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Phytochemical profile and antioxidant potential of extracts of *Callicarpa macrophylla* Vahl. from Uttarakhand Himalayan region

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ABSTRACT

The medicinal traits of *Callicarpa macrophylla* Vahl. have been used in traditional and folklore medicinal system, however, scientific evaluation of such medicinal plants have not been much explored yet. Different solvent extracts of the whole plant of *C. macrophylla* from the Himalayan region are the key subject of interest to evaluate and exhibit their antioxidant properties concerning its phytochemical composition. The different polarity extracts of *C. macrophylla* were prepared using the Soxhlet apparatus and both of their GC-MS profile outlined their individual components. The antioxidant activity of both extracts was evaluated for DPPH radical scavenging activity, metal chelating activity, and reducing power activity. Thirty-two compounds were identified in the hexane extract, accounting for 63.56% of the total extract composition with tetratetracontane (9.78%), as the prominent compound. A sum of 26 compounds making up 79.74% of the total methanolic extract composition was identified and the major compound was a diterpenoid, trachylobane (31.87%). The methanolic extract exerted potent antioxidant properties when compared, in vitro, to hexane extract as it showed better IC_{50} values of DPPH radical scavenging activity (91.97 ± 0.33 µg/ml), metal chelating activity (10.90 ± 1.40 µg/ml) and reducing power activity (56.72 ± 0.67 µg/ml). The antioxidant potential of the methanolic extract was also comparable to standards. Therefore, the methanolic extract exhibited better results as compared to hexane extract and had comparable potential with standards in vitro, if further explored might be utilized as an antioxidant agent in various pharmaceutical and cosmetic industries.

1. Introduction

All the essential needs of human life, including food, clothing, shelter, and medicine, are provided by nature. Rural areas, where plants are used for their therapeutic benefits, are still using traditional medicines. The therapeutic qualities of plants are thought to be caused by the bioactive chemicals found in them. Most medicinal plants have a distinctive feature of aroma as it indicates the presence of essential oil in the plant (Samuelsson, 2004). The Indian subcontinent is home to many medicinal plants, with the Great Himalayan Range thought to be a significant source of economically significant medicinal herbs. The biodiversity may vary according to these ranges' varied altitudes, soil types, and climatic conditions. India, one of the world's 12 mega-diversity countries, is home to a treasure trove of medicinal plants, earning it the nickname "Medical Garden of the World" for its extensive variety (Kala, 2006). Some of the

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medicinally important families are Leguminosae, Apiaceae, Malvaceae, Ranunculaceae, and Lamiaceae. The mint family, Lamiaceae, has a major presence of herbs and shrubs with a distinctive aroma. Tulsi, thyme, rosemary, and spearmint are some of the herbs representing this family. About 260 genera and 3200 species collectively make up the Lamiaceae family, with the following numbers: *Salvia* (900), *Scutellaria* (360), *Coleus* (325), *Plectranthus* (300), *Hyptis* (280), *Teucrium* (250), *Thymus* (220), *Nepeta* (200), and *Callicarpa* (158) (Raja, 2012).

About 120 species of shrubs and trees belonging to the genus *Callicarpa*, sometimes known as the beautyberry, are found across tropical and subtropical climates. Approximately 20 species have previously been identified as having ethnomedicinal uses for treating conditions like hepatitis, fever, dyspepsia, and headaches, among others. Skin cancers, as well as intestinal tumors, have been successfully treated with extracts from *Calliacrpa americana* and *C. rubella* (Singh et al., 2010). Twenty substances, including apigenin 7-*O*-rutinoside, apigenin 6-C-glucoside-8-C-glucoside, acacetin 7-*O*-glucuronide, etc., were found when the polar extracts of *C. maingayi* were studied. Previous research revealed that these components were responsible for the biological activities displayed by *C. maingayi* and might be exploited for nutraceutical preparations (Ado et al., 2016). Additionally, it was discovered that the aqueous and alcoholic extract prepared from the aerial components of *C. macrophylla* Vahl. showed hepatoprotective potential when it was examined using several biochemical criteria (Patel & Jawaid, 2014). The plant *C. macrophylla* has widespread growth across the Northeastern parts and Himalayan states of India and yet not much data is available accounting for its medicinal and therapeutic properties. The present study examines the antioxidant activities of the hexane and methanolic extracts prepared from the aerial parts of *C. macrophylla* while also analyzing the phytochemical makeup of both extracts and establishing chemical profiling of the plant.

2. Materials and methods

2.1. Sample collection

The plant of interest, *C. macrophylla*, was garnered from the hilly regions of Uttarakhand (at an elevation of 1394 m) during the autumn of 2021. The identification of the collected specimen was done at G. B. Pant University of Agriculture and Technology, Pantnagar. The plant with the specimen voucher number GBPUH-1549 was identified as *C. macrophylla* by Dr. D. S. Rawat (Assistant Professor and Plant Taxonomist), Department of Biological Science, C.B.S.H, G. B. Pant University of Agriculture and Technology, Pantnagar.

2.2. Extract preparation

The aerial parts of the plant were shade dried for 7-10 days and further subjected to fine grinding. This fine powder, weighing 3.945 g (methanolic extract) and 5.460 g (hexane extract) was used for extract preparation with the help of the Soxhlet apparatus. Two different extracts were prepared using two different polarity solvents; hexane (non-polar) and methanolic (polar). These two extracts were evaluated for their potential biological activities (Roy et al., 2020).

2.3. Phytochemical profiling

The phytochemical profile of both extracts was obtained by following the protocol of Gas Chromatography-Mass Spectrometry (GC-MS), with the aid of GCMS-QP 2010 Plus equipment. The GC conditions were set as follows; with the oven temperature at 80 °C, the total flow rate of the carrier gas (Helium) at 16.3 ml/min, whereas the column flow rate at 1.21 ml/min. The pressure was maintained at 81.9 kPa with a split ratio of 10:1. The purge flow was 3 ml/min along with a linear velocity of 40.5 cm/sec. Splitter hold, carrier gas saver, and high-pressure injection were switched off as the oven temperature raised to 210 °C (isotherm for 2 min) and further 6 °C/min up to 280 °C (isotherm for 2 min), at last being held for 11 min, flame thermionic detector (FTD).

The Kovats indices (KI) obtained from the peaks on Innowax fused silica capillary column was observed and compared with the values available in the standard libraries. This comparison aided in the identification of the individual compounds. Along with this, the fragment design of the obtained mass spectra via GC-MS was also compared with the available database in various published literature (Chandra et al., 2017).

2.4. Biochemical analysis

2.4.1. Total phenolic estimation

The Folin-Ciocalteu technique was used to determine the total phenol content (Shetty et al., 1995). For this, 0.5ml of 50% (v/v) FCR (Folin-Ciocalteu Reagent) was added to 1ml of the sample after it had been diluted in 5ml of distilled water. An aliquot of 2 ml of 5% Na_2CO_3 was added after 3 minutes of incubation and incubated for 60 min in the dark. At 650 nm, the absorbance was measured. The same procedure was used for the standard Gallic acid. Gallic acid concentrations were plotted against absorbance on a standard graph, and the amount of phenol in the sample extract was calculated as µg/mg gallic acid.

2.4.2. Total flavonoid estimation

To estimate the total flavonoid, the aluminum chloride colorimetric technique was followed (Djeridane et al., 2006). For this test, 1.5 ml of ethanol and 0.1 ml of each aluminum nitrate and potassium acetate were added, and the mixture was then given 40 min to incubate. At 415 nm, the absorbance was measured. The standard was quercetin, and methanol served as blank. The actual flavonoid content was expressed as µg/mg quercetin by plotting the standard curve between varied quercetin concentrations and their corresponding absorbances.

2.4.3. Total antioxidant estimation

The phospho-molybdenum technique was used to assess the extract's overall antioxidant capability (Rouzbahan et al., 2016). The experiment was carried out by combining 3 ml of freshly made reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate) with 1 ml of the sample. For 90 min, this combination was allowed to react at 95 °C. After the tubes had reached room temperature, the absorbance at 695 nm was measured. The total antioxidant capacity was expressed as being equivalent to ascorbic acid, which was used as the benchmark.

2.5. Antioxidant activities

2.5.1. DPPH radical scavenging activity

The pre-established protocol was followed to evaluate the DPPH radical scavenging property of both hexane and methanolic extracts (Sharma et al., 2021). A stock solution of DPPH (0.1 mM) was prepared freshly and stored in an Amber bottle at 4 °C. The test

samples (hexane and methanolic extract) were evaluated in a dose-dependent manner with varying concentrations of 10-100 µg/ml. An aliquot of 2.9 ml DPPH solution (prepared in methanol) was added to 0.1 ml of each sample concentration. This reaction mixture was incubated for 30 min in dark conditions. Then, the absorbance was recorded at 517 nm using a UV spectrophotometer, and the evaluation was done by comparing the results with that of the standard ascorbic acid (following the same protocol). The following formula was used to calculate the percentage of DPPH scavenging activity *(Eq. 1)*:

$$Percentage\ scavenging\ (\%) = 1 - \frac{A_t}{A_0} x\ 100 \qquad Eq.\ 1$$

Where A_0 is the absorbance of the DPPH solution and A_t is the absorbance of the test sample (hexane and methanolic extracts). The percentage DPPH scavenging value was used to calculate the IC_{50} value by plotting it against the varying concentrations of the study. IC_{50} defines as the concentration of the test sample at which 50% of the free radical scavenging took place.

Table 1. Chemical composition of *C. macrophylla* hexane extract (CMHE)

No	Name of compound	KI value	% Composition	Molecular formula	Methods of identification (M/Z)	Class of compound
1	Tetradecane	1413	0.20	$C_{14}H_{30}$	M+:-198 M/Z:-41, 43, 57, 71, 85	Alkane
2	Cetane	1612	0.11	$C_{16}H_{34}$	M+:-226 M/Z:-41, 43, 57, 71, 85	Alkane
3	6,10,14-Trimethylpentadecan-2-one	1754	0.29	$C_{18}H_{36}O$	M+:-268 M/Z:-43, 71, 85, 109, 124	Sesquiterpenoid
4	Phyllocladene	1789	1.04	$C_{20}H_{32}$	M+:-272 M/Z:-55, 69, 123	Diterpenoid
5	Valerenyl acetate	1829	0.14	$C_{17}H_{26}O_2$	M+:-262 M/Z:-43, 91, 119	Sesquiterpenoid
6	Tetramethylheptadecane	1852	0.18	$C_{21}H_{44}$	M+:-296 M/Z:-57, 71, 85, 99	Alkane
7	Palmitic acid methyl ester	1878	0.57	$C_{17}H_{34}O_2$	M+:-270 M/Z:-41, 74, 101	Fatty acid methyl ester
8	Nonadecane	1900	0.13	$C_{19}H_{40}$	M+:-268 M/Z:-41, 43, 57, 71, 85, 99	Alkane
9	Cembrene	1959	0.25	$C_{20}H_{32}$	M+:-272 M/Z:-41, 67, 159	Diterpenoid
10	Palmitic acid	1968	0.87	$C_{16}H_{32}O_2$	M+:-256 M/Z:-41, 43, 73, 85, 98	Long-chain fatty acid
11	Eicosane	2009	0.79	$C_{20}H_{42}$	M+:-282 M/Z:-41, 43, 57, 71, 99	Alkane
12	Phytol	2045	1.64	$C_{20}H_{40}O$	M+:-296 M/Z:-41, 57, 71, 95, 111	Diterpenoid
13	Methyl stearate	2077	0.26	$C_{19}H_{38}O_2$	M+:-298 M/Z:-41, 74, 101	Fatty acid methyl ester
14	Methyl oleate	2085	1.19	$C_{19}H_{36}O_2$	M+:-296 M/Z:-41, 55, 123	Fatty acid methyl ester
15	Methyl linoleate	2108	3.27	$C_{19}H_{34}O_2$	M+:-294 M/Z:-55, 67, 95, 109	Fatty acid methyl ester
16	Heneicosane	2109	2.19	$C_{21}H_{44}$	M+:-296 M/Z:-41, 43, 57, 85, 99	Alkane
17	Phytol, acetate	2168	0.29	$C_{22}H_{42}O_2$	M+:-338 M/Z:-41, 43	Diterpenoid
18	Palmitoleic acid	1976	3.25	$C_{16}H_{30}O_2$	M+:-254 M/Z:-41, 55 (100%), 69, 83, 97, 98, 123	Fatty acid
19	Geranylgeraniol	2192	0.32	$C_{20}H_{34}O$	M+:-290 M/Z:-41, 55, 69	Alcohol
20	Docosane	2200	1.38	$C_{22}H_{46}$	M+:-310 M/Z:-41, 43, 57, 71, 85	Alkane
21	Stearic acid	2681	0.16	$C_{21}H_{42}O_4$	M+:-358 M/Z:-83	Fatty acid
22	17-oxo-6.Beta.-Pentyl-4-nor-3,5-secoandrostan-3-oic acid	2756	8.31	$C_{23}H_{38}O_3$	M+:-362 M/Z:-67, 107, 119	Fatty acid methyl ester
23	Octacosane	2804	0.13	$C_{28}H_{58}$	M+:-394 M/Z:-41, 43, 57, 71, 85, 99	Alkane
24	α-Amyrin	2873	2.68	$C_{30}H_{50}O$	M+:-426 M/Z:-203, 218	Triterpenoid
25	β-Amyrin	2886	1.07	$C_{30}H_{50}O$	M+:-426 M/Z:-55, 119 135, 203, 218	Triterpenoid
26	Nonacosane	2900	1.93	$C_{29}H_{60}$	M+:-408 M/Z:-41, 43, 57, 71, 85	Alkane
27	Squalene	2914	7.62	$C_{30}H_{50}$	M+:-410 M/Z:-41, 55, 69, 81, 95	Triterpenoid
28	Tetratriacontane	3401	3.17	$C_{34}H_{70}$	M+:-478 M/Z:-43, 57 (100%), 71, 85, 99	Alkane
29	Pentatriacontane	3500	7.15	$C_{35}H_{72}$	M+:-492 M/Z:-43, 57 (100%), 71, 85, 99	Alkane
30	Hexatriacontane	3600	1.68	$C_{36}H_{74}$	M+:-506 M/Z:-41, 43, 57, 71, 85	Alkane
31	Tetracontane	3997	1.52	$C_{40}H_{82}$	M+:-562 M/Z:-41, 43, 57, 71, 85	Alkane
32	Tetratetracontane	4395	9.78	$C_{44}H_{90}$	M+:-618 M/Z:-41, 57, 99, 113	Alkane
	Total		63.56			

2.5.2. Metal chelating activity

The metal chelating activity of the hexane and methanolic extract was evaluated by taking different concentrations of the extracts varying from 10 µg/ml to 100 µg/ml. To each concentration of the test sample, 0.05 ml of 2 mM $FeCl_2 \cdot 4H_2O$ was added; followed by the addition of 0.2 ml of 5 mM of ferrozine. The final volume was made up to 5 ml with the help of methanol. The reaction mixture was allowed to withstand room temperature for 10 min. The absorbance was recorded at 562 nm (Gairola et al., 2021). The standard metal chelator used for this study was EDTA. The following formula was used to calculate the % inhibition of metal chelation *(Eq. 2)*:

$$Metal\ chelating\ inhibition\ (\%) = \frac{A_0 - A_t}{A_0} x\ 100 \qquad Eq.\ 2$$

Where A_0 is the absorbance of control A_t is the absorbance of the test sample. The % inhibition values were plotted against the different concentrations of extracts and the IC_{50} value was calculated.

2.5.3. Reducing power activity

Previously standardized protocols were followed to assess the reducing power activity of the hexane and methanolic extracts in a dose-dependent manner in which varying concentrations of plant extracts were taken (10-100 µg/ml) (Singh et al., 2016). To each concentration of 2.5 ml of extract, 2.5 ml of freshly prepared 200 mM phosphate buffer (pH 6.6) was added. This was followed by the addition of 2.5 ml potassium ferricyanide (1% w/v) and the reaction mixture was left for an incubation period of 20 mins at 50 °C maintained with the help of a water bath. Then, 2.5 ml of trichloroacetic acid (10% v/v) was added and centrifugation was done at 3000 rpm (453.2 g) for 10 min. Afterward, 5 ml of supernatant was mixed with an equal volume of distilled water, and 1 ml of ferric chloride was added. The absorbance of the color change observed in the reaction mixture was recorded at 700 nm. The standard taken for this evaluation was gallic acid. The following formula was used to calculate the reducing power % *(Eq. 3)*:

$$Reducing\ power\ (\%) = \frac{A_0 - A_t}{A_0} x\ 100 \qquad Eq.\ 3$$

Where, A_0 is the absorbance of control, A_t is the absorbance of the test sample. The half maximal reducing power (RP_{50}) value was calculated by plotting a graph of the reducing power % against the varying concentrations of the plant extracts.

2.6. Statistical analysis

SPSS16.00 software was used to determine the mean as well as the standard deviation of hexane and methanolic plant extracts, taken in triplicates. The results obtained for each test were subjected to the Duncan post hoc test at 5% for One Way Analysis of Variance (ANOVA) to test significance within the groups ($p < 0.05$).

3. Results and discussion

3.1. Chemical composition of C. macrophylla hexane extract (CMHE)

A total of 32 compounds were identified in the hexane extract of the plant sample using GC-MS equipment. These compounds made up 63.56% of the total composition of *C. macrophylla* hexane extract as described in Table 1. The chromatogram shown in Figure 1 shows the separation spectra of the identified individual compounds. The major compound identified was tetratetracontane (9.78%) (Figure 2). Following this, further other compounds were also identified that had a higher contribution to the composition of the hexane extract, as follows: 17-oxo-6.Beta.-Pentyl-4-nor-3,5-secoandrostan-3-oic acid (8.31%), squalene (7.62%), pentatriacontane (7.15%), methyl linoleate (3.27%), palmitoleic acid (3.25%), tetratriacontane (3.17%), α-amyrin (2.68%), heneicosane (2.19%), nonacosane (1.93%), hexatriacontane (1.68%), tetracontane (1.52%), phytol (1.64%), docosane (1.38%), methyl oleate (1.19%), β-amyrin (1.07%), phyllocladene (1.04%), and many other present in less amount. Figure 3 depicts the major classes of compounds belonging to alkanes with 30.34%, followed by other contributing compounds belonging to fatty acid methyl esters (13.60%), triterpenoids (11.37%), fatty acids (3.41%), diterpenoids (3.22%), others (1.62%). There have been reports where alkanes were found abundantly in non-polar extracts of several species of the Lamiaceae family and used as chemotaxonomic characters. Alkanes function as protective wax in plants and reduce desiccation, abrasion, or resist pest attack (Maffei, 1994). The major compound, tetratetracontane, has the potential of an antioxidant (Amudha et al., 2018).

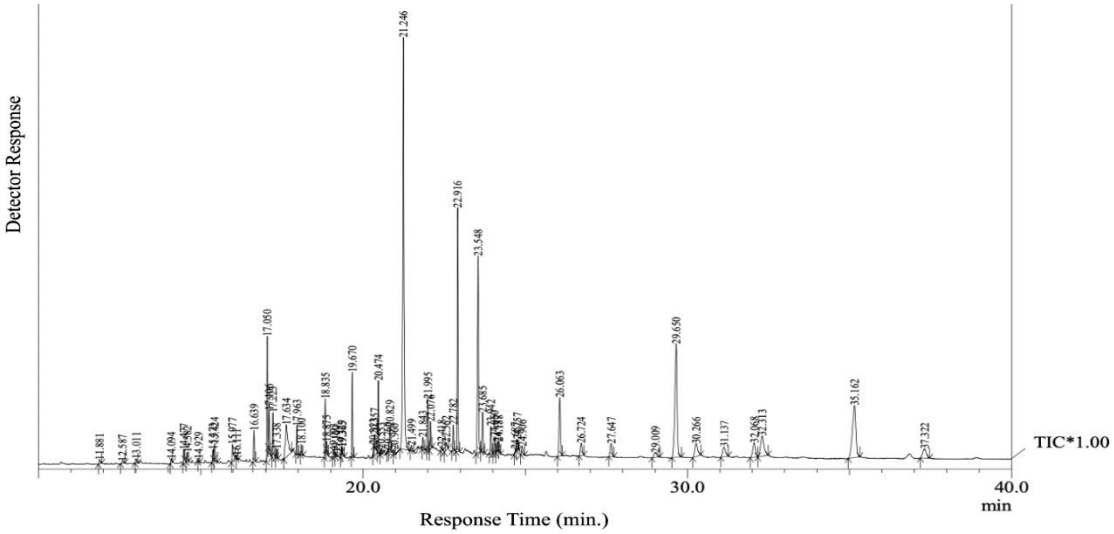

Figure 1. Gas chromatogram of C. macrophylla hexane extract (CMHE)

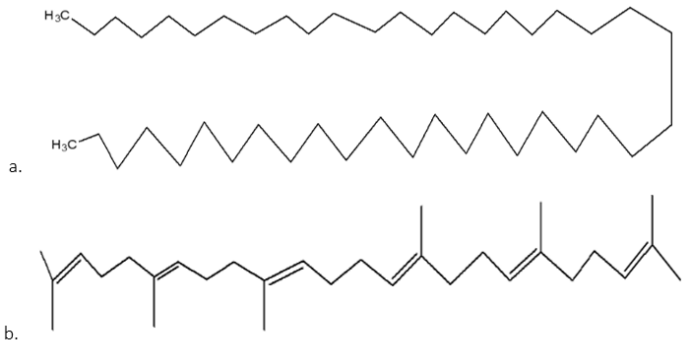

Figure 2. Chemical structures of a. Tetratetracontane and b. Squalene

3.2. Chemical composition of C. macrophylla methanolic extract (CMME)

The GC-MS profile of the methanolic extract of the interested plant sample showed the identification of 26 compounds accounting for 79.74% of the total extract composition (Table 2; Figure 4). The compounds identified were trachylobane (31.87%), palmitoleic acid (8.26%) (Figure 5), 17-oxo-6.beta.-pentyl-4-nor-3,5-secoandrostan-3-oic acid (6.41%), squalene (5.18%), methyl oleate (4.34%), methyl erucate (3.66%), dihydroabietic acid (2.96%), methyl octadeca-9,12-dienoate (2.79%), methyl palmitate (2.41%), palmitic acid (1.82%), 15-hydroxypentadecanoic acid (1.71%), methyl stearate (1.62%), and others present in trace amount. Figure 6 gives information about the classes of compounds present in the methanolic extract.

The methanolic extract is rich in diterpenoids (37.97%), followed by the presence of fatty acid methyl ester (21.93%), triterpenoids (5.18%), and long-chain fatty acid (3.53%). Terpenoids have been isolated in abundance from methanol extracts of *C. macrophylla* (Lam et al., 2021).

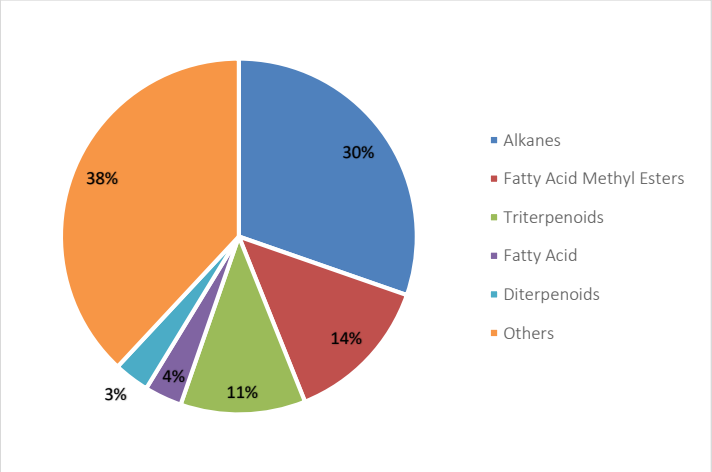

Figure 3. Classes of compounds present in *C. macrophylla* hexane extract (CMHE)

Table 2. Chemical composition of *C. macrophylla* methanolic extract (CMME)

No	Name of compound	KI value	% Composition	Molecular formula	Methods of identification (M/Z)	Class of compound
1	2-Hexanone	778	0.19	$C_{11}H_{18}O$	M+:-166 M/Z:-95, 123	Ketone
2	Linalool	1098	0.87	$C_{20}H_{34}O$	M+:-290 M/Z:-41, 69, 93	Monoterpenoid
3	Cyclododecane	1439	0.22	$C_{12}H_{24}$	M+:-168 M/Z:-41, 55, 69, 83	Cycloalkanes
4	Trachylobane	1698	31.87	$C_{20}H_{32}$	M+:-272 M/Z:-41, 55, 216	Diterpenoids
5	Hexahydrofarnesyl acetone	1754	0.23	$C_{18}H_{36}O$	M+:-268 M/Z:-43, 71, 85, 109	Ketone
6	Phyllocladene	1789	0.65	$C_{20}H_{32}$	M+:-272 M/Z:-55, 69, 105	Diterpenoid
7	Phytol	1806	0.70	$C_{20}H_{40}O$	M+:-296 M/Z:-71, 95	Diterpenoid
8	Methyl palmitate	1878	2.41	$C_{17}H_{34}O_2$	M+:-270 M/Z:-41, 74, 87, 101, 115	Fatty acid methyl ester
9	Cembrene	1959	0.76	$C_{20}H_{32}$	M+:-272 M/Z:-41, 67, 133	Diterpenoid
10	Palmitic acid	1984	1.82	$C_{15}H_{30}O_2$	M+:-242 M/Z:-41, 60, 73, 85, 98	Long-chain fatty acid
11	Methyl stearate	2077	1.62	$C_{19}H_{38}O_2$	M+:-298 M/Z:-41, 57, 74, 87, 143	Fatty acid methyl esters
12	Methyl oleate	2085	4.34	$C_{19}H_{36}O_2$	M+:-296 M/Z:-41, 55, 69	Fatty acid methyl ester
13	Methyl octadeca-9,12-dienoate	2093	2.79	$C_{19}H_{34}O_2$	M+:-294 M/Z:-67, 81 ,95, 109, 123	Fatty acid methyl ester
14	15-Hydroxypentadecanoic acid	2111	1.71	$C_{15}H_{30}O_3$	M+:-258 M/Z:-41, 98, 171, 185	Long-chain fatty acid
15	Phytol, acetate	2168	0.19	$C_{22}H_{42}O_2$	M+:-338 M/Z:-95, 123	Diterpenoid
16	Ricinoleic acid	2170	0.52	$C_{18}H_{34}O_2$	M+:-282 M/Z:-41, 55, 129	Fatty acid
17	Palmitoleic acid	1976	8.26	$C_{16}H_{30}O_2$	M+:-254 M/Z:-55, 69, 83, 97, 98, 123	Mono-unsaturated fatty acid
18	Docosane	2200	0.38	$C_{22}H_{46}$	M+:-310 M/Z:-43, 57, 85, 99	Alkane
19	Methyl 18-methylnonadecanoate	2212	0.40	$C_{21}H_{42}O_2$	M+:-326 M/Z:-41, 43, 74, 87	Fatty acid methyl ester
20	Sylvic acid	2265	0.84	$C_{20}H_{30}O_2$	M+:-302 M/Z:-41, 91	Diterpenoid
21	Dihydroabietic acid	2282	2.96	$C_{20}H_{32}O_2$	M+:-304 M/Z:-41, 81, 121, 159	Diterpenoid
22	Methyl erucate	2483	3.66	$C_{23}H_{44}O_2$	M+:-352 M/Z:-41, 55, 83, 97, 123	Fatty acid methyl ester
23	Methyl behenate	2531	0.30	$C_{23}H_{46}O_2$	M+:-354 M/Z:-43, 57, 74, 87, 101	Fatty acid methyl ester
24	Isoheptacosane	2656	0.46	$C_{27}H_{56}$	M+:-380 M/Z:-43, 57, 71, 85, 99	Alkane
25	17-Oxo-6.beta.-pentyl-4-nor-3,5-secoandrostan-3-oic acid	2756	6.41	$C_{23}H_{38}O_3$	M+:-362 M/Z:-67, 107, 119	Fatty acid methyl ester
26	Squalene	2914	5.18	$C_{30}H_{50}$	M+:-410 M/Z:-41, 69, 81	Triterpenoid
	Total		79.74			

3.3. Biochemical analysis

The total phenolic content (TPC) of both the hexane extract (CMHE) and the methanolic extract (CMME) of *C. macrophylla* was measured using a standard calibration curve of gallic acid **(Figure 7)**, and the results were represented as µg/mg gallic acid at 650 nm. According to the observations, the methanolic extract has a larger phenolic content (51.27 ± 0.61 µg/mg of GAE) than the hexane extract (40.58 ± 0.50 µg/mg of GAE). The total phenolic content of the extracts was determined by Folin-Ciocalteu reagent in an alkaline atmosphere. The principle behind this method is the formation of complex blue compounds that can be measured at a wavelength of 650 nm. Folin-Ciocalteau reagent oxidizes phenol groups to reduce the phosphomolybdate-phosphotungstate that is present in the Folin-Ciocalteau reagent into a molybdenum-tungsten complex (Shetty et al., 1995).

The total flavonoid content (TFC) of both CMHE and CMME was calculated using a standard calibration curve of quercetin **(Figure 8)**, and the results were represented as µg/mg quercetin. It was found that the methanolic extract (49.47 ± 0.34 µg/mg of QE) had a greater total flavonoid concentration than the hexane extract (34.42 ± 0.27 µg/mg of QE). The total flavonoid content was determined by the aluminum chloride colorimetric method that follows the principle based on color formation due to the formation of complexes between aluminum chloride and the keto group on C-4 atoms and hydroxy groups on neighboring C-3 or C-5 atoms of flavones. When this complex is formed, the wavelength shift is

measured at 415 nm, and the solution will turn yellow (Djeridane et al., 2006).

The total antioxidant content (TAC) was calculated using the ascorbic acid standard calibration curve, and the results were represented as micrograms per milligram of ascorbic acid equivalent (µg/mg of AAE). This assay was done by following the phosphomolybdate method. Its principle is that in the presence of plant extracts (antioxidant), Mo (VI) is reduced to Mo (V) and a green-colored phosphomolybdenum V complex is formed, which shows maximum absorbance at 695 nm (Rouzbahan et al., 2016). As compared to hexane extract (11.42 ± 0.23 µg/mg of AAE), methanolic extract (27.89 ± 0.24 µg/mg of AAE) had a higher content (Table 3). A significant amount of total phenolic content in methanolic plant extract has been observed to be higher as compared to hexane fractions, along with being rich in total flavonoid content (Zazouli et al., 2016). Previously conducted studies proved that polar plant extracts exhibit stronger antioxidant capacity when compared to other non-polar (hexane) plant extracts (Nawaz et al., 2020).

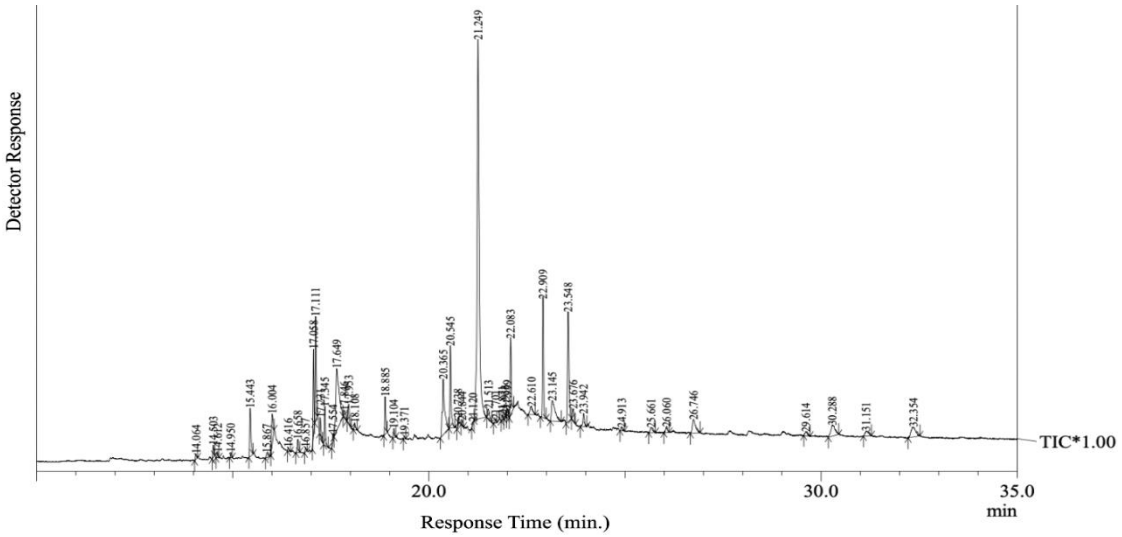

Figure 4. Gas chromatogram of *C. macrophylla* methanolic extract (CMME)

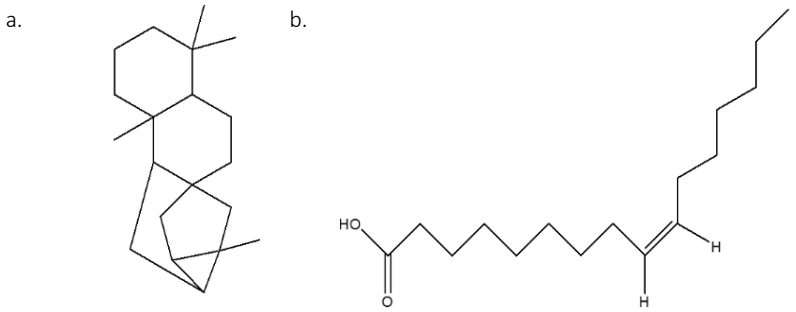

Figure 5. Chemical structures of a. trachylobane and b. palmitoleic acid

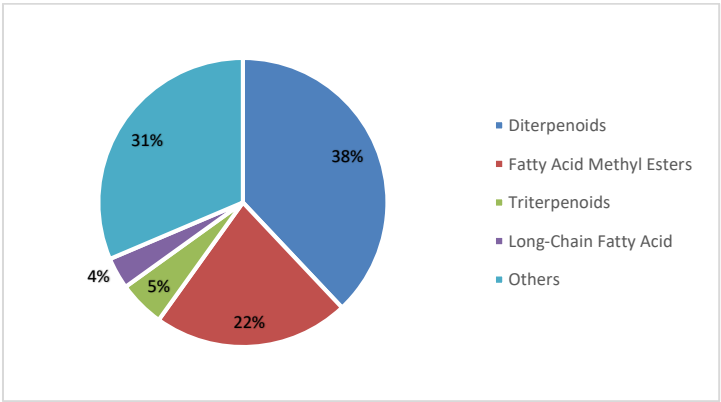

Figure 6. Classes of compounds present in *C. macrophylla* methanolic extract (CMME)

Researchers investigated the pharmacological activity of *C. attenuata* in May 2017. The leaves of the subject plant were collected from the Chittagong hill tracts and used for preparing methanolic extract. To assess its potential antioxidant ability, the Folin-Ciocalteu technique was followed to calculate the TPC value as well as aluminum chloride colorimetric technique was used to calculate the TFC value; which were 67.71 GAE/g and 341.90 QE/g, respectively. Following the observations made by these methods along with other supporting experiments, it was concluded that the methanol extract of the sample plant has good antioxidant activity and with promoting further deeper study and research, it can be used as a potential traditional medicine (Hossain et al., 2019).

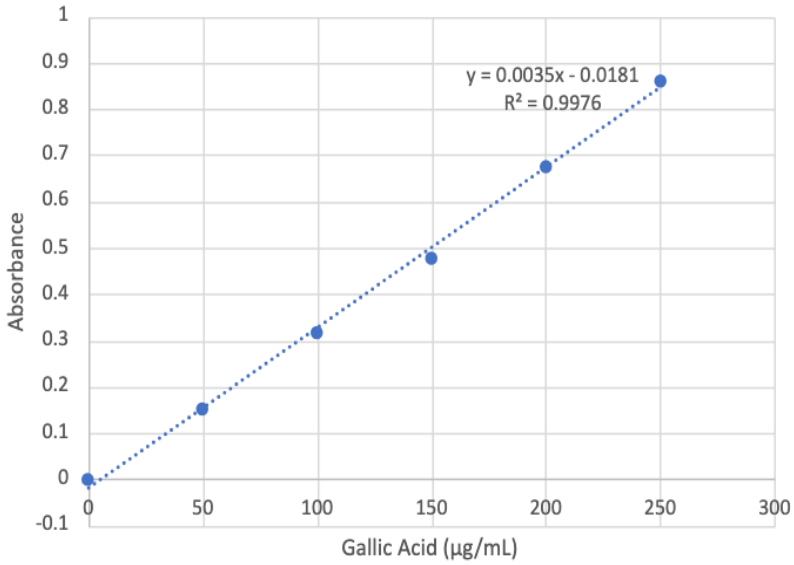

Figure 7. Calibration curve of gallic acid for total phenolic estimation

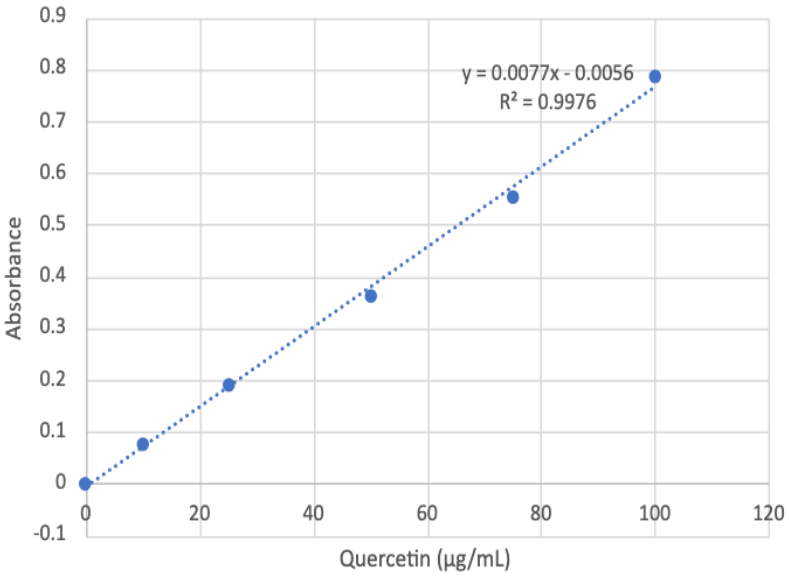

Figure 8. Calibration curve of quercetin for total flavonoid estimation

Another study was conducted in Assam to check the antidiabetic activity of hydro-alcoholic stem bark extract of *C. arborea* Roxb. with antioxidant potential in diabetic rats. In this study, the stem bark of the subject plant was collected from the forest of Dibrugarh district in December 2014. The stem barks were air-dried and then further coarsely powdered, for extraction in the ethanol: water mixture, and thus, the hydro-alcoholic extract was prepared. This prepared extract was then used to assess the total phenolic content and total flavonoid content of *C. arborea* Roxb. following the Folin-Ciocalteu colorimetric method and the aluminum chloride colorimetric method, respectively. In the end, a positive result was obtained in which the TPC and TFC values were calculated to be 52.17 ± 2.48 GE/g and 39.10 ± 2.15 QE/g, respectively. Thus, along with other supporting observations, the conclusion was made that the herbal antioxidant property is present in the hydro-alcoholic extract of *C. arborea* stem bark as indicated by the phenolic and flavonoid contents in the plant. Moreover, it may be a possible reason to treat oxidative stress-induced diabetes mellitus (Junejo et al., 2017).

In 2014, the natural antioxidants were isolated, and identified from the aerial parts of *C. kwangtungensis* Chun and further, its antioxidant activity was also assessed. In this research, the extract was prepared as petroleum ether fraction (PEF), an ethyl acetate fraction (EAF), and *n*-butanol fraction (BF). The standards used for

calculating the TPC value was pyrogallic acid and quercetin was used as the standard for calculating TFC value. The results obtained showed the same order for TPC and TFC values of the sample plant with butanol fraction showing higher values than ethyl acetate fraction and petroleum ether fraction showing negligible results. Along with other supporting experiments, the conclusion stated that the presence of phenolic compounds such as tannins, phenolic acids, and flavonoids is the major contributing factor to this plant's antioxidant activity (Cai et al., 2014).

A group of researchers studied the chemical constituents and biological activities of *C. maingayi* leaves in Malaysia. The leaves were used to prepare the methanolic extract and further, this concentrated extract was resuspended in water and partitioned into hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc), *n*-butanol, (*n*-BuOH) and aqueous (Aq) medium. The colorimetric assay using Folin–Ciocalteu's reagent was followed to calculate the total phenolic content of these prepared methanol extracts and fractions of the sample plant; with gallic acid used as standard. Results showed that methanol extract has the highest TPC value as 138.52 ± 0.02 mg GAE/g, followed by the fractions of ethyl acetate (126.39 ± 0.25 mg GAE/g) and butanol (95.59 ± 0.31 mg GAE/g). However, hexane, dichloromethane, and aqueous fractions showed lower TPC values of 24.98 ± 0.06, 34.47 ± 0.12, and 27.60 ± 0.40 mg GAE/g, respectively (Ado et al., 2016).

Table 3. IC_{50} values of various biological activities of plant extracts of *C. macrophylla*

Plant extracts	IC_{50} of various biological activities (µg/ml)		
	DPPH radical scavenging activity	Metal chelating activity	Reducing power activity
CMHE[1]	154.19 ± 1.99 µg/ml	41.60 ± 1.31 µg/ml	113.78 ± 0.99 µg/ml
CMME[2]	91.97 ± 0.33 µg/ml	10.90 ± 1.40 µg/ml	56.72 ± 0.67 µg/ml
Standard	Ascorbic acid: 57.66 ± 0.40 µg/ml	EDTA: 60.18 ± 0.39 µg/ml	Gallic acid: 74.36 ± 0.29 µg/ml

The obtained data were found to be significant at $p < 0.05$, after One-Way ANOVA

[1] *C. macrophylla* hexane extract

[2] *C. macrophylla* methanolic extract

3.4. Antioxidant activities

The antioxidant properties of the hexane and methanolic extracts of *C. macrophylla* were evaluated on three parameters: DPPH radical scavenging activity, metal chelating activity of Fe^{2+}, and reducing power activity of Fe^{3+}. The results obtained are outlined in Table 3.

3.4.1. DPPH radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical that appears violet in color and gives maximum absorption at 517 nm. It tends to form a diamagnetic molecule via accepting electrons. The molecule with potent antioxidant properties quenches this free radical and prevents it from damaging a healthy cell. As the antioxidant molecule quenches the free radical, the violet color of DPPH loses its intensity and hence the absorbance is decreased (Mishra et al., 2012). Previous studies outline the comparison between the DPPH radical scavenging activity of the polar and non-polar plant extracts. It was observed that the polar extract (butanol) showed very strong antioxidant activity with an IC_{50} value of 5.58 µg/ml whereas the non-polar (hexane) extract had an IC_{50} value of 304.37 µg/ml (Amin et al., 2015). In this study, the solvents used were methanol (polar) and hexane (non-polar) and the IC_{50} values of the plant extracts were observed in the following trend: CMME (91.97 ± 0.33 µg/ml) < CMHE (154.19 ± 1.99 µg/ml). The standard ascorbic acid showed the IC_{50} value of 57.66 ± 0.40 µg/ml. The IC_{50} value is the minimal concentration of a drug that is required for 50% inhibition in vitro. Therefore, the lesser the IC_{50} value; the better will be the inhibitory action. This indicates that in comparison to the hexane extract, the methanolic plant extract shows better DPPH radical scavenging activity. This may be due to abundantly present palmitoleic acid as shown by its GC-MS profile.

3.4.2. Metal chelating activity

A common chelator, ferrozine, can couple with ferrous ions and form a red-colored complex. This Ferrozine-Fe complex is disturbed as another competitive chelator is added to the reaction mixture. As the complex breaks, the intensity of the red color diminishes. This decrement gives the idea about the potential of the co-existing chelating agents (Gulcin & Alwasel, 2022). Earlier, in a combination of solvents used for preparing the plant extracts, both polar as well as non-polar solvents were used and it was observed that polar (butanol) extract prepared from the plant *C. kwangtungensis* Chun showed the best metal chelating activity with IC_{50} value of 0.68 ± 0.012 mg/ml, whereas the non-polar extract did not exhibit the potential metal chelating activity (Cai et al., 2014). However, in this study, both the polar (methanol) and non-polar (hexane) plant extracts showed better activity as their IC_{50} values were observed in the following order: CMME (10.90 ± 1.40 µg/ml) < CMHE (41.60 ± 1.31 µg/ml). The standard EDTA showed the IC_{50} value of 60.18 ± 0.39 µg/ml. Based on this obtained result, it is concluded that methanolic extract shows better metal chelating activity when compared to the hexane extract. This shows that methanolic extract has better antioxidant activity than hexane extract. This may be due to the higher amount of terpenoids present in the methanolic extract that has been reported to have antioxidant properties.

3.4.3. Reducing power activity

As the protocol is followed, the reaction mixture appears yellow. The molecule with potent antioxidant capacity reduces the ferric ion into ferrous ion and hence the resultant color appears Prussian blue. This reduction, indicating the electron-donating ability of bioactive molecules, is observed in the increment in the absorbances recorded at 700 nm (Patra et al., 2016). There have been reports where the methanolic and hexane plant extracts have shown comparable reducing power activity with the RP_{50} values of 10.76 ± 0.01 µg/ml and 10.04 ± 0.01 µg/ml, respectively (Bahuguna et al., 2023). In this study, the RP_{50} values of the plant extracts were observed in the following order: CMME (56.72 ± 0.67 µg/ml) < CMHE (113.78 ± 0.99 µg/ml). The standard displayed the RP_{50} value of 74.36 ± 0.29 µg/ml.

3.5. Statistical analysis

In each experiment, all the varying concentrations of the hexane and methanolic plant extracts were taken in triplicates. The results obtained from each experiment were analyzed via the Duncan test

at 5% for One Way ANOVA and were found to be significant ($p < 0.05$).

4. Conclusions

The plant *C. macrophylla* has widespread growth across the Northeastern parts and Himalayan states of India and yet not much data is available accounting for its medicinal and therapeutic properties. The obtained results show that the total phenolic content, total flavonoid content, and total antioxidant content were higher in methanolic extract when compared to hexane extract. The observation also claims to prove that the methanolic and hexane extract of *C. macrophylla* are better metal chelators than the standard EDTA. However, the hexane extract does not show much significant activity for DPPH scavenging and reducing power. The methanolic extract shows comparable DPPH scavenging activity and reducing power activity when compared to the respective standards, ascorbic acid, and gallic acid. This study aids the idea that the antioxidant and other bioactivity-related traits of the naturally available plant extracts can be utilized while eradicating the expense issues. *C. macrophylla* possesses a strong therapeutic potential and, if explored further, may prove to be a powerful antioxidant and cost-effective agent compared to synthetically derived ones and can replace various artificial synthetic agents from pharmaceutical industries.

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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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CRediT authorship contribution statement

Aradhana Arya: Methodology, Validation, Writing orginal draft, Writing review and editing
Shiv Kumar Dubey: Conceptualization, Formal analysis, Investigation, Resources, Data curation, Writing orginal draft, Writing review and editing, Supervision
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Supplementary File

None.

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