animals each.

Group 1: The native group was not subjected to ulcer induction.
Group 2: The group was subjected to ulcer induction and received 200 mg/kg of the extract
Group 3: The group was subjected to ulcer induction and received with 400 mg/kg of the extract
Group 4: The group was subjected to ulcer induction and received 20 mg/kg omeprazole as a standard antiulcer reference drug
Group 5: The group was subjected to ulcer induction and given distilled water as a vehicle control group

Treatments were done orally and experiments were performed at fixed times of the day between 9 a.m. and 1 p.m.

2.13. Water immersion stress-induced ulcer

Animals were pre-treated with test drugs for 7 days before the beginning of the experiment. The animals were fasted for 36 hours after the last day of pre-treatment with free access to water and were once more treated with the test drugs one hour before subjecting them to stress by free swimming. The animals were put into water (water depth 7 cm to avoid drowning) and allowed free

movement in the water for 6 h. After swimming, mice were removed, dried, sacrificed under ether anesthesia, dissected and their stomachs were removed (Parmar & Desai, 1993).

2.13.1 Measurement of gastric acid and pH

After dissection, the stomach of the mice was legated from its two ends; the pylorus and lower esophagus. A small incision was made for each fore stomach and the stomach contents were collected in centrifuge tubes. They were centrifuged at 3500 r/min for 15 min and the pH of the supernatant was determined using a digital pH meter. Subsequently, 1 ml of the gastric juice was withdrawn into a conical flask, and then 2 drops of phenolphthalein indicator were added and titrated against 0.01N NaOH until a permanent pink color was seen. The volume of 0.01N NaOH used was noted. The total acidity was expressed as mEq/l and calculated by the given formula (Abebaw et al., 2017).

2.14. Statistical analysis

Results were presented as mean ± standard deviation (n = 5). The comparison of mean values among groups was carried out using SPSS with one way analysis of variance (ANOVA) followed by post-hoc Dunnets's test. $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Phytochemical analysis

Phytochemical analysis of the aqueous leaf extract of *L. cupanoides* is shown in Table 1. *L. cupanoides* revealed the presence of all the tested phytochemicals. Phytochemical analysis of *L. cupanoides* revealed the presence of flavonoids as well as the phenolic constituents like tannins. Phenolic compounds have several clinical relevance. Polyphenols are beneficial against cancer, osteoporosis, cardiovascular diseases and diabetes, including peptic ulcers (Sumbul et al., 2011). Flavonoids are among the cytoprotective materials whose anti-ulcerogenic activity has been well established (Borrelli & Izzo, 2000). The presence of the aforementioned phytoconstituents could be attributed to the ability of *L. cupanoidesto* to suppress stress-induced ulcers.

Table 1. Preliminary phytochemical analysis of aqueous leaf extract (ALE)

Phytoconstituents	Methods	Aqueous extract
Alkaloids	Wagner test	+++
	Hager's test	+
	Drangendorff test	++
Flavonoids	Lead acetate test	++
	Zn.Hydrocholride test	+
Tannins & Phenols	$FeCl_3$ test	+
Saponins	Foamingtest	+++
Steroids & Triterpenoids	Salkowski test	+
Glycosides	Killer killani	+
	Leiberman Buchard's test	+
Carbohydrates	Molish test	++
Proteins	Biuret	++

3.2. Total phenolic content

As shown in Table 2, the effect of various solvents for the extraction of polyphenols present in *L. cupanioides* revealed that water had higher total phenolic content (TPC) than methanol, ethyl acetate, and DCM. This suggests that water was the best solvent for extracting phenolic compounds in the leaf of *L. cupanioides*. This is substantiated by the presence of various phytochemicals in the leaf extract as reported in Table 1. In line with other studies, most medicinal plants with higher total phenolic content were extracted with polar solvents (Alara et al., 2021).

Table 2. Effect of solvent on total phenolic content of the aqueous leaf extract (ALE) of *L. cupanioides*

Solvent	GAE/g extract
Ethyl acetate	90.50
DCM	53.84
Methanol	145.22
Water	316.61

3.3. Acute oral toxicity study

The oral toxicity result in mice revealed the LD_{50} value of *L. cupanioides* extract to be 5000 mg/kg or greater ($LD_{50} \geq 5.000$ mg/kg body weight) as it showed no mortality or any adverse effect (Table 3). This suggests that the aqueous leaf extract of *L. cupanioides* is considered to be safe or non-toxic at a human equivalent therapeutic dose. In line with this study, several medicinal plants, which are considered natural have estimated LD_{50} above 5000 mg/kg (Ebbo et al., 2020; Erhirhie et al., 2023). Usually, pre-clinical toxicity studies are used in safety investigations to identify any potential hazardous effects of any medicine (Nurudeen et al., 2021).

Table 3. Acute oral toxicity of *L. cupanioides* aqueous leaf extract (ALE)

Group	Treatment	Result
A	Control	Absence of toxicity and death
B	5000 mg/kg	Absence of toxicity and death

3.4 Effect of the L.cupanioides aqueous extract on weight change

When assessing the hazardous effects of medications, toxic chemicals, and therapies, body weight is an important parameter. One of the earliest crucial indicators of toxicity can be variations in weight. Weight index evaluation of animal growth is common practice in toxicological studies since it aids in interpreting compound-related effects (Erhirhie et al., 2023). In this study, there was no significant change in the weights of animals administered different doses of extract for 21 days compared to the control ($p > 0.05$) (Table 4). This suggests that the extract did not distort feed intake, nutrient absorption, and metabolism (Oloyede et al., 2020).

Table 4. Effect of the *L. cupanioides* aqueous extract on weight change

	Body weight (g)				
	Week 0	Week 1	Week 2	Week 3	Weight change (%)
Control	29.40 ± 1.92	29.00 ± 1.41	29.80 ± 1.91	32.60 ± 1.48	10.09 ± 2.26
Low dose	30.50 ± 1.55 ^{ns}	29.00 ± 1.22 ^{ns}	27.25 ± 1.25 ^{ns}	27.75 ± 1.43 ^{ns}	1.23 ± 9.47^{ns}
Medium dose	30.25 ± 1.38 ^{ns}	30.00 ± 0.40 ^{ns}	33.00 ± 3.34^{ns}	30.75 ± 1.43^{ns}	1.34 ± 5.22^{ns}
High dose	30.00 ± 1.22 ^{ns}	29.80 ± 1.19^{ns}	30.20 ± 1.14 ^{ns}	31.60 ± 1.30^{ns}	4.67 ± 4.87^{ns}

Values are expressed as mean ± SEM, n = 5, ns: $p > 0.05$: Not statistically significantly different from the control group. *$p < 0.05$: Statistically significantly different from the control group.

3.5. Assessment of serum biochemical parameters

Safety studies are accomplished by the implementation of general pre-clinical toxicity experiments to uncover the potential poisonous effects of any drug majorly in the liver and kidney of animals (Farzamfar et al., 2008).

Biochemical analysis showed no significant variations in urea, creatinine, aspartate transaminase (AST), and alanine transaminase (ALT) values among the different treatment groups compared to the control ($p > 0.05$) (Table 5). On the other hand, oral administration of aqueous extract of *L. cupanoides*, resulted in a significant reduction in alkaline phosphate (ALP) level at medium and high doses when compared to the control group ($p < 0.05$). AST, ALP, and ALT are considered as indicators of liver function, according to El Hilaly et al. (2004). Elevated levels of these enzymes in the serum act as a marker of hepatotoxicity (Harris, 2005). In this study, the level of AST at graded doses was not increased compared to that of the control; this is a clear indication that the extract may not be hepatotoxic. This is substantiated by a significant reduction in ALP levels in medium and high doses, which may serve as an indication of the extract's protection against liver damage. Similarly, urea and creatinine, which are biomarkers of kidney toxicity were not significantly altered when compared to the control group, suggesting that the extract is not toxic to the kidney. The absence of liver and kidney lesions in the histology results (Figures 1 and 2) strongly supports the absence of liver and kidney toxicity as revealed by the biochemical results (Table 5); this is an indication that repeated administration of the extract at concentrations of 250, 500 and 1000 mg/kg for three weeks may be safe.

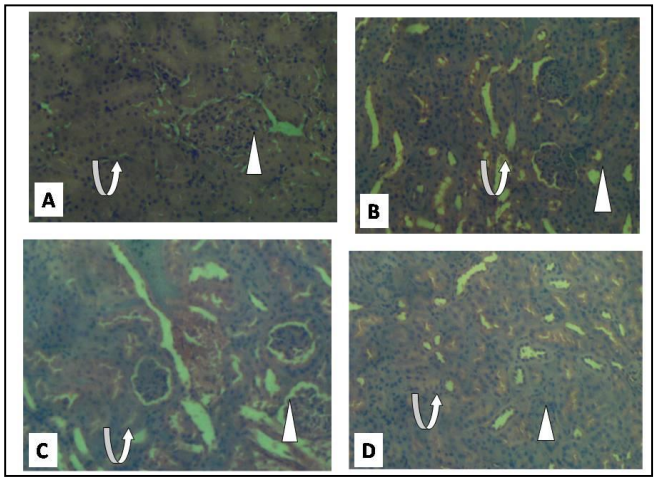

Figure 1. A, B, C, and D: Photomicrograph shows kidney tissue of albino rat with kidney histology consistent with normal morphology
The Renal capsules (arrowhead) and the tubules (curved arrow) are normal with no sign of injury (H&E, x400)

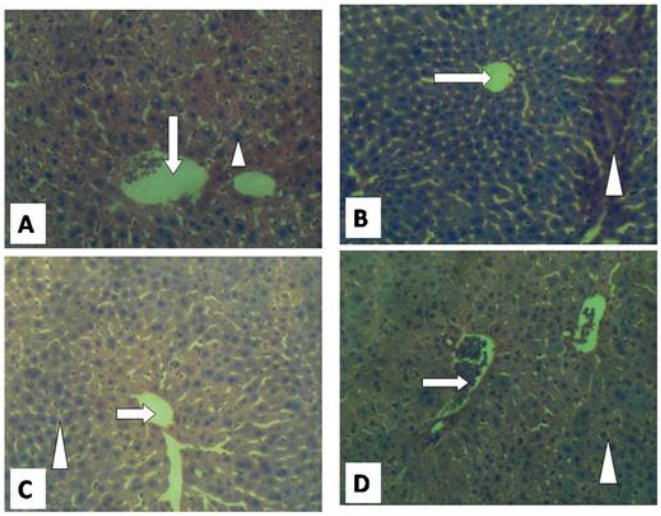

Figure 2. A, B, C, and D: Photomicrograph of liver tissue shows morphology consistent with normal liver histology
The hepatocytes (arrowhead) and central vein (arrow) are normal with no obvious sign of injury (H&E X 400).

Table 5. Effect of *L. cupanioides* aqueous leaf extract on serum biochemical parameters

	ALT (U/l)	AST (U/l)	ALP (IU/l)	UREA (mg/dl)	CREATININE (mg/dl)
Control	4.77 ± 1.19	131.74 ± 1.20	49.56 ± 11.67	37.67 ± 0.13	0.98 ± 0.13
Low dose	4.64 ± 0.66^{ns}	129.94 ± 1.31^{ns}	33.81 ± 10.28^{ns}	33.82 ± 5.60^{ns}	1.00 ± 0.20^{ns}
Medium dose	5.92 ± 1.37^{ns}	126.61 ± 2.06^{ns}	16.46 ± 4.87*	25.64 ± 5.20^{ns}	1.28 ± 0.19^{ns}
High dose	4.77 ± 0.74^{ns}	128.03 ± 1.07^{ns}	17.01 ± 4.72*	30.10 ± 5.99^{ns}	1.2 ± 0.19^{ns}

Values are expressed as mean ± SEM; n = 5, ns: $p > 0.05$: Not statistically significantly different from the control group. * $p < 0.05$: Statistically significantly different from the control group.

3.6. Assessment of hematological parameters

3.6.1 Effect of L. cupanioides aqueous leaf extract on hemoglobin, white blood cell, mean platelet volume, platelet distribution width, platelet crit, and platelet count

Hematology assessment serves as information regarding the safety profile of a substance on blood physiology (Nurudeen et al., 2021). Changes in blood parameters could be a result of alterations in cellular integrity, membrane permeability, metabolism, or even exposure to harmful compounds (Oloyede et al., 2020). The effect of the aqueous leaf extract on all the hematological parameters showed no significant effect in extract treated group ($p > 0.05$) when compared to the control (Figure 3). This suggests that the extract may not alter the homeostatic process of the blood cells by distorting the transport of oxygen, alteration of immune defense mechanisms, and alteration in platelet function. Consistent with previous studies, aqueous extract of *L. cupanioides* roots produced insignificant changes in PCV and Hb levels (Joshua & Timothy, 2011), which also suggested that the extract has no potential to cause both macrocytic and microcytic anemia (Nurudeen et al., 2021).

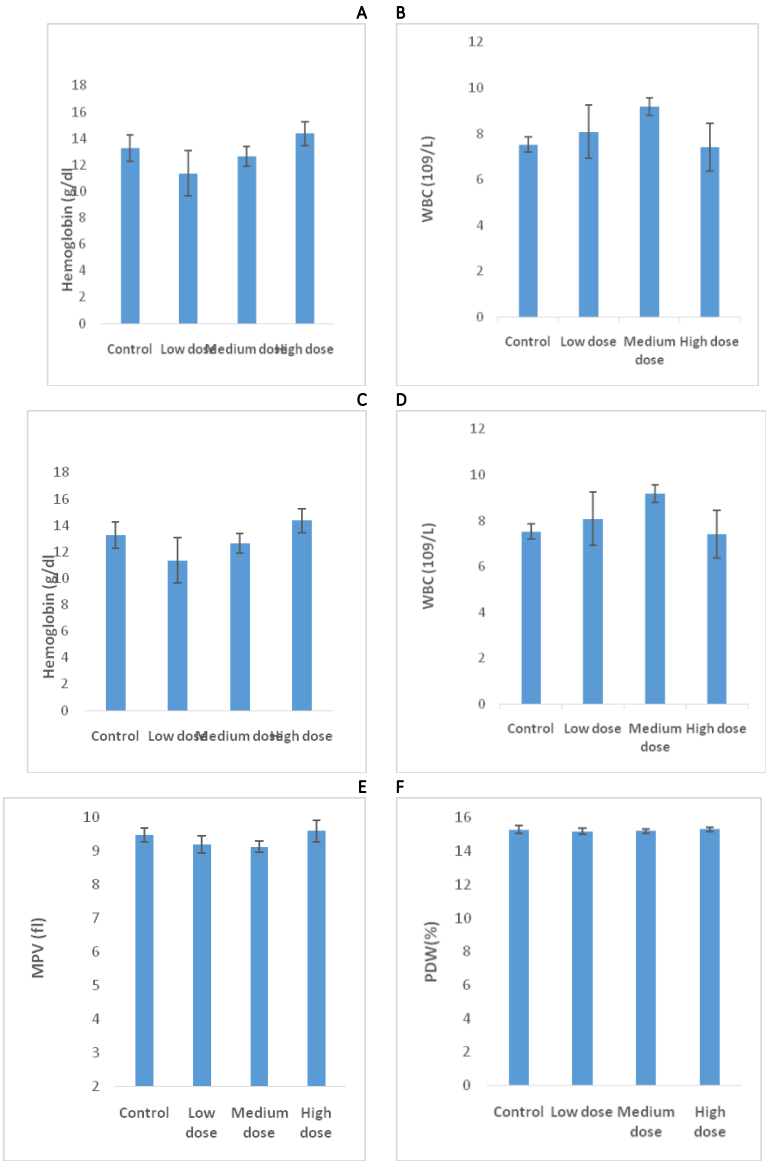

Figure 3. Effect of *L. cupanioides* aqueous leaf extract on hematological parameters of Wistar mice Haemoglobin
(A): White blood cell, (B): Mean platelet volume, (C): Mean cellular volume, (D): Platelet distribution width, (E): Plateletcrit, and (F): Platelet

3.7. Histology results

3.7.1. Kidney histology

Figure 1 shows photomicrograph of kidney tissue from control albino rats (plate A) and kidney histology results showing tests consistent with normal morphology and architecture (plates B, C, and D). The renal capsules (arrowhead) and the tubules (curved arrow) are normal with no sign of injury (H&E, x400).

3.7.2. Liver histology

From Figure 2, plate A shows the control group showing morphology consistent with normal liver histology architecture. Tissue shows congestion of the central vein (arrow), but the hepatocytes are normal (H&E X 400). The same applies to the test groups (plates B, C, and D).

3.8. Anti-ulcer activity

3.8.1. Effect of L. cupanioides aqueous leaf extract on gastric pH, ulcer severity, ulcer index, total acidity, and ulcer protection

Generally, *H. pyroli* infection is the main pathogenic cause of peptic ulcers. It has been noted that even after *H. pyroli* has been eliminated, ulcer recurrence still occurs. Psychological stress may be related to ulcer recurrence in hp-negative ulcer patients (Moriya et al., 2011). Usually, emotional stress results in a decline in mucosal defense which could alter the elements that preserve the mucosa intact (Jain, 2016; Périco et al., 2015).

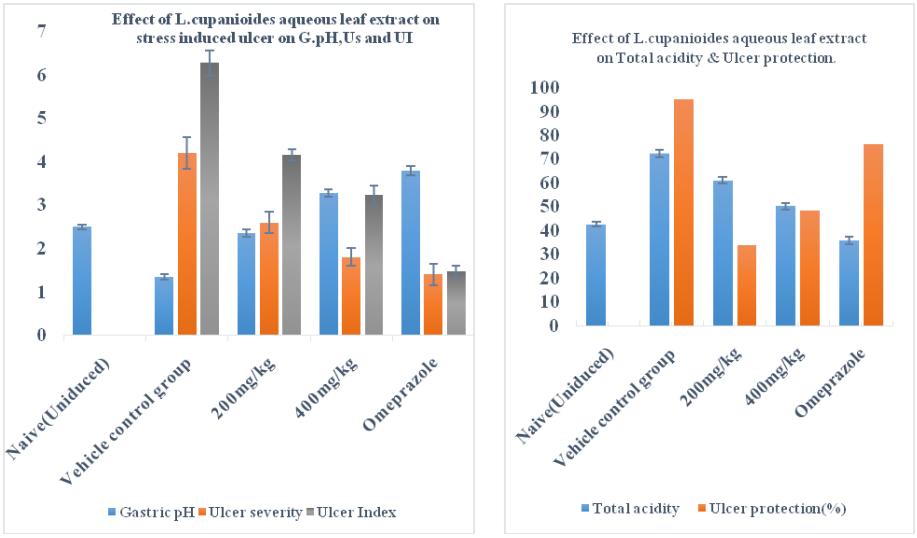

Figure 4. Effect of *L. cupanioides* aqueous leaf extract on gastric pH, ulcer severity, ulcer index total acidity, and ulcer protection

In an attempt to discover an ideal antiulcer agent with limited side effects, we also assessed the leaf extract of *L. cupanioides* for its possible antiulcer activity. The study revealed a significant influence of the extract on stress-induced ulcers at different dose levels administered when compared to the control (native, negative control, and omeprazole). When compared to both native and omeprazole, the animals treated with 200mg/kg and 400mg/kg of the extract showed a significant increase ($p < 0.05$) in ulcer score, ulcer index, ulcer severity, and total acidity, but a dose-dependent increase in ulcer protection ability of the aqueous plant extract was observed (Figure 4).

4. Conclusions

This study demonstrated that acute exposure of animals to a single dose of the aqueous leaf extract of *L. cupanioides* was safe at 5000mg/kg body weight, and did not produce obvious signs of toxicity and death. Repeated administration of 250, 500, and 1000 mg/kg doses of the aqueous leaf extract of *L. cupanioides* over 21 days revealed no deleterious effect on body weight, hematological, liver, and kidney biomarkers, and histology.

On the other hand, the aqueous leaf extract (ALE) of *L. cupanioides* significantly increased ($p < 0.05$) ulcer score, ulcer index, ulcer severity, and overall acidity, although with a dosage-dependent increase in ulcer prevention ability.

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Conflict of interest

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

Statement of ethics

All animal experiments were conducted in compliance with the 2010/63/EU Directive on the protection of animals used for scientific purposes and approved by the institution's animal ethical committee (PHACOOU/AREC/2023/019).

Availability of data and materials

All data generated or analyzed during this study are included in this published article. On request, the associated author can provide more information.

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Supplementary File

None.

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RESEARCH ARTICLE

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Antiurolithiatic activity of Indian medicinal plant: *Ocimum kilimandscharicum* Gurke (Lamiaceae)

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Ocimum kilimandscharicum

ABSTRACT

Urolithiasis is the most prevalent condition of the urinary system, characterized by the formation of stones inside the urinary tract. It is urgent to look for a natural urolithiasis therapy due to the serious side effects of conventional medications. Hydro-alcoholic (80% v/v) extract of the aerial parts of *Ocimum kilimandscharicum* (OK) and its ethyl acetate, chloroform, *n*-butanol, aqueous, and *n*-hexane fractions were subjected to in vitro antiurolithiatic screening as well as preliminary screening of phytochemicals. The in vitro antiurolithiatic activity of *O. kilimandscharicum* was studied using its hydroalcoholic extract (HAEOK). Calcium phosphate test using a colorimetric approach and calcium oxalate assay using a titrimetric model were used to determine the proportion of calcium oxalate crystals that dissolved. Total phenolic content (TPC) and total flavonoid content (TFC) were measured for the extract and fractions of OK. Ethyl acetate fraction (EAFOK) had a greater capacity to suppress crystal formation in both the calcium phosphate and calcium oxalate assays. The percent dissolution of calcium oxalate by HAEOK and EAFOK (31.48 ± 0.920% and 39.21 ± 0.903%) and calcium phosphate crystals by HAEOK and EAFOK (59.03 ± 0.820% and 66.62 ± 0.468%) was determined, respectively. At $p < 0.05$ and $p < 0.01$, differences between the results were regarded as significant. Cystone was employed as a standard drug. This study revealed that EAFOK showed significant antiurolithiatic activity. The antiurolithiatic activity of the extract/fraction was attributed to the steroids, triterpenoids, and flavonoid content of OK.

1. Introduction

Urolithiasis is an intricate mechanism that explains various physicochemical changes such as supersaturation, nucleation, aggregation, growth, and retention in kidneys (Butterweck & Khan, 2009). UL prevalence varies geographically, with 7%-13% in North America, 5%-9% in Europe, and 1%-5% in Asia. Africa's prevalence is unknown due to lack of medical facilities and research (Ondziel-Opara et al., 2022). Endogenous (calcium, phosphorous, and oxalate levels, elevated thyroid hormone levels, and difficulty in elimination of nitrogenous waste) and exogenous (excess water loss, Insufficient intake of fluid, extremely hot climate) factors are responsible for lithogenesis (Riaz et al., 2023).

Consumption of high oxalate-containing foodstuffs was also a major cause of the formation of uroliths (Aziz et al., 2024; Shabbir et al., 2023). The most common kind of urinary calculi illness is calcium oxalate (Iqbal et al., 2022). CaOx crystals adhere to renal tubular epithelial cells, forming a crucial stage in stone production. Randall's plaques (RPs) theory suggests these deposits enlarge and reach the renal papillary surface, attracting CaOx crystals, and forming CaOx stones. These theories are supported by research on reactive oxygen species and oxidative stress (Hong & Qin, 2023). The pathogenesis of urolithiasis includes the various steps involved in the emergence of stone including nucleation, growth, and aggregation of crystals

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(Kant et al., 2020; Shah et al., 2023). The nucleation phase is identified as a thermodynamically driven step in phase change wherein the dissolved supersaturated solution leads to crystallization (Bharathi et al., 2013; Rahim et al., 2023; Zahra et al., 2023). The production of urinary stones can be avoided by preventing supersaturation or subsequent phases in crystallization (Naveed et al., 2022a; Waseem et al., 2023; Zahid et al., 2022). Nowadays precautions have been taken to minimize the supersaturation of urine such as sufficient fluid intake and also by avoiding the consumption of dairy products (Smyslova et al., 2015).

As per the epidemiological studies report it affects around 12% of the population, with a reappearance rate of 70-81% in men, and 47-60% in women. Based on the chemical composition, urinary stones are further categorized into numerous types such as calcium oxalate (> 60%), calcium phosphate (10-20%), cysteine (1-5%), struvite (1-14%), uric acid (5-10%), and miscellaneous (4%) (Klein, 1996).

Advanced clinical research studies have demonstrated the therapeutic benefits of botanicals in preventing and managing of a wide range of diseases. More than 150 different species of *Ocimum* can be found in the genus, which ranges from sea level to a height of 6.000 feet and is found in temperate and subtropical areas of the world. *O. gratissimum*, *O. sanctum*, *O. basilicum*, *O. kilimandscharicum*, and *O. americanum* are examples of known important species of the genus *Ocimum* (Carović-Stanko et al., 2011). Extracorporeal shock wave lithotripsy and percutaneous nephrolithotomy are the two techniques used to diagnose this disease, but there is no evidence that these procedures will prevent stone development from returning (Geetha et al., 2010). Recently there has been a rising resurgence and revival of interest in traditional and indigenous medical practices, which are seen as being quite secure with few to no adverse effects, cost-effective, widely accessible, and easily attainable (Hayat et al., 2023; Hussain et al., 2022; Naveed et al., 2023). The use of *Ocimum* species in managing nephrotoxicity or issues related to urinary stones has been documented in an assortment of studies. An investigation of *O. basilicum* ethanol extract demonstrated nephron-protective action against cisplatin (Zaveri et al., 2011). If it refers to kidney stones, honey and the juice of basil leaves (*O. sanctum*) ingested routinely for six months will cause the stones to pass through the urinary tract (Kalyan et al., 2012). The management of urinary stones with a fresh infusion of *O. gratissimum* stem is documented in traditional medicine literature.

O. kilimandscharicum, also known as "Camphor Basil" in English and as "Kapoor Tulsi" in Hindi, is an atypical shrub that is primarily grown in South India. This plant is frequently used in traditional medicine to heal a broad range of conditions, including ulcers, abdominal discomfort, diarrhea, and microbial infection. Biologically active components in essential oils work as insect repellents, especially against mosquitoes and storage mites. The highest concentrations of camphor and oil are found in the leaves, which are followed by the blossoms. Sesquiterpenes in alcohol, terpinolene, d-pinene, d-limonene, and d-camphor are found in the stalks (Soni et al., 2012).

Literature review revealed the traditional use of various extracts of leaves and other parts of *Ocimum* species like *O. kilimandscharicum*, *O. basilicum*, *O. gratissimum* and *O. sanctum* for anti-urolithiasis (Nimavat et al., 2022), but no in vitro study has been undertaken for aerial parts of above mentioned *Ocimum* species for antiurolithiatic activity (Hussain et al., 2019). The presence of active constituents such as flavonoid and phenolic content in the extract of *Lepidagathis prostrata* whole plant (Devkar et al., 2016); terpenoids in *Bergenia ciliata* (Saha & Verma, 2013) and flavonoids, steroids, and terpenoids in *Bryophyllum pinnatum* (Nagarajan et al., 2019) were attributed to the antiurolithiatic activity. The present study aims to evaluate and compare the effectiveness of antiurolithiatic properties of extract and fractions of aerial parts of *O. kilimandscharicum*.

2. Materials and methods

2.1. Identification, authentication, and collection

The fresh aerial portions of OK were collected from Gandhi Krishi Vignan Kendra campus, Bangalore, Karnataka, India on 28/01/2020 and authenticated by Dr. Vijayakumar B. Narayanapur, College of Horticulture, Bagalkote, Karnataka, India. For reference in the future, the voucher specimen (NCP/01/2020-21) was stored in the herbarium of the Pharmacognosy Department at the National College of Pharmacy, Shimoga.

2.2. Extraction and fractionation procedure

Fresh aerial parts of OK were shade-dried for 10 days. The dried plant material was powdered and sieved through sieve no 44 to get the powder with a gritty texture. The weighed quantity (1 kg) of powdered plant material was extracted with 80% v/v hydro alcohol (5 l) using a round bottom flask for 4 days at room temperature by replacing fresh hydro alcohol daily replaced alcohol was collected, combined, and filtered. The filtrate was concentrated by using a rotary evaporator. 100 mg hydroalcoholic extract of *O. kilimandscharicum* (HAEOK) was further fractionated by using hexane and water (500 ml) as a solvent.

The hexane fraction was separated and evaporated. The aqueous layer was further partitioned using 50 ml of solvents such as chloroform, ethyl acetate, and *n*-butanol in 3 parts evaporated to dryness to get respective fractions (HFOK, AFOK, CFOK, EAFOK, and BFOK). The residual aqueous part was condensed by a rotary evaporator and finally dried at 60 °C. The dried extract/fractions were stored in the sealed container at 4 °C for the time being used (Hannan et al., 2006).

2.3. Preliminary phytochemical investigation

Standard qualitative tests were used to determine the phytoconstituents present in the HAEOK as per the procedures mentioned in the standard references (Khandelwal, 2008).

2.4. Total phenolic content

To assess the total phenolic content of the hydroalcoholic extract and its fractions of OK, the Folin-Ciocalteu reagent was used (Singleton & Rossi, 1965). In the extraction/fractions (100 mg/ml), 2.5 ml of 10% aluminum chloride solution and 2 ml of 7% sodium carbonate solution were used as neutralizing agents. The resulting reaction mixture was incubated for 30 min at room temperature leading to the development of a blue colour in the solution. The absorbance of the resulting blue-colored solution was measured at 765 nm. To estimate the phenolic content, a standard calibration curve of gallic acid was used (as the standard drug) and expressed in mg GAE per gram of dried extract/fraction.

2.5. Total flavonoid content

The concentration of total flavonoid content in hydroalcoholic extract and its fractions of OK was determined by using an aluminum chloride colorimetric assay (Chander et al., 2014). About

1 ml of extract/fractions (1 mg/ml) and 0.3 ml of sodium nitrate were added to the volumetric flask containing 4 ml of distilled water. Thereafter the solution was shaken well and it was kept aside for 5 min. After 5 min, 0.3 ml of 10% aluminum chloride was added and the volume was made up to 10 ml with distilled water and the solution was incubated for 30 min at room temperature. After mixing thoroughly, the absorbance of the solution was measured at 434 nm against a blank. The outcome of the present study was expressed as milligrams of quercetin (as a standard drug) dissolved in distilled water.

2.6. Investigation of in vitro antiurolithiatic property by titrimetry

2.6.1. Homogenous precipitation of calcium oxalate

A solution of calcium chloride dehydrate (1.47 gm) in 10 ml of 2 N sulphuric acid was allowed to react with sodium oxalate (1.34 gm) in distilled water. Calcium oxalate was precipitated and ammonia solution was used to remove traces of sulfuric acid from the solution. The precipitate was again rinsed with distilled water before being dried for 4 hours at 60 °C (Sathish & Jayebalan, 2017).

2.6.2. Synthesis of the semi-permeable membrane

Meanwhile, a semi-permeable membrane was prepared from the farm eggs by decalcifying the eggs in 2 M HCl for 24 hours until the complete removal of the shell part of the egg **(Figure 1)**. The decalcified egg was washed with distilled water and a hole was made carefully with a sharp pointer on the top and all the contents were squeezed out completely from the decalcified egg. Semi-permeable membrane was washed thoroughly with distilled water, and placed in ammonia solution, in the moistened conditions for a while, and then rinsed with distilled water. Finally, it is stored in the refrigerator at a pH of 7.0-7.4 until further need (Bhandari et al., 2021).

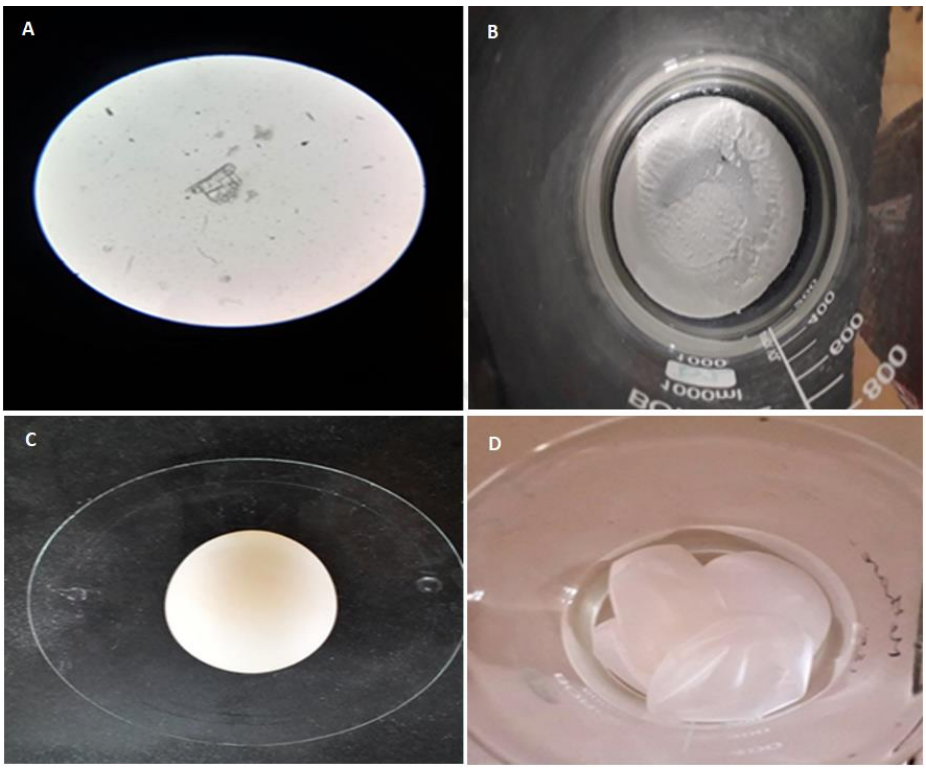

Figure 1. A) Microscopic view of calcium oxalate crystal, B) The homogenous precipitate of calcium oxalate, C) Decalcified egg, D) Semi-permeable membrane

2.6.3. Experimental method

In a semi-permeable membrane, 10 mg of the calcium oxalate and 100 mg of the sample/standard were weighed accurately and packed together. This was allowed to be suspended in a beaker containing 100 ml of 0.1 M TRIS buffer. Beakers of all groups were placed at room temperature at 37 °C for about 7-8 hours. The contents of semi-permeable were removed from each group into a test tube; 2 ml of 1 N H_2SO_4 was added and titrated against $KMnO_4$ till a light pink color endpoint was obtained. The amount of superfluous calcium oxalate is subtracted from the total quantity used at the beginning of the experiment. The percentage dissolution of calcium oxalate was calculated by using the factor 1 ml of 0.9494N $KMnO_4$ is equivalent to 0.1898 mg of calcium (Bhandari et al., 2021).

2.7. Calcium phosphate assay

2.7.1. Homogenous precipitation of calcium phosphate

An equimolar solution of disodium hydrogen phosphate (1.47 g) and calcium chloride (1.42 g) was prepared by dissolving in 2 N sulphuric acid and distilled water, respectively. Ammonia solution was used to wash out the traces of sulphuric acid from the resulting calcium phosphate precipitate. The crystals were rinsed in distilled water and dried for 4 hours at 60 °C (Sathish & Jayebalan, 2017).

2.7.2. Experimental method

Accurately weighed 10 mg of the calcium phosphate and 100 mg of extract/fractions/standard drug were packed together in a semipermeable membrane and sutured. The semipermeable membrane was prepared in the same manner as for the calcium

oxalate assay. This was allowed to be suspended in a conical flask containing 100 ml of 0.1 M TRIS buffer. All the conical flasks were placed uninterrupted at room temperature (37 °C) for 7-8 hours. The remaining contents in the semipermeable membrane were transferred into different test tubes, 4 ml of 1N H_2SO_4, 3 ml of molybdate-sulphuric acid reagent, and 1 ml of reducing solution were added to each test tube and kept aside for 2 hours; the color change was observed from dark pink to colorless. The optical densities were measured colorimetrically at 620 nm (Sathish & Jayebalan, 2017).

2.8. Statistical analysis

The outcomes of the experiment were expressed as mean ± SEM (n = 3). As per the suitability, a one-way ANOVA statistical method by using Graphpad Prism 9 software was used to evaluate the difference between the data. $p < 0.05$ was considered as significant and $p < 0.01$ was considered as very significant.

Table 1. Percentage yıeld of extract and varıous fractions of *O. kilimandscharicum*

Extract and fractions of OK	Percentage yield (%, w/w)
HAEOK	13.31 ± 0.02[a]
EAFOK	2.1 ± 0.01[a]
CFOK	1.2 ± 0.02[b]
BFOK	2.5 ± 0.02[a]
AFOK	2.2 ± 0.02[a]
HFOK	1.5 ± 0.03[b]

Percentage yield values are the mean of at least three replicates of experiments ± standard deviation; where a means $p < 0.05$, b means $p < 0.01$, sample vs standard in each group.

3. Results and discussion

The percentage yield of HAEOK, EAFOK, EAFOK, CFOK, BFOK, AFOK, and HFOK was 13.31 ± 0.02, 2.1 ± 0.01, 1.2 ± 0.02, 2.5 ± 0.02, 2.2 ± 0.02, and 1.5 ± 0.03, respectively **(Table 1)**. The preliminary phytochemical analysis of the extract reveals the fact that carbohydrates, saponins, flavonoids, steroids, triterpenoids, alkaloids, and phenolic compounds are present. Flavonoids and phenolic compounds are present in ethyl acetate fraction, triterpenoids in *n*-hexane fraction, alkaloids in the chloroform; carbohydrates, flavonoids, and phenolics in aqueous fraction; alkaloids and triterpenoids in *n*-butanol fraction **(Table 2)**. Flavonoids can prevent the adhesion of calcium oxalate crystals in the urinary tract due to its radical scavenging effect and stop further injury in the formation of kidney stones (Naveed et al., 2022a; Naveed et al., 2022b; Saleem et al., 2022). Saponins are known to disintegrate mucoproteins. Calcium oxalate crystal emergence and the dissolution of urinary stones that have already developed are inhibited by polyphenols and tannins (Bawari et al., 2018).

Table 2. Qualitative phytochemical analyses of the hydro-alcoholic extract of OK and its fractions

SL No	Chemical constituents	Test	EAFOK	CFOK	BFOK	AFOK	HFOK	HAEOK
1	Carbohydrates	Molish test	-	-	-	+	-	+
		Fehling test	-	-	-	+	-	+
		Benedict's test	-	-	-	+	-	+
2	Proteins	Ninhydrin test	-	-	-	-	-	-
		Xanthoproteic test	-	-	-	-	-	-
3	Alkaloids	Dragendroff's test	-	-	+	-	+	+
		Mayer's test	-	-	+	-	+	+
		Hager's test	-	-	+	-	+	+
		Wagner's test	-	-	+	-	+	+
4	Glycosides	Modified Brontrager's test	-	-	-	-	-	-
		Legal test	-	-	-	-	-	-
		Baljet test	-	-	-	-	-	-
5	Saponins	Foam test	-	-	-	+	-	+
6	Triterpenoids	Liebermann's Burchard's test	-	+	-	-	+	+
		Salkowski test	-	+	-	-	+	+
7	Flavonoids	Shinoda test	+	-	-	+	-	+
		Ferric chloride test	+	-	-	+	-	+
		Lead acetate test	+	-	-	+	-	+
8	Phenolics	Ferric chloride test	+	-	-	+	-	+

+ indicates present,- indicates absent

The presence of phenolic and flavonoid content was attributed to the medicinal effects of a wide range of angiosperms due to their positive pharmacological actions on human beings (Devkar et al., 2016). Polyphenols and flavonoids present in the plant extract could effectively inhibit the formation of calcium oxalate stones in vitro and in vivo, correlating with their diuretic, antioxidant, anti-inflammatory, antibacterial, and other protective effects (Ahmad et al., 2023; Monisa et al., 2023). *Persea americana* with high flavonoid content has been reported to have antilithiatic activity (Nagarajan et al., 2019). The percentage of phenolics and flavonoids was calculated using the aluminum chloride method and the Folin-Ciocalteu reagent method, respectively. The TPC and TFC of EAFOK were found to be higher when compared to other fractions (26.2 ± 0.03 mg GAE/g of fraction and 5.18 ± 0.03 mg QE/g of fraction, respectively). Quantitative estimation of the phenolics and flavonoids was carried out by calculating the percentage of phenolics and flavonoids altogether in the extract and its fractions of OK. TPC and TFC were computed in terms of gallic acid and quercetin equivalents out of each gram of extract/fractions depicted in **Table 3** respectively.

Table 3. TPC and TFC of hydro-alcoholic extract and its fractions of OK

Extract and fractions of OK	Percentage yield (%, w/w)	TFC (mg QE/g)	TPC (mg GAE/ g)
HAEOK	13.31 ± 0.02	8.18 ± 0.02[a]	30.5 ± 0.03 [a]
EAFOK	2.1 ± 0.01	5.18 ± 0.03[a]	26.2 ± 0.03 [a]
CFOK	1.2 ± 0.02	3.2 ± 0.02[a]	10 ± 0.06 [b]
BFOK	2.5 ± 0.02	4.2 ± 0.04[a]	15.6 ± 0.02 [a]
AFOK	2.2 ± 0.02	1.2 ± 0.02[b]	11.8 ± 0.02 [a]
HFOK	1.5 ± 0.03	2.5 ± 0.03[b]	8.5 ± 0.02 [b]

TPC and TFC values are the mean of at least three replicates of experiments ± standard deviation; where a means $p < 0.05$, b means $p < 0.01$, sample vs standard in each group.

Urolithiasis is a corporeal method of formation of calculi in the urinary tract (Ammara et al., 2023; Hussain et al., 2023). Oxidative stress in circumstances where stones develop is inevitable, even with advances in urology and nephrology. The structural integrity of membranes is compromised by oxalate, which causes lipid peroxidation. The by-product malondialdehyde is an important one. Antioxidant mechanisms in cells regulate lipid peroxidation; nonetheless, even with several antioxidant systems, overloading can lead to an increase in peroxidation products in tissues (Kant et al., 2020).

The concentration of Ca^{2+} in solutions was not proportionate to the dissolution of calcium oxalate. The effective percentage dissolution of calcium phosphate (59.03 ± 0.820) and calcium oxalate crystals (Figure 2) in the in-vitro antiurolithiatic investigation by HAEOK was found to be comparable to statistical studies conducted among all other fractions of OK and the standard drug cystone (48.03 ± 0.421%). The antiurolithiatic activity of the five fractions of OK's hydroalcoholic extract was assessed. With percent dissolution of calcium oxalate crystals CFOK, BFOK, AFOK, and HFOK were revealed to have considerable antiurolithiatic activity (39.21 ± 0.903%, 29.53 ± 0.414%, 21.82 ± 0.350%, 10.01 ± 0.312%, and 3.47 ± 0.670%, respectively) (Table 4).

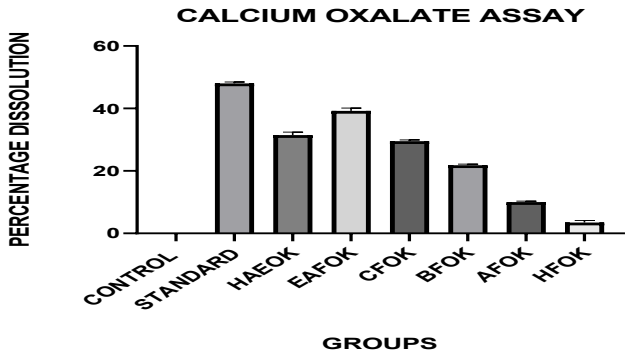

Figure 2. Calcium oxalate assay by titrimetry

The calcium phosphate calibration curve crystals at different concentrations were plotted against their respective optical densities as shown in Figure 3. The standard calibration curve of calcium phosphate was used to determine the undissolved calcium phosphate crystals. As a proportion dissolution, the study findings were analyzed. In calcium phosphate assay, HAEOK showed less significant antiurolithiatic activity than the standard drug with percentage dissolution of 59.03 ± 0.820% and 73.67 ± 0.453%, respectively (Tables 5 and 6). Among the various fractions of OK such as EAFOK was more significant than other fractions. But, it is less significant than cystone. The capability of percentage dissolution of calcium phosphate crystals by EAFOK, CFOK, BFOK, AFOK, and HFOK was found to be 66.62 ± 0.468%, 55.96 ± 0.436%, 44.67 ± 0.975%, 41.67 ± 0.642%, and 34.7 ± 0.983%, respectively (Figure 4). The EAFOK showed more significant antiurolithiatic activity but less significant than cystone drug among all other fractions of OK. HAEOK and EAFOK have a significant antiurolithiatic effect with significance levels of $p < 0.01$ and $p < 0.05$, respectively. The results of the present antiurolithiatic activity of hydroalcoholic extract and its fractions of *O. kilimandscharicum* were mainly ascribed to the inclusion of triterpenoids, phenols, and flavonoids. However, the data that has been obtained from the present investigation cannot simply provide an insight into the potential mechanism of action of tested phytoconstituents or extract. The phytochemical analysis of the extract reveals the presence of phenolics, flavonoids, triterpenoids, and other phytoconstituents in extract/fractions. The presence of these phytoconstituents may be responsible for antiurolithiatic activity. This medication can be used to treat urinary stone disease after additional study is done through in vivo experiments using experimental animals.

Table 3. TPC and TFC of hydro-alcoholic extract and its fractions of OK

Extract and fractions of OK	Percentage yield (%, w/w)	TFC (mg QE/ g)	TPC (mg GAE/g)
HAEOK	13.31 ± 0.02	8.18 ± 0.02[a]	30.5 ± 0.03 [a]
EAFOK	2.1 ± 0.01	5.18 ± 0.03[a]	26.2 ± 0.03 [a]
CFOK	1.2 ± 0.02	3.2 ± 0.02[a]	10 ± 0.06 [b]
BFOK	2.5 ± 0.02	4.2 ± 0.04[a]	15.6 ± 0.02 [a]
AFOK	2.2 ± 0.02	1.2 ± 0.02[b]	11.8 ± 0.02 [a]
HFOK	1.5 ± 0.03	2.5 ± 0.03[b]	8.5 ± 0.02 [b]

TPC and TFC values are the mean of at least three replicates of experiments ± standard deviation; where a means $p < 0.05$, b means $p < 0.01$, sample vs standard in each group.

4. Conclusions

The research outcome shows that HAEOK's antiurolithiatic action is less significant when compared to the standard. Comparing EAFOK to other fractions of OK, a very strong antiurolithiatic efficacy was observed. The extract's phytochemical assessment shows that it contains triterpenoids, flavonoids, phenolics, and other phytoconstituents. These phytoconstituents may be the cause of the antiurolithiatic action. Although *O. kilimandscharicum* has a wide range of biological uses, little scientific research has been done on the plant, and its full potential has not yet been realized. Furthermore, in vivo research has to be done on experimental animals; if more research is done, this medication may be utilized to treat urinary stone disease. Commercial use of *O. kilimandscharicum* for the production of medications intended to treat various illnesses.

Table 4. Impact of OK and its fractions on percentage dissolution of calcium oxalate crystals

Groups	Vol. of KMnO4 (ml)	Wt. of calcium estimated (mg)	Wt. of calcium reduced (mg)	% Dissolution
Control	4.2 ± 0.017	0.8034 ± 0.003	-	-
Standard**	2.2 ± 0.01	0.4176 ± 0.002	0.3859 ± 0.005	48.03 ± 0.421[a]
HAEOK**	2.9 ± 0.05	0.5504 ± 0.010	0.2531 ± 0.007	31.48 ± 0.920[a]
EAFOK**	2.6 ± 0.031	0.4884 ± 0.006	0.3151 ± 0.008	39.21 ± 0.903[a]
CFOK	3 ± 0.0153	0.5662 ± 0.003	0.2373 ± 0.004	29.53 ± 0.414[b]
BFOK	3.3 ± 0.027	0.6282 ± 0.005	0.1753 ± 0.002	21.82 ± 0.350[b]
AFOK	3.8 ± 0.027	0.7231 ± 0.005	0.0804 ± 0.002	10.01 ± 0.312[b]
HFOK	4.1 ± 0.015	0.7757 ± 0.003	0.0279 ± 0.005	3.47 ± 0.670[b]

Volume of $KMnO_4$ was measured as mean ± SD; where a means $p < 0.05$, b means $p < 0.01$, ($n = 3$).

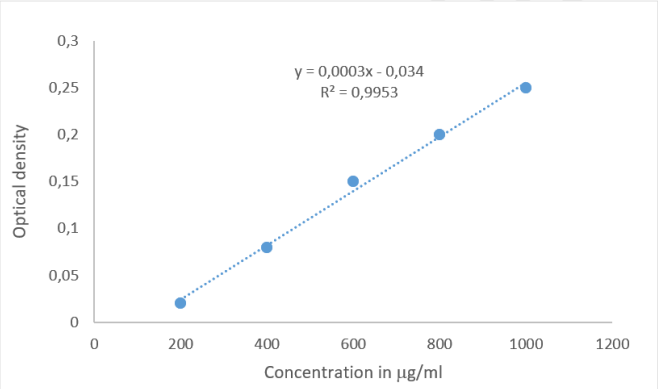

Figure 3. Calcium phosphate calibration curve

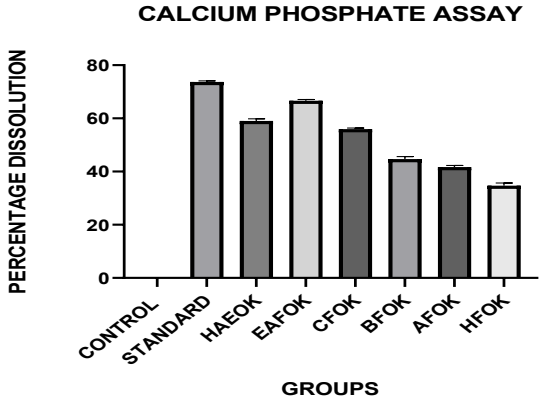

Figure 4. Calcium phosphate assay by colorimetry

Table 5. Standard calibration curve of calcium phosphate assay

Concentration (µg/ml)	Molybdic H_2SO_4 (ml)	Reducing agent (ml)	Distilled water (ml)	Optical density
200 µg/ml				0.02
400 µg/ml				0.08
600 µg/ml	2.5 ml	1 ml	q.s. to 10 ml	0.15
800 µg/ml				0.19
1000 µg/ml				0.25

Table 6. Calcium phosphate assay by colourimetry

Groups	Optical density	Calcium estimated (mg)	Calcium reduced (mg)	Percentage dissolution
Control	0.2483 ± 0.002	0.9849 ± 0.007	-	-
Standard**	0.0403 ± 0.002	0.2593 ± 0.005	0.7256 ± 0.006	73.67 ± 0.453[a]
HAEOK**	0.0817 ± 0.002	0.4035 ± 0.005	0.5814 ± 0.012	59.03 ± 0.820[a]
EAFOK**	0.060 ± 0.002	0.3349 ± 0.005	0.6561 ± 0.006	66.62 ± 0.468[a]
CFOK**	0.0903 ± 0.002	0.4337 ± 0.005	0.5511 ± 0.006	55.96 ± 0.436[a]
BFOK**	0.1217 ± 0.002	0.543 ± 0.005	0.4418 ± 0.11	44.67 ± 0.975[a]
AFOK*	0.1307 ± 0.002	0.5744 ± 0.007	0.4104 ± 0.007	41.67 ± 0.642[b]
HFOK*	0.1503 ± 0.002	0.643 ± 0.005	0.3418 ± 0.012	34.7 ± 0.983[b]

The optical densities were computed as mean ± SEM; where a means $p < 0.05$, b means $p < 0.01$, ($n = 3$)

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Conflict of interest

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

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. On request, the associated author can provide more information.

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RESEARCH ARTICLE

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Cytotoxic and apoptotic effects of Prunus spinosa fruit extract on HT-29 colon cancer line

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ABSTRACT

Colon cancer holds the position of the third most common type of cancer and stands as the third leading cause of cancer-related deaths for both men and women. Modern strategies in cancer prevention center around the use of natural compounds, which demonstrate a range of effects, including preventive, inhibitory, and latency-inducing impacts on the progression of cancer. In the present study, aqueous extracts derived from the fruits of *Prunus spinosa* L. (blackthorn, Rosaceae) are employed to assess their cytotoxic potential against the HT-29 colon cancer cell line. The fruit extract is administered to the HT29 cell line in different concentrations over 24 and 48-hours to evaluate the induction of apoptosis. The MTT cell viability test is employed to quantify the cytotoxic effect, indicating the extent of the impact. Additionally, the EB/AO (ethidium bromide/acridine orange) dual staining method is utilized to gather supplementary information regarding the cytotoxic effects. Observations after 24 hours of exposure showed no significant cytotoxic effect; however, 48-hour exposure revealed IC_{20}, IC_{50}, and IC_{80} values of 1.27, 173.7, and > 1000 µg/ml, respectively, as determined by MTT analysis. Correspondingly, values of 5.06, 123.8, and > 1000 µg/ml were recorded by the EB/AO dual staining method. Our results show that *P. spinosa* fruit water extract has an inhibitory effect on the HT-29 cell viability by exerting cytotoxic and apoptotic effects in a concentration-dependent and time-dependent manner. Toxicity studies have shown that MTT and EB/AO support each other and achieve similar results. Further extensive research into the metabolic and functional effects of *P. spinosa* could illuminate its potential and increase its economic importance in the field of anticancer treatments as a natural drug.

1. Introduction

For centuries, plants and natural products have been employed in treating diverse ailments, leading to the utilization of medicinal compounds derived from a multitude of plants (Badal et al., 2024; Chunarkar-Patil et al., 2024; Gunnar & Bohlin, 1999). Presently, numerous plant-derived compounds have been isolated, displaying activity against various cancer cell types (Nirmala et al., 2011). According to the findings from these studies, an increased intake of fruits and vegetables plays a crucial role in promoting healthy nutrition and preventing chronic diseases like heart attacks, cancer, diabetes, and neurodegenerative conditions (Liu, 2013).

Fruits and vegetables, abundant in polyphenols such as flavonoids, phenolic acids, anthocyanins, and tannins, as well as other compounds like sugars, essential oils, carotenoids, vitamins, and minerals, are recognized for their health benefits. Polyphenolic compounds, categorized as secondary metabolites in plants, exhibit potent antioxidant properties (Scalbert et al., 2005). These compounds function as antioxidants by chelating or inhibiting metal ions during the initiation of free radical formation, thereby suppressing radical species. Additionally, polyphenols act as radical scavengers by donating hydrogen molecules (Aviram et al., 2005; Halliwell, 1996).

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Polyphenols have been documented to possess both in vitro and in vivo antioxidant, anticancer, antimutagenic, anti-inflammatory, and anti-neurodegenerative properties (del Rio et al., 2013; Nile & Park, 2014). Interest in plant phenolic extracts has increased due to their potential antioxidant activity and extensive pharmacological properties, including anticancer activity (Carocho & Ferreira, 2013). Anthocyanins, a subgroup of flavonoids primarily found in fruits, contribute to the red, blue, and purple hues in many plants, particularly in grapefruits (Mazza, 1993).

Along with the beneficial effects of anthocyanins against cancer and cardiovascular disorders; antioxidant, anti-inflammatory, and antiproliferative activity is reported to affect cellular signaling pathways, cause cell cycle arrest, and activate apoptotic redox-sensitive transcription factors (Calabriso et al., 2016; Slatnar et al., 2012; Zhang et al., 2008).

The blackthorn fruit is a rich source of compounds with antioxidant and antibacterial characteristics, including flavonoids, anthocyanins, phenolic acids, vitamins, minerals, and organic acids (Natić et al., 2019; Ürkek et al., 2019). *Prunus spinosa* L., also known as blackthorn and belonging to the Rosaceae family, is grown in the north, west, and south of Türkiye and central Anatolia up to 1350 m high, grows in and at the edge of forests, the high variety between 1-3 m, perennial, shrub plant. The fruits of the *P. spinosa* grown in Türkiye are consumed in various ways for treatment among the population. It is reported that the flowers of blackthorn are used for respiratory diseases, diarrhea, stomach cramps, intestinal diseases, edema, and kidney and bladder diseases (Fraternale et al., 2009). *P. spinosa* fruits grown in our region are the best source of phenolic compounds containing flavonoid, coumarin, phenolic acid, and A-type proanthocyanins (Orhan et al., 2007), which are used as astrejan, diuretic, and purgative (Guimarães et al., 2013; Pinacho et al., 2015).

Extracts of different parts of *P. spinosa* obtained using ethanol, water, methanol, and ethyl acetate were tested on various cancer cell lines, and their antiproliferative and cytotoxic effects were studied (Condello & Meschini, 2021; Dedić et al., 2023; Guimarães et al., 2014; Meschini et al., 2017; Murati et al., 2016; Pinacho et al., 2015). It was demonstrated that ethanol extract of P. spinosa fruits on PC-3 and DU145 (Dedić et al., 2023) and methanol-water extract on MCF-7, NCI-H40, HCT-15, HeLa, and HepG2 cancer cell cultures (Guimarães et al., 2014) induced antiproliferative and cytotoxic activity. Proanthocyanidins obtained from *P. spinosa* extract were tested in GLC and COLO320 cell lines, and the cytotoxic effect of proanthocyanidins on the cancer cell line was obtained (Kolodziej et al., 1995). Phenolic compounds containing anthocyanin obtained from *P. spinosa*, *Rosa micranta*, and *R. canina* have antioxidant and antitumor effects on NCL-H460 cell lines (Guimarães et al., 2014). Furthermore, ethanol and water extracts of *P. spinosa* fruits showed antitumor effects on the colorectal cancer cell line (HT-29) (Popović et al., 2020).

In many studies with plant extracts, the MTT viability test is used to investigate the cell proliferation and cytotoxic effects of extracts or secondary metabolites on cancer cell lines. The MTT assay is a colorimetric experiment to assess cell metabolic activity. This method is based on a mitochondrial enzyme that breaks down MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium salt by using it as a substrate. Mitochondrial dehydrogenase enzymes found in the mitochondria of living cells break down MTT into formazan crystals. When the crystals were dissolved with DMSO or acid isopropanol, the purple-colored solution formed and was measured using a spectrophotometer at 560 nm and absorbance at 620 nm (Mosmann, 1983).

Ethidium bromide/acridine orange (EB/AO) cell viability test is employed in toxicology studies. AO stains both live and dead cells, while EB exclusively stains dead cells due to membrane structure disintegration. AO causes DNA to appear orange but is very weakly bound to the RNA. Although AO stains dead cells as green, EB suppresses the green color and reflects an orange-red color that can be observed by fluorescence microscopy. Cells entering necrosis stain the same as dead cells but do not have an apoptotic morphology. Early apoptotic cells are observed as green. Green cells with normal morphology observed in the cell population are alive, and red-orange cells with normal morphology are necrotic cells (Kasibhatla et al., 2006).

The water extract of *P. spinosa* fruit is known to possess a high phenolic and flavonoid content, albeit not as much as its branches. Additionally, the highest amount of anthocyanin is detected in the water extract of *P. spinosa* fruit (Pinacho et al., 2015). Studies have demonstrated that *P. spinosa* extracts were non-toxic to non-tumor cell lines (Gerardi et al., 2016; Guimarães et al., 2014; Meschini et al., 2017). This study aims to assess the cytotoxic and apoptotic effect of water extracts from *P. spinosa* fruits on HT-29 colon cancer cell lines using the MTT viability test and EB/AO staining methods.

2. Materials and methods

2.1. Extraction and isolation

P. spinosa fruit extraction and isolation were performed according to Stanković et al. (2019) with minor modifications. *P. spinosa* fruits were collected from Edirne, Türkiye. Plant material was identified by Prof. Dr. Çiler Kartal and the voucher specimen (No. 4315) was deposited at the herbarium of Trakya University, Biology Department (Republic of Türkiye Trakya University, EDTU Herbarium). *P. spinosa* fruits were extracted, homogenized and centrifuged at 9000 rpm for 2 minutes. The extract was diluted with water (1:1 ratio), infused with a magnetic stirrer for 30 minutes, and filtered through a 0.45µm filter. The filtrates were frozen and lyophilized in a vacuum freeze dryer (Virtis SP Scientific). Extracts were stored at –18 °C until tested (Stanković et al., 2019).

2.2. Chemicals used

Reagents and chemicals used in the study were Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), trypsin (Gibco, USA), MTT, phosphate buffered saline (PBS) salts, penicillin-streptomycin, AO, EB (Sigma Aldrich, USA), and Annexin V-FITC apoptosis detection kit (Biovision, USA).

2.3. Cell culture and application

HT-29 cells were cultured in DMEM medium containing 10% FBS and 1% penicillin-streptomycin and were incubated in a humidified atmosphere with 5% CO_2 and 95% air at 37 °C. HT-29 cells (5×10^3 cells/well) were grown in 96 well plates. After 24 hours, the cells showed a growth increase of 80%, and the medium was replaced with a new medium. The new medium contained herb extracts at different concentrations. No extract was added in the control group (Dewi et al., 2022). To determine IC_{20}, IC_{50}, and IC_{80} values, cells were exposed to different concentrations of the water extract of *P. spinosa* for 24 and 48 hours (43.95, 87.89, 175, 351.56, 703.13, 1406.2, 2812.5, 5625, 11250, 22500 and 45000 µg/ml). IC_{20}, IC_{50},

and IC_{80} concentrations obtained by MTT test were applied to cell cultures, and the apoptotic effects were assessed with the EB/AO method.

2.4. Determining apoptosis with the EB/AO dual staining method

EB/AO dual staining method based on the separation of viable and dead cells, was used to detect apoptosis. Viable cells were stained with EB/AO to determine the number of apoptotic cells. With fluorescence microscopy, AO enables the visualization of normal and apoptotic nuclei of living cells as green. EB allows the normal and apoptotic nuclei of dead cells to appear red (Kasibhatla et al., 2006; Petit et al., 1993). The experiment was repeated 3 times, 500 cells were counted for each concentration in each repetition.

2.5. Preparation of EB/AO dual staining solution

EB/AO staining solution used for counting live and dead cells consisted of a 100x stock solution prepared by mixing 50 mg of EB, 15 mg of AO with 1 ml of 98% ethanol, and 49 ml of distilled water and frozen in 1 ml aliquots. 1x working solution was prepared by mixing stock solution and PBS in a 1:1 ratio. The cell suspension was prepared at 1–5 x 10^6 cells/ml in a cell culture medium. Cell suspension and staining solution were mixed at a 1:1 ratio to observe dead and live cells by using fluorescence microscopy at 460 nm and 510 nm wavelengths (Parks et al., 1979). The phases of the cells in the apoptosis process were described by Gasiorowski et al. (2001) and at least 500 cells were counted. Apoptosis was determined by the deformation of the colon cancer cells. The percentage of apoptotic cells was calculated as the apoptotic index.

2.6. MTT assay

Antiproliferative effects were determined in the concentration range of 1–45000 µg/ml and dose-response curves and IC_{50} values were determined by Probit analysis. In this study, plant extracts with different concentrations (1–45000 µg/ml) were applied to evaluate their impact on cell viability on the HT-29 colon cancer cell line during 24 and 48-hour incubation periods. The experiment was repeated three times for each concentration, and average absorbance values were determined. IC_{50} values were then calculated for both the extract and negative control.

HT-29 cells were plated in 96-well plates as 5 x 10^3 per 100 µl suspension. Plates were incubated in an incubator for 24 hours to ensure adherence of colon cancer cells and duplication of cell number. Then, the medium in the wells was discarded and replaced with varying concentrations of plant extract in culture medium (43.95, 87.89, 175, 351.56, 703.13, 1406.2, 2812.5, 5625, 11250, 22500, and 45000 µg/ml). Only wells without cells containing the assay medium were evaluated as blind. The plates were incubated for 24 and 48 hours. Subsequently, 10 µl of MTT solution was introduced into each well, and the plates were incubated in the incubator for 4 hours. Following this, 100 µl of the solvent solution was added to each well, and the plates were incubated overnight. It is measured using a spectrophotometer at 560 nm and absorbance at 620 nm. Cytotoxicity levels were determined using the formula:

$$Cytotoxicity\ level = 1 - \frac{Absorbance\ of\ the\ test}{Absorbance\ of\ the\ control}\ x\ 100$$

The concentration of cytotoxic effect at 50% relative to the control was regarded as the cytotoxic dose (Ghasemi et al., 2023).

2.7. Statistical analysis

The results of the MTT and EB/AO assays were analyzed using the Probit analysis program (SPSS 17). Pearson correlation test was used to determine the relationship between concentration and cell death (statistical tests were performed at the confidence interval of $p < 0.05$).

3. Results and discussion

P. spinosa fruit extract caused the death of HT-29 cancer cells at different concentrations after 24 and 48-hour treatments. The impact of various concentrations on cell death following 24 and 48 hours of treatment, as assessed by the MTT test, is presented in Figure 1. The cell death ratio did not reach 50% after 24 hours of treatment. However, IC_{20}, IC_{50}, and IC_{80} values were determined after 48 hours of treatment, showing an increase in cell death rate inhibition up to 80%. Following 48 hours of treatment, the IC_{20}, IC_{50}, and IC_{80} values were found to be 1.27 µg/ml, 173.7 µg/ml, and > 1000 µg/ml respectively (Table 1). The MTT assay showed that the number of viable cells decreased after 24 and 48 hours of treatment compared with the control group. The apoptotic effect was observed at a maximum concentration of the extract after 48 hours.

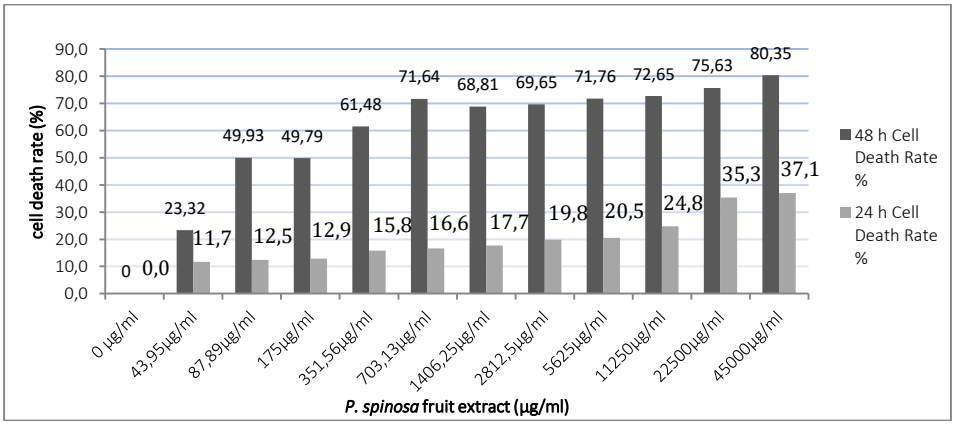

Figure 1. HT-29 colon cancer cell line proliferation inhibition after 24 and 48-hour *P. spinosa* fruit extract treatment measured by using MTT assay.

The cell death rate increased as the concentration increased.

Table 1. IC_{20}, IC_{50}, and IC_{80} inhibition concentration values after 24 and 48-hour treatment on HT-29 cell using MTT and Probit analyses

Treatment period	IC_{20}	IC_{50}	IC_{80}
24 h	> 1000 µg/ml	> 1000 µg/ml	> 1000 µg/ml
48 h	1.27 µg/ml	173.7 µg/ml	> 1000 µg/ml

The apoptotic effect was assessed through cell counting utilizing the EB/AO test method, a technique employed to distinguish between live and dead cells. Cell viability was analyzed after treating the cells with the herb extract at concentrations of 44 µg/ml, 88 µg/ml, and 45000 µg/ml for 24 and 48-hour time points. Morphological evaluations of cells were conducted, revealing the presence of viable, necrotic, and apoptotic cells. The morphological alterations of the cells are depicted in Figure 2. Table 2 provides the percentages of viable, necrotic, and dead cells after treatment with three different concentrations of *P. spinosa* extract on the HT-29 cell line over 24 and 48 hours. The results of the 24 and 48-hour treatments indicate that *P. spinosa* extract induces cell death, with the extent of cell death showing a significant increase with both concentration and duration ($p < 0.01$, $p < 0.001$). Notably, the results exhibit a substantial rise in cell death with escalating concentrations, as illustrated in Figure 3. The IC_{20}, IC_{50}, and IC_{80} values obtained from the EB/AO double coloring test, analyzed using Probit analysis software for both 24 and 48-hour treatment periods, are presented in Table 3. According to the Probit analysis, the IC_{50} value is 159.3 for the 24-hour treatment and 123.8 µg/ml for the 48-hour treatment.

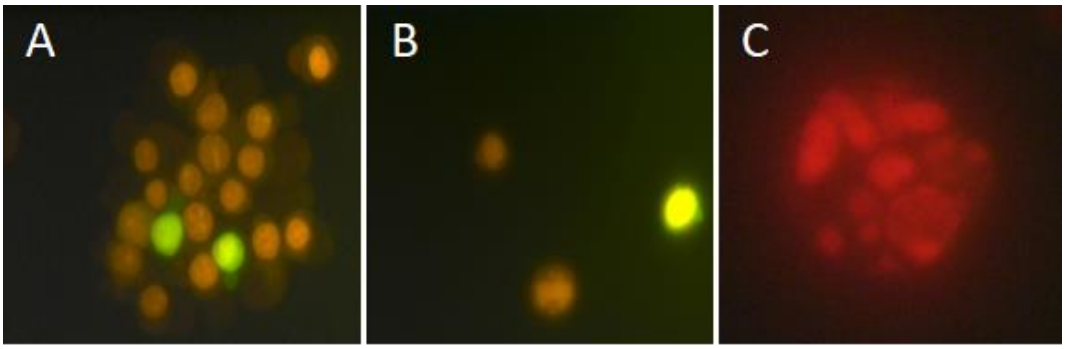

Figure 2. Fluorescence images of HT-29 cells after treatment with *P. spinosa* fruit water extract using the EB/AO dual staining method
(A) green cells are live and orange cells are necrotic (40x). (B) Orange cells were identified as apoptotic due to changes in morphology (40x). (C) apoptotic cell (100x).

Table 2. Percentage of viable, necrotic, and apoptotic HT-29 cells observed after three different concentrations of *P. spinosa* extract treatment after 24 and 48 hours, by EB/AO staining

Concentration (µg/ml)	Viable normal cells (%)	Necrotic cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)	Total cell viability (%)	Total apoptosis (%)
24 h						
Control	86.78 ± 6.05	7.7 ± 6.4	0 ± 0	5.4 ± 1.2	94.5 ± 6.2	5.44
44	58.63 ± 7.83[b]	0 ± 0	36.63 ± 5.04[b]	4.7 ± 2.7	58.6 ± 7.8[b]	41.38[b]
88	53.79 ± 5.51[b]	0 ± 0	42.97 ± 6.61[b]	3.2 ± 1.1	53.7 ± 5.5[b]	46.2[b]
45000	17.37 ± 0.26[b]	0 ± 0	81.62 ± 1.34[b]	1.01 ± 1.0	17.3 ± 0.2[b]	82.63[b]
48 h						
Control	75.74 ± 7.67	21.9 ± 8.9	0 ± 0	2.3 ± 1.3	97.6 ± 7.5	2.32
44	54.52 ± 5.64[a]	0 ± 0	42.45 ± 5.74[b]	3.0 ± 0.1	54.5 ± 5.6[a]	45.48[a]
88	52.34 ± 2.2[a]	0 ± 0	42.76 ± 2.27[b]	4.9 ± 0.6	52.3 ± 2.2[a]	47.66[a]
45000	5.51 ± 0.17[b]	0 ± 0	0 ± 0	94.4 ± 0.2[b]	5.5 ± 0.17[b]	94.49[b]

[a]= $p < 0.01$, [b]= $p < 0.001$

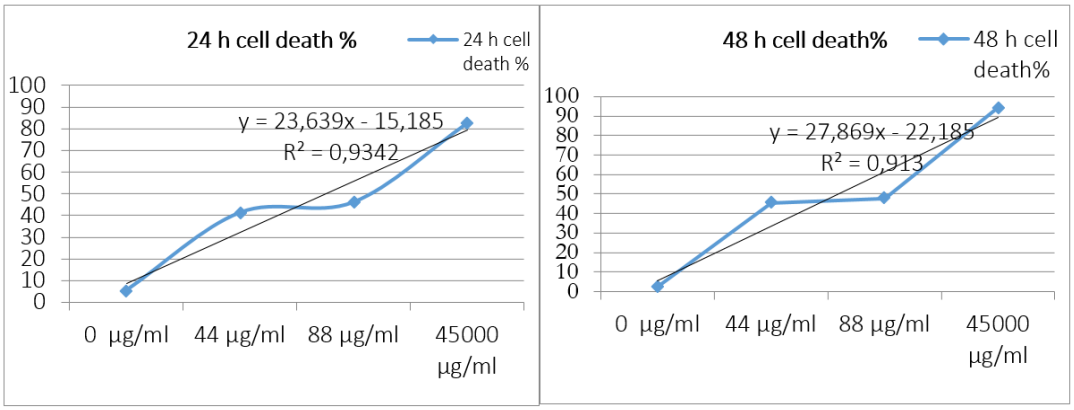

Figure 3. Apoptosis rates obtained after 24 and 48-hour treatment of *P. spinosa* fruit extract on HT-29 cells using the EB/AO dual staining method

Cell viability was detected on the HT-29 cancer cell line after exposure to different concentrations of *P. spinosa* water extract using the EB/AO dual staining method (Table 2).

The MTT viability test revealed a decrease in absorbance values after 24 and 48 hours compared to the control group. Notably, the IC_{50} value was determined to be 173.7 µg/ml following 48 hours of treatment. Similarly, employing the EB/AO method demonstrated

the maximum apoptotic effect at 48 hours, revealing an IC_{50} value of 123.8 µg/ml.

In recent years, to demonstrate the high cytotoxic effect of *P. spinosa*, extracts of different parts of the plant prepared in different solvents have been tested on cancer cell lines. *P. spinosa* fruit ethanol extract was tested on HepG2, Hepa1-6 (Murati et al., 2016), PC-3, and DU145 cells (Dedić et al., 2023), and methanol extract on MCF-7, NCI-H40, HCT-15, HeLa, and HepG2 cells (Guimarães et al., 2014), stem, leaves, fruits of *P. spinosa* with different solvents (Pinacho et al., 2015), and flowers (Karakas et al., 2019; Murati et al., 2019) on cancer cell lines. The types and quantities of phenolic compounds found in plant extracts differ, as well as their antiproliferative and cytotoxic effects on the cell lines, and the IC_{50} values detected may differ depending on the cell line used, the duration of the extract, the solvent, and the plant part. In our study, the effect on the cell viability of *P. spinosa* fruit water extract was tested by using MTT and EB/AO staining methods on the HT29 cell line. According to the probit analysis of *P. spinosa* fruit water extract, the MTT-viability test absorbance values were found to be decreased compared to control after 24 hours and 48 hours of treatment, and the IC_{50} value of 48th hour was found to be 173.7 µg/ml. Similarly, according to the EB/AO method, the apoptotic effect was detected at 48-hour treatment, and the IC_{50} value was determined as 123.8 µg/ml.

Table 3. IC_{20}, IC_{50}, and IC_{80} values obtained after 24 and 48-hour treatment by EB/AO double staining, according to Probit analysis

Incubation time	IC_{20}	IC_{50}	IC_{80}
24 h EB/AO	1.018 µg/ml	159.3 µg/ml	24908 µg/ml
48 h EB/AO	5.06 µg/ml	123.8 µg/ml	3031 µg/ml

Different solvents and different plant parts were used in other studies with *P. spinosa*. According to the part of the plant and the type of solvent; the variety, amount, antiproliferative and cytotoxic activities of the obtained phenolic compounds differ. Pinacho et al. (2015) reported that phenolic, flavonoid, and anthocyanin amounts obtained from *P. spinosa* stem, leaves, and fruits by extraction with different solvents (dichloromethane, ethyl acetate, ethanol, and water) differ in containing phenolic, flavonoid and anthocyanin amounts relating to the herb parts. The antiproliferative activities of water and ethanol extracts from different parts of plants exhibit variations (Nabende, 2015). The methanol extract from the leaves of *P. africana* was found to have higher antiproliferative activity on the mouse breast cancer cell line (4T1) and mouse colon cancer cell line (CT-26) compared to its water extract. However, body shell water extract had a higher antiproliferative effect on a colon cancer line than a breast cancer line (Nabende, 2015). Murati et al. (2016) found that alcohol extract of *P. spinosa* showed antiproliferative and prooxidant activity in mouse (Hepa1-6) and human (HepG2) liver cancer cell lines. 72nd hour IC_{50} values of *P. spinosa* ethanol extract in HepG2 and Hepa1-6 cell lines were determined as 68.2 µg/ml and 85.41 µg/ml, respectively (Murati et al., 2016). The reason behind these values being lower than those in our study might be the extended duration (72 hours), the utilization of ethanol extract, or the specific assay applied. In our investigation, the IC_{50} values of the water extract and the 48-hour results were evaluated and were found to be higher than the 72nd hour IC_{50} values.

The growth inhibition (48 hour GI_{50}) values of the non-anthocyanin phenolic compound enriched extract (methanol:water 80:20) of *P. spinosa* fruits on MCF-7 (breast carcinoma), NCI-H40 (non-small lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma) were determined as 270.65, 154.25, 220.44, 193.62, 169.56 µg/ml, respectively (Guimarães et al., 2014). These values were nearly identical to the IC_{50} values obtained in the results of the present study, where the IC_{50} values were 173.7 µg/ml and 123.8 µg/ml for MTT and EB/AO assays, respectively. This correlation could be attributed to the exclusive presence of phenolic acids and the highest levels of flavones and flavonols for *P. spinosa* phenolic extract. The researchers also confirmed that the samples were non-toxic on non-tumor liver primary culture (Guimarães et al., 2014). Dedić et al. (2023) also tested *P. spinosa* fruit extract on PC-3 and DU145 malignant prostate cell lines, indicating that the extract had dose-dependent antiproliferative activity on malignant cells. The fruit extracts exhibited 72-hour IC_{50} values ranging from 637 µg/ml to 950 µg/ml for PC-3 and from 385 µg/ml to > 1000 µg/ml for DU145 cells (Dedić et al., 2023). To compare our results, the aqueous extract of *P. spinosa* fruit IC_{50} values were lower (173.7 µg/ml and 123.8 µg/ml) indeed in the short-term (48 hours) treatments. Similar to the study of Dedić et al. (2023), a dose-dependent antiproliferative effect was also observed in the present study.

The 24-hour cell growth inhibition of HT-29 cells was found as IC_{50} 4790 µg-dw/ml after exposure to methanol/water extract of *P. spinosa* (BL1 genotype). IC_{50} values of the other genotypes ranged between 5880 and 28480 µg-dw/ml (Popović et al., 2020). In our study, 24-hour IC_{50} was found at over 1000 µg/ml, consistent with the study. Popović et al. (2020) also found a positive correlation between HT-29 cell toxicity and the 3-pCoQA hydroxycinnamic acid component of the extract. Hydroxycinnamic acids are the largest class of phenolic compounds, scavenge free radicals, and are associated with reduced cancer risk (Herrmann & Nagel, 1989; Huang et al., 1986; Manach et al., 2004; Spencer et al., 2008). It is also suggested that these compounds might modulate the activity of specific enzymes and inhibit cell proliferation (Manach et al., 2004). The anticancerogenic effects of hydroxycinnamic acids were studied on several cancer cell lines (Janicke et al., 2011; Kurata et al., 2007; Puangpraphant et al., 2011). It was shown that diCQA fractions inhibited the proliferation of RKO and HT-29 human colon cancer cells by inducing apoptosis rather than arresting the cell cycle. Apoptosis occurs via induction of Bax and Bcl-2 protein expression and diCQA induced the cleavage of procaspase-3 to active caspase-3 (Puangpraphant et al., 2011), which might explain the apoptotic activity in the present study. Similar results were obtained in our study; *P. spinosa* fruit water extract showed antiproliferative effects on the HT-29 colon cancer cell line according to the MTT test, and it was found to induce apoptosis according to the EB/AO test. IC_{50} values were obtained after 24 and 48 hours of treatment, as examined according to the EB/AO dual staining method; after 48-hour treatment, late apoptotic cells were observed more than at 24 hours. Remarkably, there was a significant decrease in the number of cells at high concentrations. It was found that the antiproliferative effect increased and showed a statistically significant difference compared to the control cells.

4. Conclusions

Our findings reveal that the water extract from *P. spinosa* fruit exerts an inhibitory impact on cell viability of the HT-29 cell line, demonstrating cytotoxic and apoptotic effects in a concentration- and time-dependent manner. Leveraging more advanced extraction methods to isolate metabolites from fruit extracts and conducting further investigations to elucidate the activities of these metabolites have the potential to enhance the economic value of *P. spinosa* and position it as a natural remedy in the anticancer pharmaceutical industry. This study highlights the cytotoxic impact of *P. spinosa* fruit water extract on the HT29 colon cancer cell line and its capacity to

impede cell viability. In toxicity-focused examinations, it has been observed that MTT and EB/AO methods complement each other, yielding comparable outcomes.

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Conflict of interest

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

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. On request, the associated author can provide more information.

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CRediT authorship contribution statement

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Supplementary File

None.

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RESEARCH ARTICLE

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Citrus reticulata fruit peel extract ameliorates testesterone-induced benign prostatic hyperplasia-like phenotypes in rats

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ABSTRACT

Benign prostatic hyperplasia is a major pathophysiologic event that presents a high risk for prostate cancer (the second most frequently diagnosed cancer in men). The prognosis of conventional therapy for BPH remains poor due to treatment failures. Thus, natural remedies such as botanical drugs remain a promising alternative therapy to be explored for the treatment of BPH and prostate cancer. *Citrus* fruits, specifically fruit peels of *Citrus reticulata* (CRE) contain bioactive compounds that exhibit anti-inflammatory properties and have been used in crude form in traditional settings to manage benign prostatic hyperplasia and prostate cancer, however, scientific scrutiny of this ethnobotanical claim remains incomplete. This study assessed the protective effect of CRE in testosterone-induced benign prostatic hyperplasia-like phenotypes in rats. Male Wistar rats (n = 30, 150-200 g) were randomly assigned into six groups (n = 5), and treated for 28 days as follows: control group (normal saline, 5 mg/kg s.c.), model group (testosterone,5 mg/kg, i.p.), the finasteride (1 mg/kg, p.o.), and CRE (50, 100, and 200 mg/kg, p.o.) groups received testosterone (5 mg/kg, i.p.) in the morning and their respective treatments (either finasteride or CRE). All rats were given chow and water ad libitum. On the 28th day, the rats were sacrificed following deep anesthesia. Blood and the prostate gland were collected. Full blood count, serum levels of prostate-specific antigen (PSA), testosterone, C-reactive protein (CRP), and histology of the prostate gland were assessed. Compared to the model, treatment with *C. reticulata* peel extracts markedly reduced prostate weight, attenuated atresia of the prostatic glands, stromal fibrosis, and mast cell infiltration, and increased glandular secretion. Additionally, serum levels of testosterone, CRP, PSA, and white blood count were reduced in the high-dose *C. reticulata* peel extract-treated group. Fruit peels of *C. reticulata* exhibited a protective effect against BPH partly by attenuating inflammatory activity. Thus, this finding provides a rationale for further exploration of CRE for novel anti-BPH molecules that could be used to develop therapeutics against prostate cancer.

1. Introduction

Benign prostatic hyperplasia (BPH) is a major pathophysiologic event that presents a high risk for prostate cancer. Prostate cancer is the second most frequently diagnosed cancer in men and the fifth major cause of death worldwide (Rawla, 2019; Wang et al., 2022). Phenotypic hallmarks of BPH include difficulty in initiating urination, painful micturition, weak or interrupted urine flow, incomplete urinary bladder emptying, haematuria, hematospermia, and orgasmalgia (Drudge-Coates et al., 2018; Leslie et al., 2023). BPH is normally screened using a serum prostate-specific antigen (PSA) test (Armstrong et al., 2017; Tikkinen et al., 2018) and digital rectal examination (Jones et al., 2018). However, BPH is normally confirmed histologically through prostate biopsy (Streicher et al., 2019). Aside from hereditary (Ni Raghallaigh & Eeles, 2022; Vietri et al., 2021) and testosterone imbalance (Parsons et al., 2005; Xu et al., 2015), available evidence suggests that the pathogenesis of BPH involves dysregulated cell death and

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proliferation (Campbell & Leung, 2021; Kyprianou et al., 2000), inflammation (Archer et al., 2020; Shafique et al., 2012) and oxidative stress (Battisti et al., 2011; Oh et al., 2016). Thus, agents with the ability to counter the aforementioned pro-BPH defective cellular processes may hold therapeutic potential for therapy against BPH and prostate cancer. Conventionally, drugs used to manage BPH and prostate cancer in particular include anti-androgens [5-α-reductase inhibitors and α-1-adrenergic receptor antagonists] (Rashid et al., 2020; Sarkar et al., 2019; Wade et al., 2019), microtubule inhibitors [tamoxifen, vinblastine, docetaxel (Taxotere) and cabazitaxel (Jevtana)] (Clarke et al., 2019; Pienta et al., 1995; Yamamoto et al., 2023), anti-inflammatory drugs [mitoxantrone (Novantrone)] (Doat et al., 2017; Hatano et al., 2020), and DNA-modifying drugs [estramustine (Emcyt), carboplatin, oxaliplatin and cisplatin] (Corn et al., 2019; Ravery et al., 2011). Although these anti-BPH and anti-prostate cancer drugs have proven relatively effective for managing BPH and prostate cancer over the years, however, they present many setbacks. For example, these drugs do not completely cure BPH and prostate cancer. Also, these drugs have serious side effects such as reduced libido, erectile dysfunction, and nasal congestion which significantly limit their therapeutic usefulness. Similarly, the use of surgery and radiation also cause serious side effects that significantly impair the quality of life after treatment (Miernik & Gratzke, 2020). The difficulties identified with the currently available therapies for BPH and prostate cancer necessitate the need for alternative therapies preferably therapies that are not only relatively safe and organic but also easily available and cost-effective such as those derived from plants.

Plants belonging to the genus *Citrus* and family Rutaceae have several applications in ethnomedicine and the utility of various parts of these plants in ethnomedicine is gaining scientific attention lately. The genus comprises about seventeen species including *Citrus lemon* L. (lemon), *C. sinensis* L. (sweet orange), *C. reticulata* Blanco (mandarin orange, tangerine), *C. aurantium* L. (bitter orange), and *C. paradise* M. (grapefruit). Morphologically, the genus *Citrus* includes plant species (shrubs and trees) with heights spanning 3 to 15 m (Klimek-Szczykutowicz et al., 2020). The leaves are leathery and lanceolate. Depending on the species, the stems may have several branches with spines. Also, the flowers develop in leaf axils. Every flower is penta-petalous and has either white or red color. A frequently used part of *Citrus* plants is their fruits, which are hesperidium berries. *Citrus* fruits have diverse applications due to their nutritional and extra-nutritional (cosmetic and pharmaceutical) qualities. Geographically, *Citrus* sp. are distributed naturally in warm and tropical ecological regions, including Africa and the Mediterranean (Klimek-Szczykutowicz et al., 2020).

Different parts of *Citrus* plants, such as fruits, fruit peels, leaves, and seeds, are used traditionally to treat various forms of diseases. The fruit peels of *C. reticulata* are traditionally used as tonic, stomachic, astringent, carminative, and skin care. Also, the dried fruit peels of *C. reticulata* are used to improve digestion and reduce phlegm (Lv et al., 2015). The medicinal uses of *C. reticulata* are attributed to its rich phytochemicals which have diverse bioactivities including anti-fungal, anti-bacterial, anti-hyperalgesia, anti-oxidant, and anti-inflammatory properties (Klimek-Szczykutowicz et al., 2020). Extracts from *C. reticulata* fruit peels were shown to antagonize lipopolysaccharide (LPS)-induced production of nitric oxide in macrophages (Zhang et al., 2022). This observation is indicative of the potential anti-inflammatory properties of *C. reticulata* fruit peels. Additionally, a polymethoxylated flavone, nobiletin, derived from the fruit peels of *C. reticulata* exhibited neuroprotection in a rat model of Parkinsonism (Jeong et al., 2015) and ameliorated memory impairment in a rat model of Alzheimer's disease (Kimura et al., 2018), highlighting the potential of mitigating age-related disorders. Also, two flavonoids, tangeretin, and nobiletin, that are derived from the fruit peels of *C. reticulata*, inhibited cancer growth in vivo, and also effectively inhibited the proliferation and blocked cell cycle progression at the G1 phase in colon and breast cancer cell lines (Morley et al., 2007). Furthermore, tangeritin caused apoptosis in HL-60 cells (human promyelocytic leukemia cells) but not in human peripheral mononuclear cells (Hirano et al., 1995), highlighting selective toxicity of the flavonoid against cancerous cells. This observation suggests that the fruit peels of *C. reticulata* could prevent prostate cancer initiation. Therefore, this study investigated the effect of *C. reticulata* fruit peel extract (CRE) against testosterone-induced BPH-like phenotypes in rats as well as the possible phytochemical composition of CRE.

2. Materials and methods

2.1. Drugs and chemicals

Testosterone propionate, finasteride, and ethanol were obtained from the Center for Plant Medicine Research (CPMR), Akuapem-Mampong, Eastern Region, Ghana. The animal experimentation and extraction were carried out at the laboratories of the CPMR. Biochemical assays and hematological analyses were performed at the laboratories of the University of Ghana Medical Center, Accra, Ghana.

2.2. Collection and identification of plant material

Fruits of *C. reticulata* were obtained from local farmers in Mankesim village, Central Region, in January 2022. The sample was identified, confirmed, and authenticated by Mr. Francis Otoo, a botanist at the Herbarium of the School of Biological Science, University of Cape Coast where a voucher specimen was deposited (https://ir.ucc.edu.gh/xmlui/handle/123456789/8598).

2.3. Preparation of C. reticulata fruit peel extract

The *C. reticulata* fruit peel extract was prepared as described previously (Boye et al., 2024) with slight modifications. Briefly, the peels of the *Citrus* fruits were removed and dried at room temperature after washing the fruits under tap water. A known mass of the dried peels was grounded using an electrical blender (Philips HL7777-00) after which soxhlet extraction was performed on the resultant powder. The powder was placed in the thimble, which was then placed in a distillation flask containing ethanol (200 ml). The ethanol was heat-refluxed for 12 h during which the vapor extracted solutes from the powder into the bulk ethanol in the distillation flask. The ethanol-turned extract was placed in a desiccator. After complete drying, the final crude ethanol extract of *C. reticulata* fruit peel was code-named CRE. The extraction process was repeated several times to obtain more CRE. CRE was stored in a refrigerator at –20 °C until use.

2.4. Animal acquisition and husbandry

Thirty (30) male Wister albino rats aged 10-15 weeks and weighing 150-200 g were purchased and housed in cages at the Animal Holding Facility of CPMR. The rats were kept in well-ventilated cages at normal room temperature (35-37 °C) and humidity and fed with regular laboratory chow (Grower mash, Sankofa) in a sipper bottle/spill-proof bowl. Animal experiments, procedures, and techniques were done according to institutional, national, and

international guidelines concerning the use of animals in scientific experimentation.

2.5. Establishment of testosterone-induced prostatic hyperplasia in rats

Benign prostatic hyperplasia was induced in rats as previously described by Cai et al. (2018) with slight modifications. The rats were randomly assigned into six groups. Except for the control rat group, the five rat groups were anesthetized with phenobarbital injection (50 mg/kg, i.p.), right and left testicles were removed aseptically. The rats were then intraperitoneally injected with 5 mg/kg testosterone propionate for 28 days and concurrently treated with either CRE or standard drug (Finasteride, 1 mg/kg, p.o.). Additionally, rats that did not receive testosterone injection served as controls. The experimental groups were:

- Control group: Rats in the control group received normal saline (5 mg/kg, i.p.) daily in the morning for 28 days.
- Model group: Rats in this group were intraperitoneally injected with testosterone propionate (5 mg/kg, i.p.) in the morning for 28 days without any other treatment.
- Finasteride (1mg/kg, p.o.) group: Rats in this group received intraperitoneal testosterone propionate (5 mg/kg, i.p.) injection in the morning and finasteride (1mg/kg, p.o.) in the afternoon for 28 days.
- CRE (50 mg/kg, p.o.) group: Rats received testosterone propionate injection (5 mg/kg, i.p.) in the morning and CRE (50 mg/kg, p.o.) in the afternoon for 28 days.
- CRE (100 mg/kg, po) group: Rats received testosterone propionate injection(5 mg/kg, i.p.) in the morning and CRE (100 mg/kg, p.o.) in the afternoon for 28 days.
- CRE (200 mg/kg, po) group: Rats received testosterone propionate injection(5 mg/kg, i.p.) in the morning and CRE (200 mg/kg, p.o.) in the afternoon for 28 days.

2.6. Body weight measurement

The body weight of rats was determined weekly throughout the study. On the last day, following overnight fasting and testosterone propionate injection, the rats were anesthetized before sacrifice.

2.7. Biochemical analysis of blood

Blood was collected into labeled EDTA anti-coagulant tubes for full blood count using (Mindray BS200) and labeled Gel tubes for biochemical analysis namely testosterone, C-reactive protein, and prostate-specific antigen (PSA).

2.8. Serum PSA measurements

Serum PSA was determined using the Enzyme-Linked Immunosorbent Assay (ELISA) kit as described previously (Dalal et al., 2022). Serum samples or standards (0, 2.5, 5.0, 10, 25, and 50 ng/ml of PSA) (v = 25 µl) were placed in wells of 96-well plate pre-coated with HRP-labeled anti-mouse PSA (v = 100 µl) after which the plate was incubated at 20-25 °C for 30 min. The wells were washed thrice with 300 µl of a reconstituted washing buffer. A 100 µl substrate-reagent mixture consisting of TMB (3,3′, 5,5′-tetramethylbenzidine) (1.2 mM) and hydrogen peroxide (≤ 6.0 mM) was then added to each well and the total mixture was thereafter incubated for 15 min at 20-25 °C. The reaction was halted using 100 µl of a stop solution (0.5 M sulphuric acid) and the absorbance of the reaction mixture was measured subsequently at a wavelength of 450 nm using a Urit 680 microplate analyzer. The straight-line graph was plotted using the various standard concentrations (as abscissa) and their respective absorbance (as ordinate). The serum PSA concentration was subsequently determined using an equation of the linear graph.

2.9. Serum testosterone level

Serum testosterone level was determined using enzyme immunoassay as described previously (Njoroge et al., 2015). Goat anti-rabbit IgG-coated wells of a 96-well plate were filled with 150 µl of a reaction mixture, consisting of rabbit anti-testosterone reagent and testosterone-HRP conjugate (1:2). A 100 µl of either testosterone standards or serum was added to the wells after which the plate was incubated for 90 min at 37 °C. The wells were rinsed thrice with washing buffer (1x). TMB substrate(100 µl) was added to the reaction mixture and after 10 seconds, the plate was incubated for 20 min at room temperature (18-22 °C). A 100 µl stop solution was added to the wells and the absorbance of the reaction mixture was read within 15 min at 450 nm using a microfilter well reader.

2.10. Histological assessment of prostate tissues using Gleason score

Prostate glands were dissected out, weighed, and preserved in 10% formalin solution. The fixed prostate gland was sliced and the slices were dehydrated in increasing gradient concentrations of ethanol, cleared with xylene, and embedded in paraffin wax. Thin tissue sections (4-5 µm) were cut from the paraffin-embedded tissue blocks using a microtome (Leica Histocare Autocut), treated with hematoxylin and eosin stains and mounted on the slide using DPX. Images of the five fields of the dorsal lobes of the mice's prostate glands were captured using a bright field binocular microscope connected to a high-resolution camera. After the histological assessment, the micrographs were graded by a pathologist who did not know the different treatment groups. The International Society of Urological Pathology (ISUP)-modified Gleason grading (Epstein et al., 2016) was used to grade the histology of the prostate gland according to the criteria described in Table 1.

Table 1. The International Society of Urological Pathology (ISUP)-modified Gleason grading

Characteristics	Score
Small invariable glands	1
More interglandular stroma	2
Distinctively invasive margins	3
Asymmetrical masses of neoplastic glands	4
Only seldom gland formation	5

Briefly, the most common pattern (primary or dominant) was graded after which the next common pattern (secondary or subdominant) was graded. The grades for the two patterns were summed to get the Gleason score for the prostate gland.

2.11. Statistical analysis

Graph Pad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analyses. Except for the data on the Gleason scores which were reported as medians, all other data were expressed as mean ± standard deviation. Comparisons of group data were done using one-way ANOVA followed by a post hoc test (Dunnett's test). The Kruskal-Wallis test was used to assess the differences among the median scores. *p*-value less than 0.05 was considered statistically significant.

3. Results and discussion

C. reticulata fruit peel extract demonstrated prophylactic potential against experimentally-induced androgen-dependent prostate cancer, which is one of the most frequent malignancies in older men. Testosterone propionate was used to induce prostatic hyperplasia phenotype in Wistar rats. Testosterone binds and activates the androgen receptor (AR) in the prostate gland. A testosterone-androgen receptor complex forms which is then translocated to the nucleus to activate genes that promote the survival and proliferation of prostatic cells (Gerald & Raj, 2022). Additionally, prostate gland pathophysiology involves inflammation, which is inducible by hypertestosteronemia. In this study, the anti-BPH effect of CRE was assessed by measuring serum levels of testosterone, PSA, and C-reactive protein (CRP), determining full blood count (FBC), and also assessing the histological architecture of the prostate gland. The findings from this study suggest that *C. reticulata* fruit peel extract could delay the progression of prostate cancer.

3.1. Phytochemical composition of CRE

The bioactivity of plant extracts is attributable to myriads of secondary metabolites that are present. Phytochemical screening of CRE showed the presence of secondary metabolites, namely flavonoids, tannins, alkaloids, terpenoids, and saponins. The anti-inflammatory, antioxidant, and anti-tumor properties of all the identified secondary plant metabolites in CRE (Table 2) have been demonstrated, but in other *Citrus* plants. These suggest that the observed phytochemical signature may be a shared characteristic of all the plant species in the genus *Citrus*. More so, prostate cancer pathophysiology involves oxidative stress, inflammation, and proliferation (Archer et al., 2020; Battisti et al., 2011; Campbell & Leung, 2021; Kyprianou et al., 2000; Oh et al., 2016; Shafique et al., 2012), and thus CRE have the potential to mitigate prostate cancer progression. The prophylactic prostate-protective effects of CRE were expected given its phytochemical signature.

Table 2. Phytochemical composition of CRE

Secondary plant metabolites	Phytochemical test	Reference	Results
Alkaloids	Dragendorff's test	Tanzey et al. (2020)	+
Tannins	$FeCl_3$	Kuntal et al. (2018)	+
Flavonoids	Sodium hydroxide test	Barupal et al. (2019)	+
Terpenoids	Salkowski test	Abdel-Rahman et al. (2019)	+
Saponins	Foam test	El Hazzam et al. (2020)	+

+ = qualitatively detected, CRE = *C. reticulata* fruit peel extract

3.2. Effect of CRE treatment on changes in body and prostate gland weights

Benign prostatic hyperplasia is characterized by an increase in prostate weight, which could be detected in a digital rectal examination during the screening process. An increase in prostatic weight is a crucial indicator of the start of benign prostatic hyperplasia (Akanni et al., 2020). Rats in the model group significantly recorded a higher percentage loss in body weight relative to the rats in the control group. However, relative to the model group, the finasteride-treated and CRE-treated testosterone-injected rats gained weight. The mean weight of the prostate gland was increased in the model group relative to the control group. Relative to the model group, treatment with finasteride and CRE decreased the weight of the prostate gland (Figure 1). Prostate weight and size increases are a direct result of testosterone's crucial role in the development of the prostate gland (Li et al., 2018). In this study, the model group exhibited an increase in prostate weight, which was attenuated after treatment with CRE, highlighting the anti-prostate cancer potential of *C. reticulata* fruit peel extract.

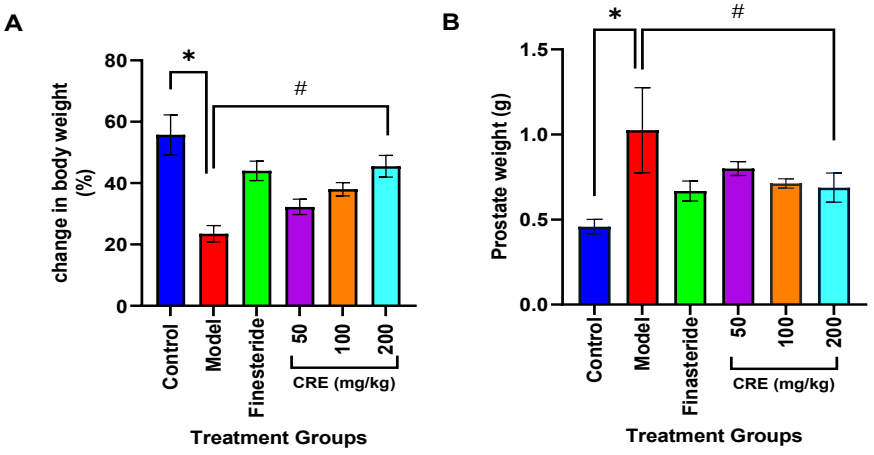

Figure 1. Effect of CRE treatment on body weight and prostate weight

3.3. Effect of CRE on the histology of testosterone-induced BPH

Benign prostatic hyperplasia is confirmed via histological assessment of a prostate biopsy. In this study, there were marked changes in the histological architecture of the prostate gland in the model group relative to the control. Relative to the control group, the model showed shrunken prostate glands with scanty secretions, increased interstitial fibrosis, and mast cell infiltration, indicative of inflammatory processes in the model group (Figure 2). Additionally, the epithelia of the prostate glands were markedly different from

that of the normal rats, indicative of poorly differentiated prostate glands. CRE dose-dependently preserved the prostate gland histology. In the peel extract-treated group, the prostate gland exhibited increased prostatic secretion and reduced stromal fibrosis, indicative of the therapeutic potential of CRE. Additionally, the columnar cells lining the prostate gland appeared closer to the control relative to the model, indicative of at least a moderately differentiated prostate gland (Figure 2). These observations suggest a histoprotective potential of the peel extract in prostate cancer.

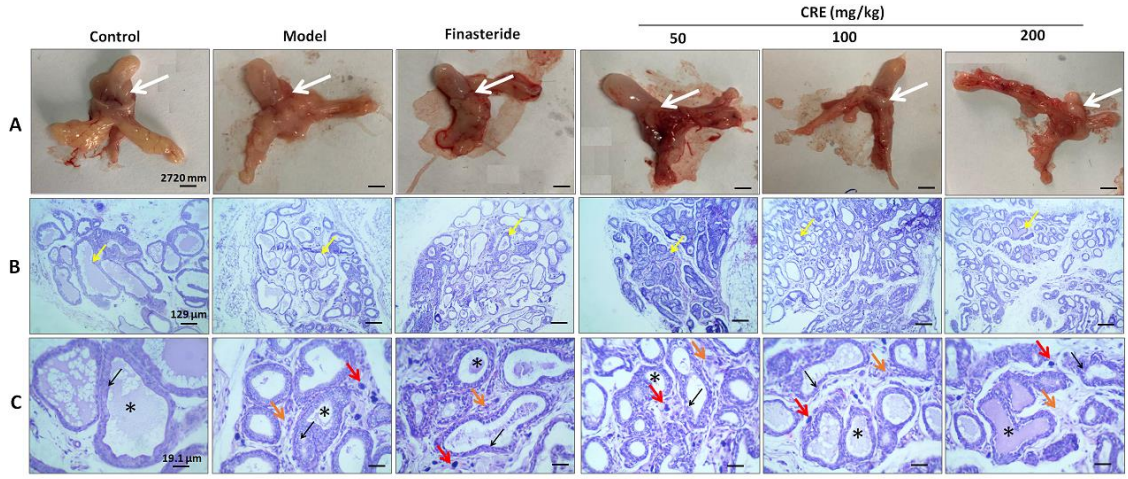

Figure 2. Effect of treatments on gross (A) and histopathological appearance (B&C) of the prostate

Treatment groups: Control group, model group, finasteride group, CRE group (50 mg/kg), CRE group (100 mg/kg), CRE group (200 mg/kg). The yellow arrow represents prostatic glands, The black asterisk (*) represents prostatic secretion, the black arrow represents epithelial cells of the gland, the red arrow represents mast cells, and the brown arrow represents lymphocytic infiltration. CRE: *C. reticulata* fruit peel extract.

Also, histological grading of prostate cancer is a powerful prognostic indicator for clinically localized prostate cancer and is one of the most vital factors in determining the course of patient management. In this study, the 2014 ISUP-modified Gleason grading system was used to assess the histology of the prostate glands (Epstein et al., 2016; Humphrey, 2017). The grading system reports the sum of the scores for the dominant gland pathology and the subdominant gland pathology (Delahunt et al., 2012; Humphrey, 2017), highlighting the usefulness in tracking the progress of the disease. The model group had a Gleason score of 10, with scores of 5 and 5 for dominant and subdominant pathologies, respectively. The finasteride-treated group had a Gleason score of 6, with scores of 3 and 3 for dominant and subdominant pathologies, respectively. The low-dose, middle-dose, and high-dose CRE-treated groups had Gleason scores of 9, 7 and 7, respectively. The dominant and subdominant pathologies of CRE-treated groups were 5 and 4 for low dose, 4 and 3 for middle dose, and 3 and 4 for high dose (Figure 3).

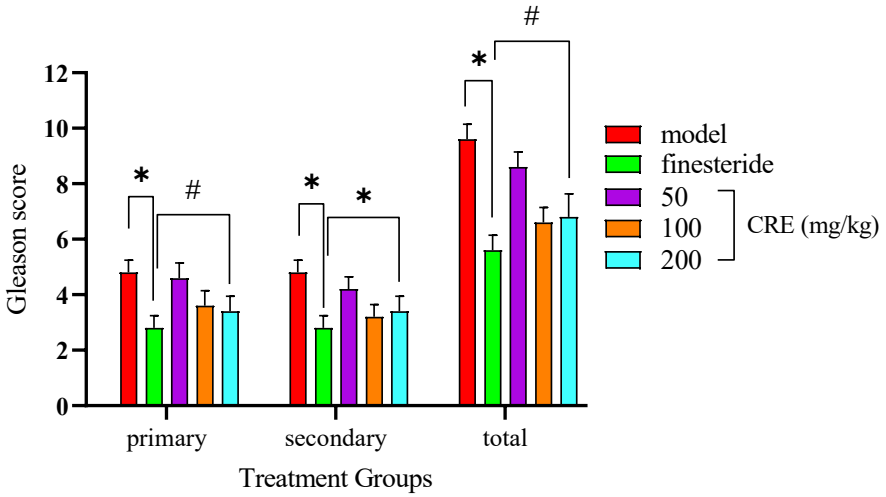

Figure 3. Assessment of the effect of CRE on prostate histology using Gleason score

In this study, the model group recorded median scores of 5 for both dominant and sub-dominant pathologies. The median scores for dominant and subdominant pathologies of CRE-treated groups were 5 and 4 for low dose, 4 and 3 for middle dose, and 3 and 4 for high dose, indicating the histoprotective potential of CRE (Figure 3).

3.4. Effect of CRE on full blood count

Clinicians monitor prostate cancer using a plethora of serum markers including testosterone and prostate-specific antigen (PSA). Testosterone is the predominant male androgen and high serum levels of testosterone increase the risk of developing prostate

cancer; hypertestosteronemia promotes the proliferation of the prostate gland. Additionally, extraprostatic organ damages, involving the kidney and liver, occur in hypertestosteronemia.

PSA is a glycoprotein enzyme secreted by the epithelial cells of the prostate gland (Duskova & Vesely, 2015). Assaying serum levels of PSA is useful in prostate cancer detection and patient treatment and monitoring. The serum PSA levels of men with healthy prostates are minute, but is often elevated in prostate cancer or other prostate disorders (Mazzucchelli et al., 2000). In this study, the model groups recorded high levels of serum testosterone and prostate-specific antigen (PSA) (Figure 4), highlighting the increased risk of the model group to developing prostate cancer and hyper testosterone-associated extra-prostatic damage. Treatment of the model rats with CRE decreased the serum levels of testosterone and PSA, indicative of the protective effect of CRE.

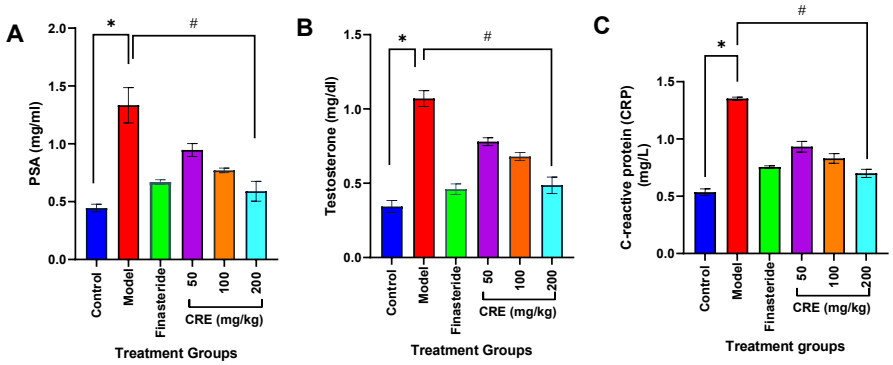

Figure 4. Effect of CRE on PSA, testosterone, and C-reactive protein levels
Each value is the mean ± SD, $n = 3$. # $p < 0.05$ (treatment versus model groups); * $p < 0.05$ (control versus model groups)

Inflammation is one of the mechanisms that underlie the pathophysiology and progression of prostate cancer and other cancers (Libby, 2007). Several indicators, including C-reactive protein (CRP), a ring-shaped pentameric protein, and white blood cell count are used in the clinical setting to assess the inflammation status of cancer patients. Serum CRP levels rise in response to inflammation, which is associated with an increased risk of developing BPH (O'Brian et al., 2021). In this study, serum CRP level was increased in the model group after testosterone induction. Additionally, the stroma of the prostate gland from the model group exhibited massive infiltration of mast cells (Figure 2) and higher counts of WBC (Figure 5), which were attributable to elevated counts of lymphocytes, monocytes, and neutrophils (Figure 5). The changes in the aforementioned variables underscore the involvement of inflammation in prostate cancer pathophysiology, thus reduction in inflammation could delay cancer progression. CRE decreased mast cell infiltration of the prostate gland stroma, and reduced blood counts of leukocytes, neutrophils, lymphocytes, and monocytes as compared to the model group, highlighting the anti-inflammatory potential of CRE in mitigating BPH.

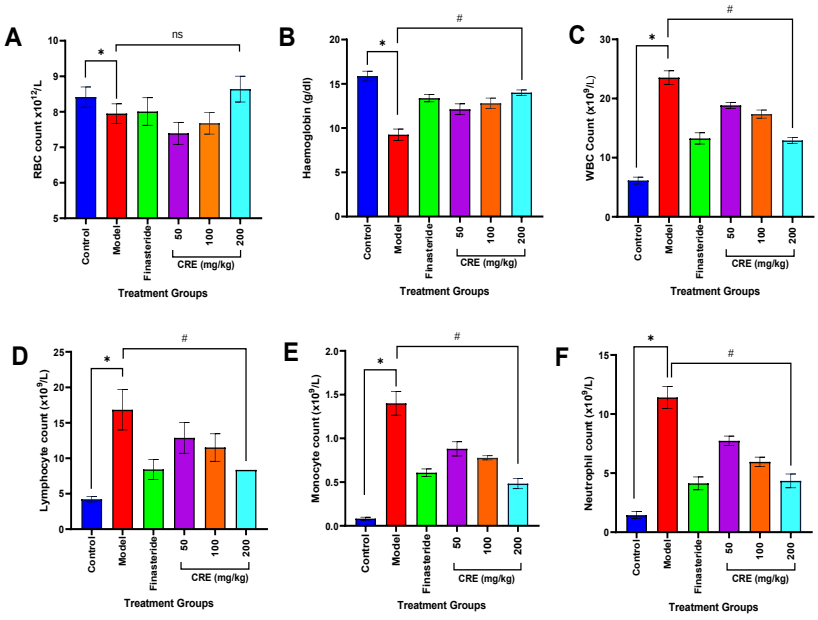

Figure 5. Effect of CRE on full blood count (FBC)
Hemoglobin, red blood cell (RBC) count, white blood cell (WBC) count, lymphocyte count, monocyte count, neutrophil count. Each value is the mean ± SD, $n = 4$. # $p < 0.05$ (control versus model groups); * $p < 0.05$ (treatments versus model groups). CRE: *C. reticulata* fruit peel extract

The findings of this study provide a scientific context and rationale for further incremental studies on CRE concerning BPH and prostate cancer even though the study could not benefit from additional assessments such as immunohistochemistry of the prostate gland, use of flow cytometry to monitor cell proliferation, and cell cycle arrest status. Notwistandingly, the present finding provides the basis for additional future studies as well as the generation of new scientific questions to drive the direction of future studies.

4. Conclusions

C. reticulata fruit peel extract prevented the exercabation of testosterone-induced BPH-like phenotypes in rats. The anti-BPH activity of CRE could be attributed partly to its anti-inflammatory phytochemical components. Thus, this finding provides a rationale for further exploration of CRE for novel anti-BPH molecules that could be used to develop therapeutics against prostate cancer.

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Conflict of interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

Statement of ethics

All animal experiments and procedures used in this study were in full compliance with standard institutional (UCCIRB/CHAS/2022/90), national, and international guidelines (Guide for the Care and Use of Lab Animals, NIH publication No. 85-23) regarding the use of animals in scientific experimentation.

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RESEARCH ARTICLE

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Therapeutic effect of the solvent fraction of hexane leaf extract of *Tapinanthus bangwensis* (Engl. & K. Krause) (Loranthaceae) in alloxan-induced pathology in diabetic rats

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ABSTRACT

Plant-based products are gradually replacing pharmaceuticals in treating ailments, including diabetes, due to their safety, cost-effectiveness, potency, and availability. Therefore, the current study looked into the therapeutic effect of the solvent fraction of hexane leaf extract of *Tapinanthus bangwensis* (HEXETACF) (Loranthaceae) in alloxan-induced pathology in diabetic rats. The biochemical parameters were estimated using analytical grade kits via spectrophotometric method. The laboratory rats were distributed into group W (five rats on feed and water), group X (seven rats + 150 mg/kg alloxan solution only), group Y (seven rats + 150 mg/kg alloxan solution + 200 mg/kg BW silymarin for 21 days), and group Z (seven rats + 150 mg/kg alloxan solution + 250 mg/kg BW HEXETACF for 21 days). The results showed that HEXETACF and silymarin (SILY) reduced blood glucose concentration by 33.77% and 34.80%, respectively, after the 21st day of treatment ($p < 0.05$). Additionally, alkaline phosphatase (ALP) and alanine aminotransferase (ALT) activity in SILY and HEXETACF were significantly decreased compared to the diabetic group ($p < 0.05$), but no significant decrease in aspartate aminotransferase (AST) activity was observed between the test samples and the diabetic group. Furthermore, the test samples lowered malondialdehyde (MDA) levels, by improving glutathione, superoxide dismutase (SOD), and catalase (CAT) activity. The HEXETACF and SILY significantly decreased triglyceride levels (TG) compared to the diabetic group at $p < 0.05$. They also reduced low-density lipoprotein (LDL) and cholesterol levels and increased the high-density lipoprotein levels compared to the diabetic group. Additionally, no significant decrease in serum electrolytes (Na^+, K^+, and Cl^-), urea, and creatinine (including albumin and total protein) values was observed in HEXETACF and SILY, while hematological indices increased compared to the diabetic group. Histology results revealed that the test samples had normalized glomeruli, β-islet cells, and hepatocytes. However, a trace of mild congestion was noticed in the STDG. But edemic blood congestion was observed in the diabetic group. In conclusion, the current result demonstrated that HEXETACF may be a promising antidiabetic agent that could replace mSILY.

1. Introduction

The global record indicated that annually, diabetic cases rise to about 321.000 in sub-Saharan Africa and about 1.5 million worldwide. This outcome had been attributed to poor nutrition, alcoholism, a sedentary lifestyle, and drug abuse (International Diabetes Federation, 2015; Wang et al., 2015). Furthermore, the International Diabetes Federation predicted that diabetic cases in adults (18 – 90 years) would increase from 10.5% (451 million) in 2021 to approximately 12.4% (532 million) by 2045 (Cho et al., 2018; Sun et al., 2022). Considering the statistics above, researchers and funding partners are called upon to upscale the fight against diabetes mellitus, by developing effective treatment models for reducing or eradicating the disease. According to records, diabetes is the third killer disease, including a chronic metabolic

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and non-communicable disease, characterized by persistent hyperglycemia caused by defective insulin secretion or insensitivity/resistance or, in some cases, by both factors, and therefore the blood system exceeds the normoglycemic level and condenses with glucose (Ononamadu et al., 2019; Wong et al., 2024). Diabetologists categorized diabetes mellitus (DM) as type 1 diabetes (insulin-dependent diabetes mellitus, IDDM), type 2 diabetes (non-insulin-dependent diabetes mellitus, NIDDM), and pregnancy-related diabetes/gestational diabetes mellitus (PRDM or GDM). However, NIDDM is considered the most prevalent. In IDDM, the glucose level persistently increases, owing to the absence of insulin, because the islet cell has been immunologically destroyed. However, in type 2 diabetes, insulin is either not secreted sufficiently, its activity is resisted, or sometimes synergy occurs (Feyisayo & Victor, 2019; Ihegboro et al., 2020b), while gestational diabetes is triggered during pregnancy and normalizes after delivery. In the bid to find lasting solutions to this metabolic disorder, Ihegboro et al. (2022) submitted that inhibiting key metabolic pathways/enzymes, and/or the use of synthetic drugs (metfonin, glibenclamine, acarbose), may be a potent approach.

However, the fact that medicinal plants have a unique pharmacological potential compared to synthetic drugs has led to increased interest in ethnomedicine (Kolhe & Rachh, 2018; Wang et al., 2022). There are already available results comparing the antidiabetic potential of medicinal plants with standard drugs (methonin or glibenclamine), but results on silymarin are limited despite its anti-cancer, anti-inflammatory, antioxidant and antidiabetic properties (Tuorkey et al., 2015).

As far as we are aware, no result shows the antidiabetic capacity of *Tapinanthus pangenesis* with silymarin. The current research looked into the therapeutic effect of solvent fraction of hexane leaf extract of *T. bangwensis* in alloxan-induced pathology in diabetic rats, about silymarin. Briefly, *T. bangwensis*, normally found on the *Citrus* tree as a parasite, belongs to the family of the Loranthaceae. It is ecosystem-friendly in African regions and exhibits several ethnomedicinal properties. The countries where the plant is domicile have designated local names, but in Nigeria, the Hausa, Igbo, and Yoruba call it Kauci, Awurusie, and Afomo onisana, while English tacked it (all purpose herb, healing tree, life-giving tree, or bird lime), respectively (Ihegboro et al., 2020a).

2. Materials and methods

2.1. Materials

Silymarin tablets (Silybon-140 mg, India), alloxan monohydrate (Aldrich-Sigma, United Kingdom), silica gel (60-120 mesh, England), glucose strip (ACHUCHEK, USA), hexane, ethylacetate and formaldehyde solvents (BDH, England), biochemical analytical kits (Randox Laboratory Limited, United Kingdom), but not limited to the aforementioned. All the materials were of quality and analytical grade.

2.2. Plant material identification

The plant's fresh leaves were acquired in March 2022 from Mushin situated at 6°32'6.84''N and 3°20'56.28''E co-ordinate of Lagos State. The plant material was identified at the University of Lagos (Department of Pharmacognosy) by Mr. Adeleke, a taxonomist. He facilitated the issuance of the registration number (LUH 4323) and requested that a sample be kept in the institution's herbarium to ensure its traceability and authenticity.

2.3. Preparation of plant material

The leaves were washed, air-dried, and pulverized into powdered mass. About 1500 g was soaked in 5000 ml of hexane solvent and allowed to stand for 2 days, with stirring at intervals. After filtration, it was exposed to the atmosphere for evaporation, and 62.04 g of solid hexane extract was recovered. Furthermore, the recovered extract was loaded onto a column glass already packed with a mixture of silica gel and hexane solvent. After washing the column with the different combinations of the eluting solvents (hexane and ethylacetate), approximately eighty-eight fractions were collected, which were later pooled into three fractions using the TLC plate, taking into account their retention factors. The above protocol was used by Ihegboro et al. (2020a).

2.4. Familiarization of animals with the new environment

Twenty-six healthy rats (males) were purchased from the University of Lagos' animal breeding laboratory. Their weight ranges were between 100 – 110 g. They were conditioned to the new environment (temperature: 25 °C, humidity: 55 °C, and illumination: 12 hours day/night cycle) for 2 weeks, before commencing the experiment, while feeding with commercially formulated rat feed and clean water (Ihegboro et al., 2020a).

2.5. Initiation of diabetic condition in the animals

The method of Emordi et al. (2018) was used. After acclimatization, the rats had become physiologically stable. Type 2 diabetes was then induced by administering a freshly prepared alloxan solution of 150 mg/kg body weight into the intraperitoneal region and leaving it for three days. When blood glucose was checked with a glucometer (ACHUCHEK, USA), a hyperglycemic condition (> 300 mg/dl) developed compared to normoglycemic rats that had 85 mg/dl.

2.6. Animal groupring and treatment

In this section, the method applied by Ihegboro et al. (2020a) was used. The animals were distributed as follows:

Group W had five rats that were fed with food and water only (normal control). Group X had seven rats that received 150 mg/kg BW alloxan solution only (diabetic group). Group Y had seven rats that were treated with 200 mg/kg BW silymarin for 21 days, after receiving 150mg/kg alloxan solution, while group Z had seven rats that were treated with 250mg/kg BW of the fraction for 21 days, after receiving 150mg/kg alloxan solution. After the 21st day of treatment, the Wistar rats were anesthetized using chloroform, and blood was collected by puncturing the jugular vein in the neck region.

2.7 Analysis of the oxidative stress markers

The liver was washed in 1.15% potassium chloride solution shortly after excision, then homogenized in phosphate buffer (pH 6.2) and then centrifuged at 1593 × g for 5 min. The collected supernatant was used to measure glutathione and malondialdehyde levels, including the activities of catalase and superoxide dismutase enzymes.

2.7.1. Quantification of reduced glutathione level

In this section, the method applied by Fatima et al. (2016) was found suitable. The method involves the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) also called Ellman's reagent, by a

sulfhydryl group to produce 2-nitro-5-mercaptobenzoic acid. The process started by diluting 50 ml of the liver homogenate into 1.0 ml of 0.1 M phosphate buffer (pH 8.0). Furthermore, the resulting mixture (3.0 ml) was added to a 20 ml of 0.01 M DTNB, and the yellow-colored product was measured spectrophotometrically at 412 nm after 5 min.

2.7.2. Quantification of superoxide dismutase (SOD) activity

In this section, the experimental protocol of Katrenčíková et al. (2021) was followed. The liver homogenate (0.05 ml) was added to a mixture of 0.186 mM methosulfate phenazine (0.1 ml), 0.3 mM nitroblue tetrazolium chloride (0.3 ml), 1.2 ml of 0.05 M sodium pyrophosphate buffer (pH 8.3), and 0.2 ml of 0.78 mM reduced nicotinamide adenine dinucleotide (NADH). After 1 min and 30 seconds, the reaction was halted by adding glacial acetic acid. In addition, 20 ml butanol was added later to remove the chromogen formed. The supernatant recovered from centrifuging the mixture at 1593 x g for 10 min was measured spectrophotometrically at 500 nm.

2.7.3. Quantification of catalase activity

To measure the ability of catalase to degrade hydrogen peroxide, the method followed by Katrenčíková et al. (2021) was used. Briefly, the liver homogenate (0.05 ml) and 1.95 ml of 0.05 M phosphate buffer (pH 7.4) were thoroughly mixed, after which 1.0 ml of 19 mM H_2O_2 was added. The entire mixture was left for a while before reading the absorbance spectrophotometrically at 240 nm.

2.7.4. Quantification of malondialdehyde level

The method of Kolagal et al. (2009) was used to measure the color intensity (pink color) formed when MDA reacted with two molecules of acidified thiobarbituric acid (TBA) at 40 °C. In a nutshell, a resulting mixture containing 0.5 ml of 20% tricarboxylic acid (TCA), 1.0 ml of 0.67% TBA, and the liver homogenate (0.5 ml) was incubated for 15 min. Later, 2.0 ml of *n*-butanol was added, followed by centrifugation at 1593 x g for 15 min. The spectrophotometric measurement of the supernatant was taken at 532 nm. To quantify the MDA level, a calibration curve was plotted by preparing different concentrations of 1,1,3,3-tetraethoxypropane, from which the MDA level would be extrapolated.

2.8. Analysis of liver enzymes activity

2.8.1. Estimation of alanine and aspartate aminotransferases

In this section, the method of Adeyemi and Orekoya (2014) was used. After mixing the both serum (0.1 ml) and 0.5 ml of the chemical reagent (containing L-alanine, oxoglutarate, and phosphate buffer, pH = 7.4), they were incubated at 37 °C. After cooling (30 min), 0.5 ml of 2 mM 2,4-dinitrophenylhydrazine was added and the entire content was mixed vigorously, and left for 25 min, before adding 0.4 mM sodium hydroxide (0.5 ml) to produce a color change, which was measured spectrophotometrically at 546 nm as against the blank.

The above procedure is also valid for estimating aspartate aminotransferase activity, except that the chemical reagent used includes L-aspartate, oxoglutarate and phosphate buffer.

2.8.2. Estimation of serum alkaline phosphatase

To estimate the serum alkaline phosphatase, the method of Tietz (2006) was used. This is a unique method in which the reaction between ALP and phenolphthalein monophosphate produces a pink colored product called *p*-nitrophenol. Shortly, after obtaining the resulting mixture, which contained the serum (0.1 ml) and 0.5 ml of the reagent [containing phenolphthalein monophosphate (63 mM) and 2-amino-2-methyl-1-propanol (pH = 7.9), it was incubated at 37 °C for 10 min. Moreover, 0.5 mL of 80 mM disodium hydrogen phosphate was added and left to stand for 20 min, before introducing 5 ml of sodium hydroxide. After 5 min, the absorbance was measured at 546 nm.

2.9. Analysis of liver function indices

2.9.1. Estimation of serum creatinine concentration

To estimate the serum creatinine concentration, the method of Jung (2008) was employed. The serum sample (0.1 ml) was mixed with 0.05 ml of the starting reagent that had 10 mM picric acid, 10 mM sodium borate, sodium hydroxide, and 240 mM surfactant, and 0.5 ml of the creatinine standard (5.0 mg/dl) was introduced and the absorbance was measured at 20^{th} and 80^{th} seconds against the reagent blank at 540 nm. Furthermore, two test tubes labeled as standards A and B were prepared, in which A contained the starting reagent (0.5 ml) and 0.1 mL of an equal volume of picric acid and creatinine standard, while B had creatinine standard (0.5 ml) and 0.1 ml of an equal volume of picric acid and creatinine standard, respectively. The absorbance was measured at 20^{th} and 80^{th} seconds at 546 nm.

2.9.2. Estimation of serum urea concentration

The Ezeugwunne et al. (2017) method, which involves the use of urease Berthelot, was used. In a test tube, 10 µl of the serum was introduced alongside 100 µl of an initial reagent (containing 116 mM EDTA, 6.0 mM sodium nitroprusside, and 1.0 g/l urease) and then incubated at 25 °C for 10 min. Afterward, 2.5 ml of 12 mM phenol was added, accompanied by the addition of 2.5 ml of 27 mM sodium hypochlorite. The final solution was thoroughly mixed, and incubated at 37 °C for 15 min, and the absorbance was measured at 546 nm.

2.9.3. Estimation of serum albumin concentration

The bromocresol green method described by Macrelli et al. (2013) was used. Briefly, 10µl of the serum and 3 ml of the BCG concentrate (comprising of succinate buffer at pH 4.2 and 0.15 mM bromocresol green) were mixed vigorously and then incubated at 37 °C for 2 min. The absorbance was read spectrophotometrically at 630 nm against the reagent blank.

2.9.4. Estimation of serum total protein concentration

The biuret method as outlined by Asuk (2018) was employed. A mixture containing 20 µl of the serum and 10 ml of the biuret reagent was thoroughly mixed and kept for 10 min at 37 °C. The entire content was spectrophotometrically measured at 540 nm against the reagent blank.

2.10. Analysis of lipid profile/markers

2.10.1. Determination of triglyceride concentration

To determine the triglyceride concentration, the method of Adaramoye et al. (2013) was followed. The principle is based on the peroxidation of 4-aminophenazone in the presence of 4-chlorophenol, under which peroxidase acts to form a quinoneimine product. Briefly, 1.0 ml of trichloroacetic acid (TCA) was added to 0.1 ml of the serum sample, and centrifuged at 1106 x g for 10 min. Furthermore, three test tubes labeled as blank, sample, and standard were prepared, such that each contained water + 0.5 ml TCA, 1.0 ml supernatant, and 0.5 ml standard solution + 0.5 ml TCA, respectively. To each, 1.0 ml of cholesterol was added, and were left to stand for 20 min before measuring the absorbance at 540 nm.

2.10.2. Determining the cholesterol concentration

The method of Adaramoye et al. (2013) was good enough for estimating the serum cholesterol level. The principle involves oxidation and enzyme hydrolytic reactions in the reaction of hydrogen peroxide, 4-amino antipyrine and *p*-hydroxybenzoic acid catalyzed by peroxidase, forming a colored product called quinoneimine. 10 µl of serum and 1000 µl of cholesterol reagent, after being thoroughly mixed, was allowed to stand for 10 min. The absorbance was read within 60 min at 500 nm. Two other test tubes were prepared and labeled as standard and blank, respectively. The labeled standard contained 10 µl of standard sample and 1000 µl of cholesterol, while the blank contained 10 µl of distilled water and 1000 µl of cholesterol

2.10.3. Determining the serum low-density lipoprotein

To determine the serum low-density lipoprotein, the method of Bachorik (2000) was found appropriate. To the serum (0.02 ml), three drops of the precipitating solution were added. After being mixed and incubated for 15min, the mixture was centrifuged at 1593 x g for 10 min. The supernatant (0.01 ml) and 1.0 ml of cholesterol were put into three test tubes labeled as sample, standard 1 and standard 2, respectively, and were left for 10 min at ambient temperature. In addition, 0.01 ml of the standard reagent was pipetted into standard 1 and 2, respectively, and were left to stand for 10 min. Finally, the absorbance was measured at 505 nm.

2.10.4. Determining the serum high-density lipoprotein

To determine the serum high-density lipoprotein, the method described by Ighodaro and Ighodaro and Omole (2012) was used. The method is a catalytic reaction, in which HDL-cholesterol is degraded by PEG-cholesterol oxidase to form H_2O_2, and combines with sodium-N-(2-hydroxyl-3-sulfopropryl)-3,5-dimethoxy aniline, HSDA) and 4-amino-antipyrine catalyzed by peroxidase to form a purple/blue pigment. Briefly, the precipitating solution (0.1 ml) and the serum sample (0.3 ml) were mixed thoroughly and left for 15 min. It was centrifuged at 1106 x g for 15 min, after, the supernatant's absorbance was measured at 600 nm.

2.11. Analysis of kidney function indices

2.11.1. Estimation of serum sodium concentration

Serum sodium was estimated by the colorimetric method outlined by Igwe et al. (2020). Briefly, 1.0 ml of filtrate reagent was pipetted into test tubes labeled as blank, standard, and sample. Additionally, 50 µl of standard reagent and 50 µl of serum were added to the standard and sample tubes, respectively. The blank contained distilled water only. All the test tubes were mixed and left to stand for 3 min, followed by centrifugation at 1593 x g for 10 min. Test tubes were labeled and 1.0 ml of acid reagent was added to all tubes. Then, 50 µl of the supernatant was added to the corresponding tubes and appropriately mixed. Finally, 50 µl of color reagent was added to all tubes, and mixed, and absorbance was measured at 550 nm.

2.11.2. Estimation of serum potassium concentration

To estimate the serum potassium concentration, the turbidometric method of Egbung et al. (2020) was used. Shortly after pipetting the serum (25 µl) into the test tube, 100 µl sodium tetraphenylborate was added and the entire contents were incubated for 5 min. After incubation, the absorbance was recorded at 578 nm.

2.11.3. Estimation of serum chloride concentration

To estimate the serum chloride concentration, the method of Egbung et al. (2020) was followed. Two test tubes were prepared, labeled as calibrator and sample, respectively. 1.5 ml of chloride reagent (containing mercuric nitrate, mercuric chloride, ferric nitrate, and mercuric thiocyanate) was introduced into each test tube, while the sample test tube had an additional 0.01 ml of serum and mixed vigorously before incubation at 28 °C for 5 min. The spectrophotometric measurement of the sample was determined at 480 nm.

2.12. Analysis of haematological indices

A full package hematological analysis was carried out on the serum using a hematology analyzer (Sysmex XE-5000, SYSMEX, Japan).

2.13. Histopathological examination of some organs

The procedure outlined by Mazani et al. (2018) was used. After the organs were harvested, they were washed in a solution of physiological saline, and fixed in 10% formalin solution. In an ethanol solution (50 - 100%), the samples were dehydrated, cleared in xylene and embedded in paraffin. Afterward, hematoxylin and eosin dye were applied to a cross-sectional area of 5 µm thickness of the sample, and subsequently examined under a microscope (Olympus IX71) for possible pathological changes.

2.14. Statistical analysis

Triplicate data generated were converted to mean ± SD using SPSS (version 25.0), while both the one-way ANOVA and Tukey's post hoc tests were also conducted at a significant limit of $p < 0.05$.

3. Results and discussion

3.1. Percentage yields of fractions from crude hexane extract of T. bangwensis

Table 1 indicates that the percentage yields of the solvent fractions for CF1, CF2, and CF3 are 0.71%, 8.21%, and 4.75%, respectively.

3.2. Hypoglycemic effect of the fractions of hexane extract of T. bangwensis

According to Figure 1, the blood glucose concentration was significantly elevated (> 300 mg/dl) at $p < 0.05$, after the third day of alloxan induction, compared to the normoglycemic rats (85 mg/dl).

However with consistent oral administration of the HEXETACF and silymarin (SILY) for 21 days, the blood glucose concentration reduced significantly (≥ 90 mg/dl) compared to the diabetic group. (270 mg/dl) at $p < 0.05$

Table 1. Percentage yields of the fractions of crude hexane leaf extract of *Tapinanthus bangwensis*

Samples	Eluting solvents	Collected fractions	Nature of sample after dryness	Sample yield	Prep TLC band
CF1	100% HEX	25 (F_1-F_{25})	Yellowish oily extract	0.372 g (0.74%)	-
CF2	90% HEX:10% ETAC	15 (F_{26}-F_{40})	Yellowish solid extract	4.106 g (8.21%)	1
CF3	85% HEX:15% ETAC 80% HEX:20% ETAC	47 (F_{41}-F_{88})	Brownish solid extract	2.373 g (4.75%)	6

CFs stands for column fractions, HEX represents hexane while ETAC stands for ethyl acetate

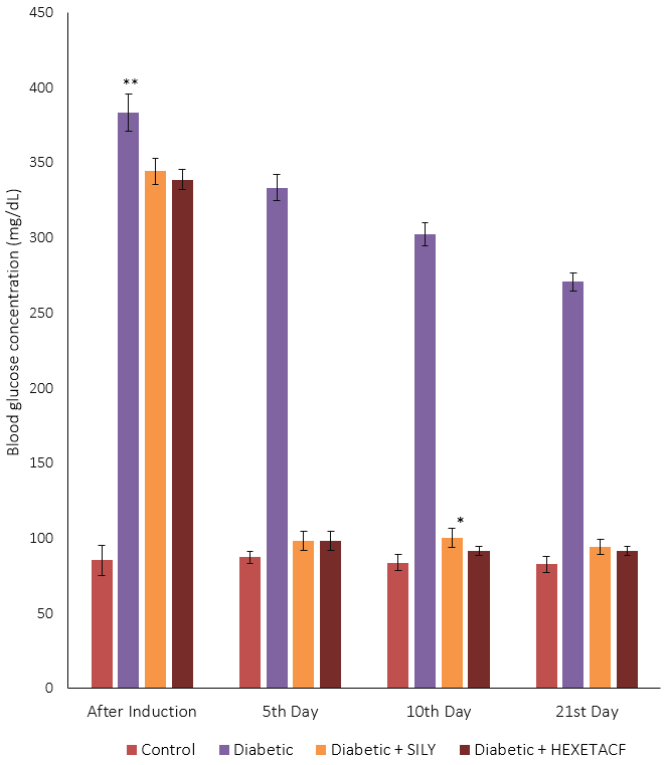

Figure 1. Hypoglycemic effect of the fraction of the hexane extract of *T. bangwensis* in alloxan-induced diabetic rats

The same * connotes no significance, while different * connotes significance at $p < 0.05$. The SILY and HEXETACF stand for silymarin and hexane-ethylacetate fraction.

Diabetes mellitus (DM), formally considered a trivial issue, has become a critical health discourse, due to the heightened annual statistical data of diabetic patients worldwide (International Diabetes Federation, 2015). Notably, DM is a severe metabolic disorder with significantly elevated plasma glucose levels that confer negative consequences on the retina, nerves, liver, kidney, heart, and reproductive cells. One key therapeutic approach to ameliorating postprandial hyperglycemia is simply to inhibit carbohydrate hydrolytic enzymes (α-amylase and α-glucosidase), thereby preventing glucose absorption after carbohydrate ingestion (Ben Salem et al., 2017; Emordi et al., 2018). Ihegboro et al. (2024) reported that the presence of 1,2-benzene dicarboxylic acid, butyl-2-ethylhexyl ester could have influenced the antidiabetic outcome, by inhibiting the metabolic activity of α-amylase and α-glucosidase (Elavarasi et al., 2020; Hassan et al., 2022; Ihegboro et al., 2024).

In addition, neophytadiene and squalene were reported to promote β-islet regeneration, thereby enhancing peripheral glucose metabolism (Alabi & Oyeku, 2017; Ferdosi et al., 2021; Ihegboro et al., 2024). The significant reduction in blood glucose concentration, after the 21st day of administration may be attributed to the hypoglycemic properties of these compounds in the plant.

3.3. Anti-Oxidative effect of fraction of the hexane extract of T. bangwensis

In Figure 2, GSH (glutathione) level improved in the treated groups compared to the diabetic untreated group (DUTG). However, the GSH level was higher in the HEXETACF-treated diabetic group (HTDG) (11.82 ± 0.5 µmol/ml) compared to the silymarin-treated diabetic group (STDG) (10.99 ± 1.50 µmol/ml). However, no significant difference was noticed between the DUTG and the treated groups. Moreover, there was no significant increase in superoxide dismutase (SOD) and catalase (CAT) activity in the treated groups. In addition, the result reveals that no significant decrease in MDA level exists in HTDG (2.21 ± 0.1 µmol/ml), and STDG (2.33 ± 0.1 µmol/ml), compared to the DUTG (2.84 ± 0.1 µmol/ml).

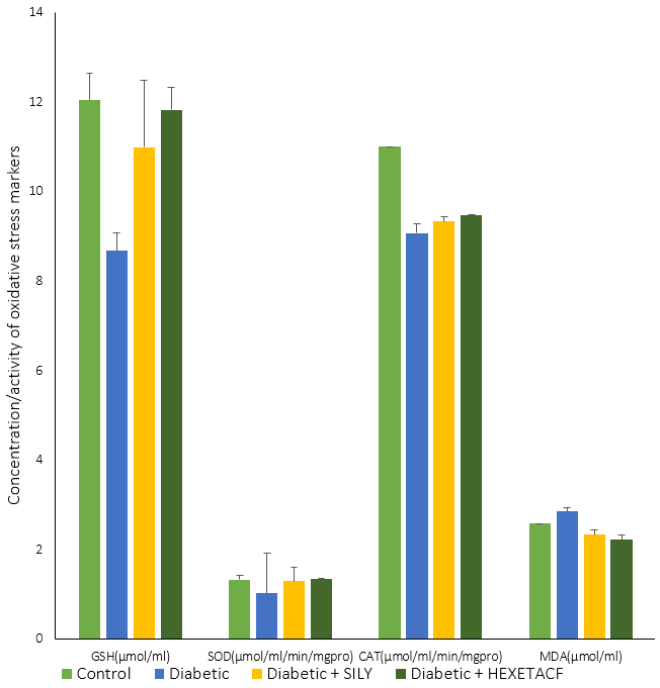

Figure 2. Anti-oxidative effect of the fraction of hexane extract of *T. bangwensis* in alloxan-induced diabetic rats

The same * connotes no significance, while different * connotes significance at $p < 0.05$. The SILY and HEXETACF stand for silymarin and hexane-ethylacetate fraction.

Diabetic-associated type of oxidative stress occurs when lipid peroxyl radicals-byproducts of lipid peroxidation, protein glycation, and glucose auto-oxidation deleteriously damage the lipid-rich membrane, leading to an increase in membrane rigidity and MDA synthesis, as well as decrease erythrocytes lipid fluidity and antioxidant enzyme activity (CAT, SOD, GPx, GST) (Ananthan et al., 2004). In the current study, the HEXETACF appeared to have exerted anti-oxidative potential, by lowering MDA level and enhancing glutathione level, CAT, and SOD activity, respectively.

3.4. Effect of fraction of the hexane extract of T. bangwensis on liver enzymes cctivity

Figure 3, reveals the effect of HEXETACF and SILY on the activities of ALT, AST, and ALP. The ALT activity reduced significantly in the HTDG (89.40 ± 12.65 U/l) and STDG (62.30 ± 12.41 U/l) compared to the DUTG (140.90 ± 19.80 U/l) at $p < 0.05$. But no significant reduction was found between the STDG and HTDG, respectively.

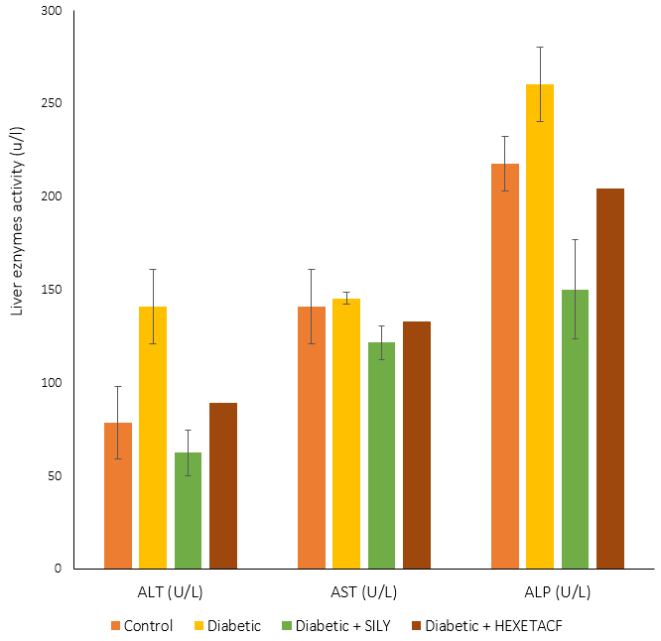

Figure 3. Effect of the fraction of hexane extract of *T. bangwensis* on the activity of liver enzymes in alloxan-induced diabetic rats
The same * connotes no significance, while different * connotes significance at $p < 0.05$. The SILY and HEXETACF stand for silymarin and hexane-ethylacetate fraction.

While AST activity increased in the DUTG (145.37 ± 3.05 U/l), it decreased in the STDG (121.43 ± 8.81 U/l) and HTDG (132.90 ± 10.41 U/l). However, no significant difference exists between the DUTG and treated groups. Finally, ALP activity significantly decreased in the STDG (150.067 ± 26.75 U/l) compared to the HTDG (204.267 ± 27.21 U/l) at $p < 0.05$, additionally significant reduction exists between the treated groups and DUTG (260.30 ± 20.10 U/l) at $p < 0.05$.

Alloxan metabolism in the liver facilitated by cytochrome P450 enzymes, produces excess radical byproducts that destroy the integrity of the pancreatic β-islet cells, affecting insulin secretion. It also has an adverse effect on hepatocyte distribution, the hematopoietic system, and the nephron (Ben Salem et al., 2017; Ihegboro et al., 2022). Interestingly, research studies have established a positive correlation between diabetes and increases in AST, ALT, and ALP activity. Notably so, because free radicals cause hepatocellular lesions, increase the permeability of the liver, and this leads to the release of liver enzymes into the circulatory system. Worthy of note, the ALT and AST perform a transamination reaction, where amino acids are converted to ketoacids, before being transformed to the corresponding amino acids. The HEXETACF's hepatoprotective potential in this study may likely be attributed to the effect of squalene (Ihegboro et al., 2024).

3.5. Hepato-Renal Effect of fraction of the hexane extract of T. bangwensis in diabetic rats

Although CREAT (creatinine) and urea levels were reduced in STDG and HTDG compared to the DUTG, no significant difference was observed between the DUTG and treated groups. Furthermore, there was no significant decrease in ALB (albumin) level, between the DUTG (≥ 30.15 g/l) and the treated groups (≥ 27 g/l). Lastly, the concentration of total protein (TP) was found to be high in the DUTG (64.30 ± 1.84 g/l) but, became reduced with 58.17 ± 9.10 g/l (HTDG) and 61.60 ± 2.43 g/l (STDG), respectively, but no significant difference occurred between the DUTG and the treated groups (Figure 4).

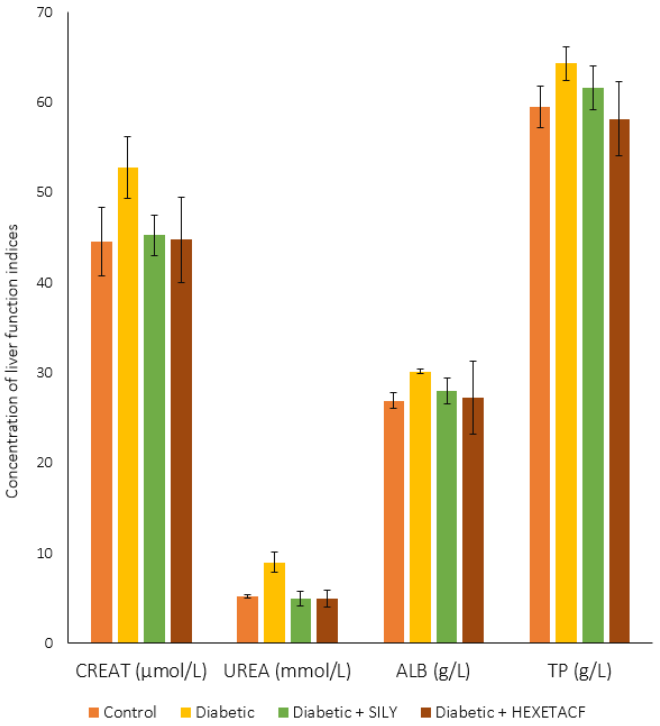

Figure 4. Anti-hepato-renal effect of the fraction of hexane extract of *T. bangwensis* in alloxan-induced diabetic rats
The same * connotes no significance, while different * connotes significance at $p < 0.05$. The SILY and HEXETACF stand for silymarin and hexane-ethylacetate fraction.

The hyperproteinemia and hyperalbuminemia observed in the DUTG had been previously reported by Num-Adom et al. (2022). However, the positive outcome shown by the HEXETACF may suggest its capacity to improve hepato-renal parameters (Emordi et al., 2018).

3.6. Hypolipidemic effect of fraction of the hexane extract of T. bangwensis

Figure 5 reveals the effect of HEXETACF and SILY on lipid markers in diabetic rats. Looking at the result, serum triglyceride (TG) levels decreased significantly in the treated groups compared to the DUTG at $p < 0.05$. Nevertheless, no significant difference exists between the treated groups. After treatment, serum cholesterol (CHOL) was reduced in the treated groups, compared to the DUTG, but no significance occurred between the DUTG and treated groups. The concentration of serum low-density lipoprotein (LDL) was found to be lower in HTDG (0.327 ± 0.03 mmol/l) compared to STDG (0.59 ± 0.21 mmol/l). However, no significant decrease was observed between the treated groups. Also, there was no significant difference in LDL concentration between the DUTG compared to the

treated groups. Finally, serum high-density lipoprotein (HDL) concentration decreased in DUTG but subsequently increased in the treated groups. Nevertheless, no significant difference was found between the DUTG and the treated groups.

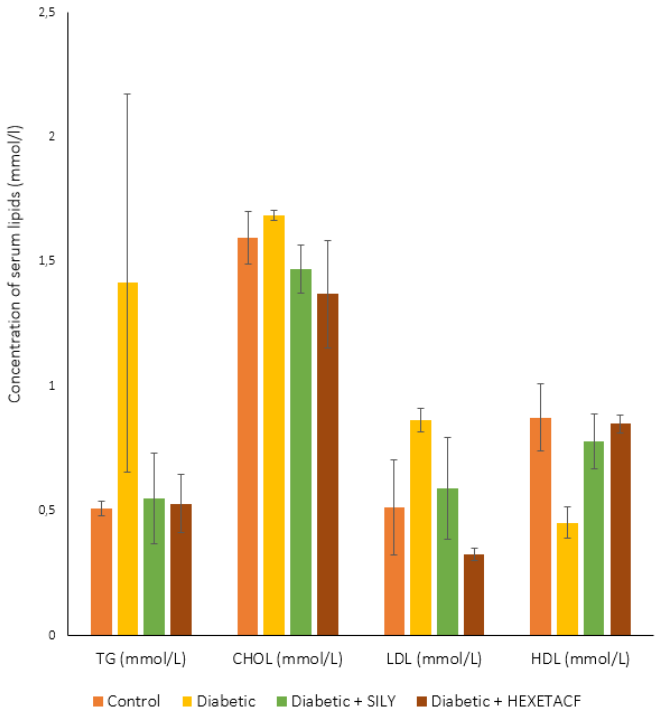

Figure 5. Hypolipidemic activity of the fraction of hexane extract of *T. bangwensis* in alloxan-induced diabetic rats

The same * connotes no significance, while different * connotes significance at $p < 0.05$. The SILY and HEXETACF stand for silymarin and hexane-ethylacetate fraction.

In diabetic conditions, a decrease in insulin secretion increases serum TG and cholesterol levels by down-regulating the activity of pancreatic lipase (lipolytic enzyme) and hydroxylmethylglutaryl-CoA reductase (HMG-CoA reductase) (Shah & Khan, 2014). This is because during insulin deficiency, free fatty acids (FFAs) are displaced from adipose tissue for biosynthesis of fatty acid and ketone bodies. But as insulin secretion improves, glucagon activity is inhibited (that is, the recruitment of FFAs from the adipose tissue), hence, depleting TG and cholesterol levels. The present results reveals that the lipid-lowering outcome may likely be attributed to the presence of hexadecanoic acid, ethyl ester, *cis*-vacenic acid, and squalene, which inhibits the activity of lipase, cholesterol esterase and HMG-CoA reductase – a key enzyme involved in LDL-cholesterol metabolism (Ihegboro et al., 2024; Lozano-Grande et al., 2018; Mirmiranpour et al., 2018; Semwal et al., 2018). Consequently, the reduction in the LDL level, and the subsequent increase in HDL level, suggests that the HEXETACF may facilitate the antiport transport of cholesterol and triacylglycerol from the liver to the peripheral tissues, and from the peripheral tissues to the liver (Donald & Judith, 1990).

3.7. Effect of fraction of the hexane extract of T. bangwensis on serum electrolytes

Considering **Figure 6**, serum sodium (Na^+) and chloride (Cl^-) increased in the DUTG. However, after treatment with HEXETACF and SILY, their concentrations were reduced. However, no noticeable significance exists between the DUTG and the treated groups. Again, serum potassium (K^+) increased in the DUTG (15.17 ± 3.20 meq/l), but decreased in both the STDG (9.23 ±1.06 meq/l) and HTDG (10.023 ±1.05 meq/l). There was no significant difference between the DUTG and the treated groups.

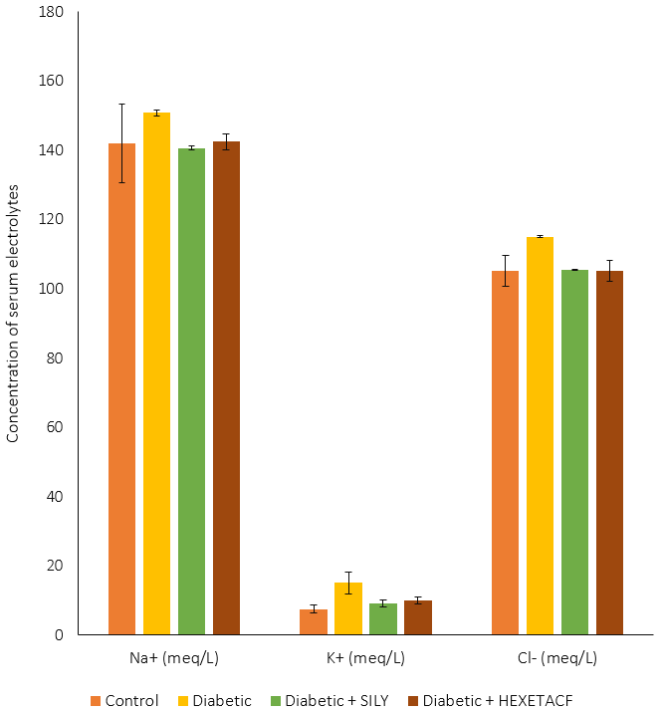

Figure 6. Effect of the fraction of hexane extract of *T. bangwensis* on serum electrolytes in alloxan-induced diabetic rats

The same * connotes no significance, while different * connotes significance at $p < 0.05$. The SILY and HEXETACF stand for silymarin and hexane-ethylacetate fraction.

Many multi-factorial reasons contribute to the electrolyte imbalance in diabetic patients. However, diabetic ketoacidosis and hyperglycemia seem to be predominant (Oyesola et al., 2020). Diabetic ketoacidosis occurs when intracellular fluid-potassium (ICF-K^+) exchanges with excess extracellular fluid-proton (ECF-H^+). Furthermore, reticulocytosis in the peripheral circulation also results in elevated K^+ concentration in the reticulocytes. Finally, in the event of diabetic acidosis, hyperchloridaemia develops via the loss of bicarbonate ions (Esievo, 2017; Navya et al., 2018). The current results indicate that the HEXETACF may improve nephritic performance in diabetic animals.

3.8. Histological effect of fraction of the hexane extract of T. bangwensis on some organs

Figure 7, indicates that in STDG and HTDG, there were normal hepatocytes with central vein (CV), portal vein (PV), basophilic portion with nuclei, and acidophilic cytoplasm of acinar cells, without any abnormalities. It also showed normocellular glomeruli disposed on a background containing renal tubules **(Figure 8)**, while the pancreas had normal exocrine acini with islets and no inflammatory cells **(Figure 9)**. However, the DUTG had congested blood vessels with edema observed in the organs.

3.9. Erythrocytopoietic effect of fraction of the hexane extract of T. bangwensis

According to **Table 2**, the HEXETACF and SILY improved the levels of RBC, MCH (mean cell hemoglobin), HGB (hemoglobin), mean cell hemoglobin count (MCHC), and as well as the MCV (mean cell volume), HCT (packed cell volume) and MPV (mean platelet volume) compared to the diabetic rats. Furthermore, the DUTG (18.75 ±

3.89%) had a higher % RDW-CV compared to the HTDG (16.77 ± 1.70%) and STDG (17.43 ± 3.13%). Finally, the result highlighted that the HEXETACF had substantial haematologic activity compared to the SILY.

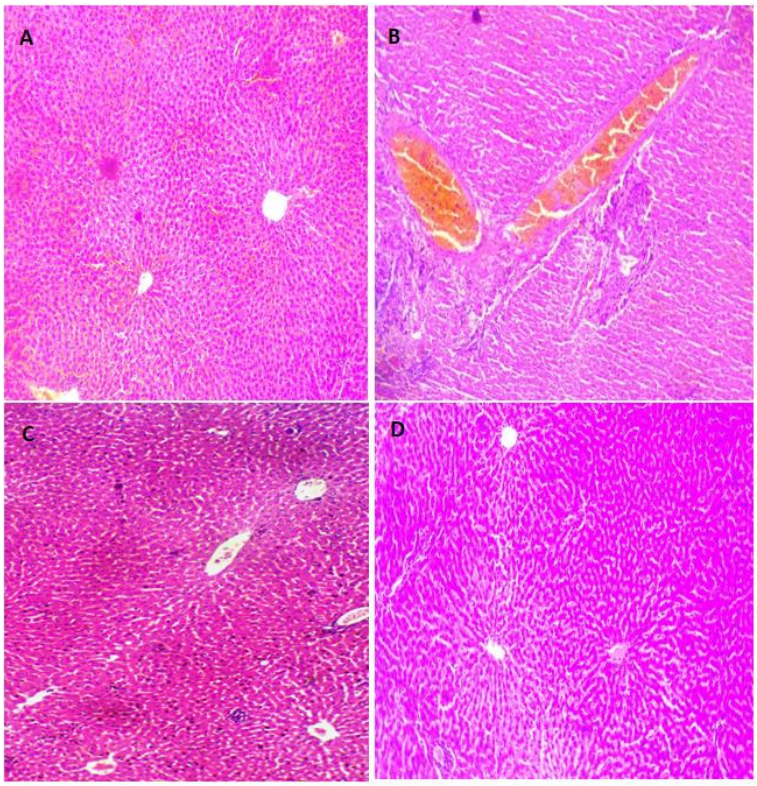

Figure 7. Histological sections of the liver tissue of the different groups
A) Control group (normal hepatocytes), B) Diabetic group (edemic vascular congestion), C) Diabetic + SILY (normal hepatocytes), D) Diabetic + HEXETACF (normal hepatocytes)

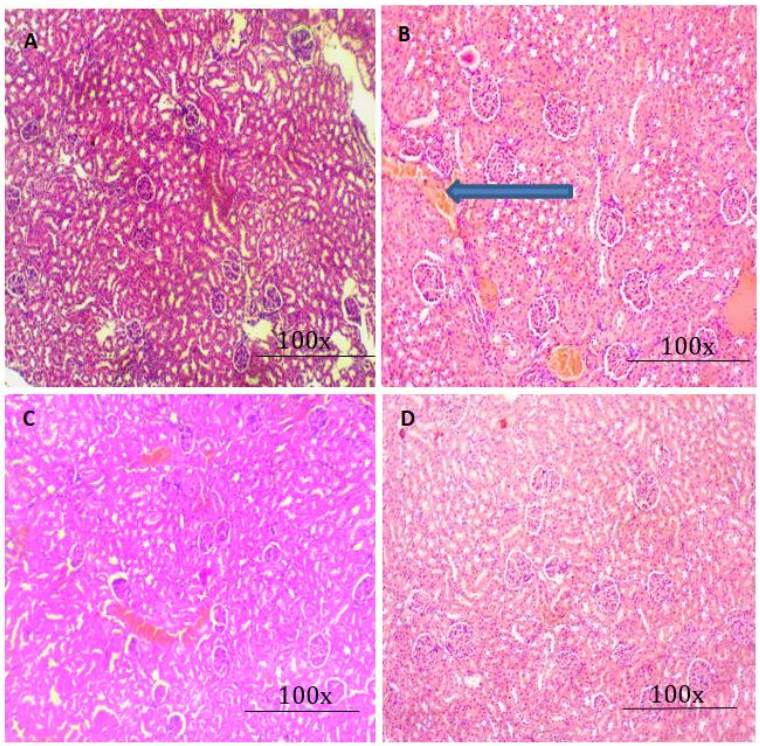

Figure 8. Histological sections of the kidney tissue of the different groups
A) Control group (normal glomerular), B) Diabetic group (edemic vascular congestion), C) Diabetic + SILY (normal glomerular with mild congestion), D) Diabetic + HEXETACF (Normal glomerular)

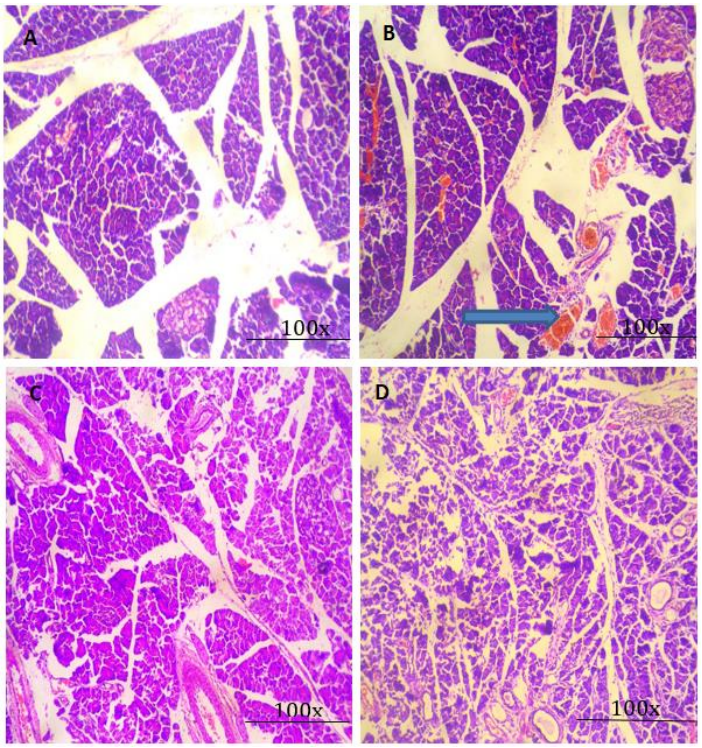

Figure 9. Histological sections of the pancreatic tissue of the different groups
A) Control group (normal islet cells), B) Diabetic group (edemic vascular congestion), C) Diabetic + SILY (normal islet cells), D) Diabetic + HEXETACF (normal islet cells)

Table 2. Effect of the fraction of the hexane leaf extract of *T. bangwensis* on erythrocytic indices in alloxan-induced diabetic rats

Parameters	Control	Diabetic only	Diabetic + SILY	Diabetic + HEXETACF
RBC (x 10^{12}/l)	8.66 ± 0.74^{a}	7.59 ± 2.32^{ab}	7.70 ± 0.47^{a}	8.32 ± 0.61^{a}
MCH (pg)	22.90 ± 0.28^{a}	18.80 ± 1.41^{ab}	19.67 ± 1.41^{a}	21.87 ± 0.76^{a}
MCHC (g/l)	33.30 ± 0.71^{a}	31.65 ± 1.63^{ab}	31.77 ± 0.78^{a}	32.13 ± 1.12^{a}
HCT (%)	45.10 ± 0.14^{a}	44.60 ± 8.06^{ab}	44.80 ± 1.04^{a}	46.23 ± 4.22^{a}
HGB (g/dl)	15.55 ± 1.06^{a}	14.20 ± 3.25^{ab}	14.43 ± 0.50^{a}	14.90 ± 1.54^{a}
MCV (dl)	54.00 ± 1.98^{a}	49.80 ± 7.50^{ab}	58.40 ± 3.31^{a}	55.60 ± 0.95^{a}
MPV (dl)	7.20 ± 1.13^{a}	6.70 ± 0.71^{ab}	6.83 ± 0.12^{a}	7.03 ± 0.31^{a}
RDW-CV (%)	15.45 ± 1.77^{a}	18.75 ± 3.89^{ab}	17.43 ± 3.13^{a}	16.27 ± 1.70^{a}

Values were in triplicates and were expressed as mean + standard deviation. ^{a} indicates no significant difference exists between the control group and the other groups. ^{b} indicates a significant difference exists between the diabetic group and the treated groups at $p < 0.05$. RBC: Red blood cell, MCH: Mean cell hemoglobin, MCHC: Mean cell hemoglobin count, HCT: Packed cell volume, HGB: Haemoglobin, MCV: Mean cell volume, MPV: Mean platelet volume, RDW-CV: Red blood cell distribution

Table 3. Effect of the fraction of the hexane leaf extract of *T. bangwensis* on leucocytic indices in alloxan-induced diabetic rats

Parameters	Control	Diabetic only	Diabetic + SILY	Diabetic + HEXETACF
WBC (x 10^{9}/l)	11.55 ± 1.485^{a}	9.45 ± 4.031^{ab}	9.93 ± 4.46^{a}	10.30 ± 5.23^{a}
LYMP (%)	37.70 ± 0.42^{a}	30.60 ± 6.36^{ab}	34.37 ± 6.87^{a}	36.73 ± 5.28^{a}
GRAN (%)	42.05 ± 4.88^{a}	47.35 ± 3.47^{ab}	40.47 ± 6.60^{a}	45.83 ± 7.77^{a}
MID (%)	20.25 ± 5.30^{a}	18.55 ± 6.29^{ab}	25.17 ± 3.53^{ac}	19.43 ± 2.63^{a}
PLT (x 10^{9}/l)	776.00 ± 24.04^{a}	612.50 ± 71.41^{ab}	700.00 ± 123.24^{a}	675.33 ± 83.72^{a}

Values were in triplicates and were expressed as mean + standard deviation. ^{a} indicates no significant difference exists between the control group and the other groups. ^{b} indicates a significant difference exists between the diabetic group and the treated groups at $p < 0.05$. WBC: White blood cell, LYMPH: Lymphocyte, GRAN: Granulocyte, MID: Combined values of other WBCs not classified as lymphocytes or granulocytes, PLT: Platelet count

The decrease in RBC count and HGB, suggests insufficient production of erythropoietin – a glycoprotein hormone (Ohlsson & Aher, 2006; Thomas, 2008; Thomas et al., 2003). Moreover, the decrease in mean HGB concentration per erythrocyte and per volume of packed red blood cells (MCH and MCHC), HCT, and MCV – which represents the average volume of RBC indicate pathological condition with anemia (Hoffman et al., 2013). Our finding implies that the HEXETACF, enhanced erythropoietin production, inhibits ROS-induced RBC hemolysis, or reduces RBC osmotic fragility (Ben Salem et al., 2017; Muhammad et al., 2012).

3.10. Effect of fraction of the hexane extract of T. bangwensis on leucocytic indices

Table 3 reveals that WBCs (white blood cell count) and the % LYMPH (lymphocyte) were higher in the HTDG compared to the STDG, but lower in the DUTG. Also, the result indicates that the DUTG (18.55 ± 6.29%) and STDG (25.17 ± 5.33%) had the lowest and highest % MID, respectively. However, the HTDG (19.43 ± 2.68%) had a higher % MID compared to the DUTG. Finally, the STDG (700.00 ± 123.24 x 10^{9}/l) had platelet count (PLT) higher when compared to the HTDG (675.33 ± 83.72 x 10^{9}/l), while the DUTG (612.50 ± 71.41 x 10^{9}/l) had the lowest PLT count. Considering all the parameters, no significant difference exists between the DUTG and the treated groups.

Queiroz et al. (2021) reported decreased WBCs and LYNF and increased GRAN (granulocytes) level in alloxan-induced animals, while Ben Salem et al. (2017) reported a decrease in PLT count in diabetic states. The study further established that these pathological changes affect immunity and blood homeostasis (Uhuo et al., 2022). Interestingly, our results indicate that HEXETACF could improve the immune system and thrombocytopoietic activity, by reversing these pathological abnormalities in the diabetic rats.

4. Conclusions

The current result reveals that blood glucose concentration reduced between 33-34%, indicating an improved insulin secretion. Moreover, the hepatocellular membrane appears to have been restored, considering the decreases in the activity of the liver enzymes, compared to the diabetic rats. Additionally, the plant exhibited antioxidative potential, by increasing GSH concentration, and SOD and CAT activity, which culminated in MDA reduction. In comparison with the diabetic rats, the serum TG, cholesterol, and LDL decreased, with an increase in HDL level. Finally, the haematologic indices increased, suggesting an improvement in the secretion of the haematopoietin hormones. In conclusion, the results demonstrate that HEXETACF looked promising as an alternative antidiabetic agent in the absence of SILY.

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Conflict of interest

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

Statement of ethics

Ethical approval for this study was obtained from the Nigerian Police Academy, Kano, Ethics Committee (Date: March 15, 2023, Number: NPA/ETC/03/2023).

Availability of data and materials

All data generated or analyzed during this study are included in this published article. On request, the associated author can provide more information.

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Supplementary File

None.

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