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RESEARCH ARTICLE

Phytochemical screening, GC-MS profiling and *in vitro* antioxidant activity of leaves of *Dysoxylum malabaricum* Bedd. ex C. DC.

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Abstract

Plants are a rich source of phytocompounds, have remained an integral part of traditional medicine and serve as alternatives to modern medical treatments. They are powerful sources of antioxidants and the bioactive compounds in plants are associated with a wide range of pharmacological activities. *Dysoxylum malabaricum* is a species of medium to large-sized trees from the Meliaceae family that is widely found in the Southern Western Ghats and its bark and fruits are used in traditional medicine. Even though it is widely used as ethnomedicinal plant, limited research has been done on its phytochemical constituents, especially the phytocompounds present in the leaves. Therefore, this study aims to extensively explore and identify the phytocompounds and bioactive elements found in the leaf extracts of *D. malabaricum.* Extract was prepared from leaves of *D. malabaricum* using soxhlet extraction method in different solvents (methanol, water and chloroform). Quantitative estimation of phytochemicals and *in vitro* antioxidant assays were carried out, followed by chemical profiling of the extracts using GC-MS, which revealed the presence of many important secondary bioactive compounds. The methanolic extract showed a higher concentration of phenolics (67.88 ± 0.26 mg GAE/g) and flavonoids (57.55 ± 0.23 mg QE/g) when compared to aqueous and chloroform extracts. The methanolic extract also demonstrated remarkable DPPH scavenging (with IC_{50} value 32.45 ± 0.22 µg/mL) and ferric reduction activities. The results demonstrate that *D. malabaricum* is an effective source of bioactive and antioxidant compounds.

Keywords: antioxidants; bioactive compounds; dysoxylum; GC-MS analysis

Introduction

Plants have always been a plentiful source of secure and efficient medication as they are abundant in phytochemicals or active secondary metabolites (1) such as alkaloids, phenolic compounds, flavonoids, fatty acids and saponins. Plant polyphenolics are extremely significant because they provide hydroxyl groups that can scavenge free radicals. Many plant species have been studied recently for the management and treatment of a variety of illnesses due to their strong antioxidant qualities (2). Phytochemical profiling of plant extracts plays a significant role in identifying pharmacologically important bioactive compounds and help in drug design and discovery.

Dysoxylum is a genus of woody plants belonging to the mahogany family, Meliaceae, that have been extensively used in agriculture, industrial purposes and traditional medicine. The genus Dysoxylum mostly consists of medium to large sized trees that are widely used as ethnomedicine and exhibit excellent pharmacological properties (3). A wide range of bioactive secondary metabolites have been reported from the plants belonging to the genus Dysoxylum which includes alkaloids, phenolics, saponins, diterpenoids, triterpenoids and sesquiterpenoids (4). Important anticancer agents like Rohitukine, the key precursor for synthesis of the anticancer drug flavopiridol (5) and camptothecin (6) have been successfully isolated from the plant parts of other Dysoxylum species. Rohitukine isolated from the bark extracts of *D. binectariferum* was found to show significant cytotoxic activity against ovarian and breast cancer cell lines (7) . Leaf and bark extracts of *D. binectariferum* were reported to exhibit notable anti-inflammatory and immunomodulatory properties. Dysobinin, a tetranortriterpenoid isolated from the ethanolic fruit extract of *D. binectariferum,* was found to have CNS depressant and anti-inflammatory activities (8). The pharmacological potency of the phytocompounds isolated from the *Dysoxylum* genus is well-known, exhibiting properties like anti-inflammatory, anti-malarial, anti-tumor and antimicrobial activity (9).

D. malabaricum also referred to as white cedar is a commercially important species native to the Southern Western Ghats (Kerala and Karnataka) of India. It is an evergreen canopy tree species. It has unique morphological characteristics like white fragrant flowers and smooth greyish or pale white bark. Many plant parts of *D. malabaricum* like bark, fruits and seeds are being used in traditional folklore and Ayurveda. *D. malabaricum* is an excellent reservoir of a wide variety of terpenoids, especially triterpenes with remarkable biological properties. Cycloartane triterpenoids isolated from bark extract of *D.malabaricum* were found to cause inhibition of cell cycle in MCF-7 cell lines (10) and induce programmed cell death in MDA-MB-231 cells (11). A novel triterpenoid called Mahamanalactone A was identified from the bark extract of *D. malabaricum* which demonstrated potent cytotoxic effect against BT549 breast cancer cells (12). Methanolic leaf extract of *D. malabaricum* showed potent larvicidal, pupicidal and anti -ovipositional properties against *Anopheles stephensi* (13).

To date, studies on *D. malabaricum* leaves remain limited even though other parts of the plant especially the bark is being extensively explored and a complete screening of the phytochemical compounds present in the leaves of *D. malabaricum* has not been carried out. Leaves serve as a renewable source when compared to the bark as removal of the bark can sometimes be a threat to the plant. So, this study aims at thoroughly investigating and identifying the phytoconstituents and bioactive compounds present in the leaf extracts of *D. malabaricum* through phytochemical screening, chemical profiling using GCMS and further evaluation of the *in vitro* antioxidant activity of the plant extracts in different solvents.

Material and Methods

Collection of plant materials

Fresh leaves of *D. malabaricum* (Fig. 1) were obtained from Kerala Forest Research Institute (KFRI), Peechi, Thrissur, Kerala. The plant specimen was identified at the Department of Silviculture, KFRI, Thrissur and the voucher specimen (Voucher no: FRLHT 6807) was submitted to Foundation for Revitalisation of Local Health Traditions (FRLHT), Bengaluru. After collection, the leaves were cleaned properly using distilled water and were stripped from the stems. The leaves were dried using a hot air oven at temperatures lower than 40 °C for 2-3 days until they became well dried. After drying, the leaves were ground well using mechanical blender into fine powder.

Fig. 1. *Dysoxylum malabaricum.*

Preparation of extract

Using a Soxhlet apparatus, 25 g of dried *D. malabaricum* leaf powder was extracted with 250 mL of methanol, water and chloroform until the reflux became clear. The extract was then evaporated with a rotary evaporator until concentrated extract was left behind (14). The acquired crude extract was weighed and the yield percentage was determined as follows:

% Yield = weight of crude extract / weight of dried plant sample x 100 (Eqn.1)

The crude extract obtained was then stored at 4 °C until further use.

Phytochemical analysis

Determination of Qualitative phytochemical composition

Qualitative phytochemical composition was evaluated for the leaves of *D. malabaricum* extracted using methanol, water and chloroform. The phytochemical screening for alkaloids (Wagner's test), flavonoids (Ammonia test), saponins (Froth test), tannins (Lead acetate test), terpenoids (Salkowski test), steroids (Salkowski test) and phenolics (Ferric Chloride test) was performed using standard protocol (15, 16).

Determination of quantitative phytochemical composition

Total phenolic concentration

The total concentration of phenolic compounds present in the *D. malabaricum* leaf extracts was measured using the Folin and Ciocalteau (FC) reagent method (17). First, a mixture was prepared by combining 0.5 mL the extract with 1 mL of FC reagent and 2.5 mL of distilled water. After allowing the mixture to react for 6 min, 20 % Na_2CO_3 (sodium carbonate) solution was added to the mixture, which was then kept in darkness for 30 min to develop colour. The optical density (OD) of the final solution was taken at 760 nm using Shimadzu UV-1800 Spectrophotometer. Triplicate measurements were carried out. Gallic acid was utilised as standard, with a calibration curve prepared in the range of 20-100 µg/mL ($y = 0.0096x + 0.0114$, $R^2 = 0.9985$). Gallic acid equivalents (mg GAE/ g) per gram of the dry sample were used to express the results.

Total flavonoid concentration

Spectrophotometric measurement of the total concentration of flavonoid present in the plant extracts was done using Aluminium chloride method with minor changes (18). After combining, 0.5 mL of plant extract and 2 mL of 100 % methanol together, 10 % Aluminium chloride and 1M Sodium acetate were added. Measurement of optical density was done at 415 nm after keeping the mixture in darkness for 40 min. Using Quercetin as standard, a calibration curve in the range of 20-100 µg/mL ($y = 0.0084x - 0.0285$, $R^2 = 0.9928$) was prepared. Quercetin equivalents (mg QE/g) per gram of the dry sample were used to express the results.

Gas Chromatography and Mass Spectroscopy (GC-MS) analysis

The bioactive chemical compounds found in the leaf extracts of *D. malabaricum* were detected using Gas Chromatography and mass spectroscopy (Model: Shimadzu GCMS TQ8040NX). The system utilized a capillary column measuring 30meters in length, with an internal diameter of 0.25 mm and a film thickness of 0.25µm. Helium was utilised as the carrier gas

(99.9995 %). A volume of 1 μL of samples was administered through the GC-MS device. The column was initially held at a temperature of 70 °C for 3 min and then increased to 300 °C and maintained constant for 15 min. Electron ionization (EI) mode was used at 70eV with a scan time of 0.5 sec for detection and the fragments were captured at 50-550 m/z range. The retention time of the compounds was compared with the standard NIIST mass spectra database to recognize the bioactive components of the extracts (19).

In vitro antioxidant assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

The free radical scavenging potential of the leaf extracts in various solvents was assessed using the DPPH method. Different dilutions of the extracts (10-200 μg/mL) were prepared by combining 0.5 mL of each dilution with 2.5 mL of methanol and 1 mL of 0.004 % DPPH solution. The reaction mixtures were then vortexed and kept in dark condition for 30 min (20). The sample absorbance readings were recorded at 516 nm. The standard used was ascorbic acid. Radical scavenging capacity was found out using the following equation:

Scavenging % = (OD of Control - OD of Sample / OD of Control) x 100 (Eqn.2)

IC_{50} values were obtained by plotting dose-response curves (using the percentage of radical scavenging potential), which represent the antioxidant potential of the extracts.

Ferric Reducing Antioxidant Power (FRAP) Assay

The potassium ferricyanide method, with some modifications (21) was adopted to analyse the ferric reducing antioxidant power of the leaf extracts. Initially, 1 mL of the plant extract was combined with 2.5 mL of phosphate buffer and 1 % potassium ferricyanide. After incubation at 55°C for 20 min, 10 % TCA (Trichloro acetic acid) and 2.5 mL of distilled water were added to the reaction mixture, along with 0.5 mL of ferric chloride solution. The optical density of the final solutions was measured at 700 nm. Standard used was ascorbic acid.

Statistical analysis

Triplicate measurements were done for all the tests. Any significant changes between the three solvents used were compared using ANOVA (Analysis of Variance) test and post hoc analysis- Duncan's test with a significance level set at $p \leq 0.05$. The results are presented as Mean ± standard deviation (SD).

Results

Qualitative phytochemical analysis

The percentage yield of the crude extracts after extraction using different solvents by Soxhlet extraction method is depicted in Table 1. Methanolic leaf extract showed a relatively higher yield percentage of 18 % followed by aqueous extract (13.6 %) and chloroform extract (10 %).

The preliminary phytochemical screening of the leaf extract in methanol, water and chloroform revealed the presence of bioactive phytoconstituents like alkaloids, phenolics, flavonoids, tannins, terpenoids, saponins and steroids. The results of the preliminary screening are given in Table 2.

Table 1. Percentage yield of leaf extracts of *D. malabaricum* in different solvents

Solvent	Weight of dried plant material (g)	Weight of crude extract (g)	% Yield	Colour of extract	Texture of extract
Methanol	25	4.5	18	Light green	Sticky
Water	25	3.4	13.6	Brown	Dry powder
Chloroform	25	2.5	10	Dark green	Sticky

Table 2. Preliminary phytochemical screening profile of leaf extract *D. malabaricum* leaves in different solvents

Phytocompounds	Solvents used		
	Methanol	Water	Chloroform
Alkaloids (Wagner's test)	+	+	+
Flavonoids (Ammonia test)	+	+	+
Tannins (Lead acetate test)	+	+	-
Saponins (Froth test)	+	+	-
Terpenoids (Salkowski test)	+	+	+
Steroids (Salkowski test)	+	-	+
Phenolics (Ferric chloride test)	+	+	+

"+" = Present **"-"** = Absent

Quantitative Phytochemical analysis

The results of quantitative phytochemical composition (phenolic and flavonoid concentration) of the plant extracts in different solvents are mentioned in Table 3. Methanolic extract had the maximum phenolic content (67.88 ± 0.26 mg GAE/g), whereas chloroform (46.13 ± 0.31 mg GAE/g) had the lowest.

Table 3. Quantitative phytochemical concentrations of the leaf extracts of *D. malabaricum*

Solvent	Phenolic concentration (mg GAE/g)	Flavonoid concentration (mg QE/g)
Methanol	67.99 ± 0.26^{a}	57.55 ± 0.23^{a}
Water	54.4 ± 0.31^{b}	42.51 ± 0.24^{b}
Chloroform	46.13 ± 0.31^{c}	39.18 ± 0.58^{c}

The data is represented as mean ± standard deviation (n=3) and the values followed by different alphabets in the same column represents significant differences ($p<0.05$)

Total content of flavonoid was higher in methanolic extract (57.55 ± 0.23 mg QE/g) followed by aqueous extract (42.51 ± 0.24 mg QE/g) and lowest in chloroform extract (39.18 ± 0.58 mg QE/g).

Gas Chromatography and Mass Spectroscopy (GC-MS) analysis

Gas chromatography and mass spectroscopy (GC-MS) profiling was performed (Fig. 2) to identify the major bioactive compounds present in the leaves of *D. malabaricum* and the results are given in Table 4. Many biologically important compounds like phenolics, diterpenoids, triterpenoids, sesquiterpenoids, fatty acids, organic and aromatic compounds, etc were identified

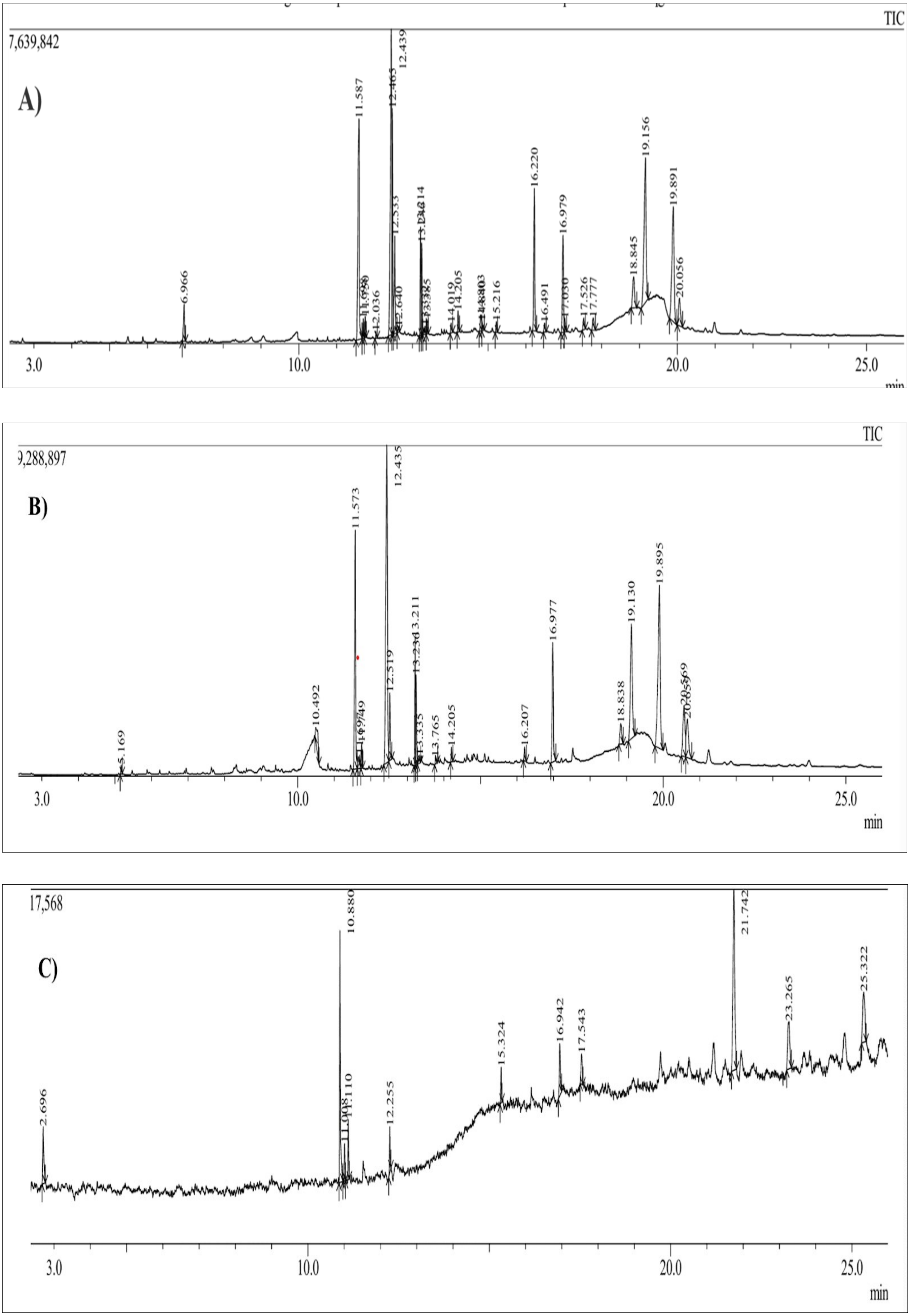

Fig. 2. GC-MS profile of the (A) methanolic, (B) aqueous and (C) chloroform extracts of leaves of *D. malabaricum.*

Table 4. Bioactive compounds identified through GC-MS analysis of the leaf extracts of *D. malabaricum*

S No	Name of compound	Retention time	Peak area (%)	Molecular formula	Biological activity	Reference
1	5-hydroxymethylfurfural	6.966	1.72	$C_6H_6O_3$	Antioxidant, Antiproliferative, Anti -inflammatory	(22,23)
2	n-hexadecanoic acid	11.587	14.23	$C_{16}H_{32}O_2$	Anti-inflammatory, Antioxidant, Antimicrobial	(24,25)
3	Heptadecanoic acid	12.036	0.22	$C_{17}H_{34}O_2$	Antiproliferative	(26)
4	Oxazole, 4,5-dihydro-2-pentadecyl-	12.439	18.5	$C_{19}H_{37}NO$	Antibacterial, Antifungal, Antitumour	(27)
5	9,12,15-Octadecatrienoic acid	12.465	7.34	$C_{18}H_{30}O_2$	Anti-inflammatory, Anticancer, Hepatoprotective, Antioxidant	(28)
6	Octadecanoic acid	12.533	4.44	$C_{18}H_{36}O_2$	Antibacterial, Antioxidant, Antiviral	(29,30)
7	Docosanoic acid	14.205	0.64	$C_{22}H_{44}O_2$	Anticancer	(31)
8	Methyl eicosa-5,8,11,14,17-pentanoate	14.84	0.28	$C_{25}H_{48}O_2$	Anti-inflammatory	(32)
9	13-Docosenamide, (Z)-	15.216	0.38	$C_{22}H_{43}NO$	Antimicrobial, Anticancer	(33)
10	delta. -Tocopherol	16.22	6.25	$C_{20}H_{29}O_2$	Antioxidant, Anticancer	(34)
11	beta. -Tocopherol	16.979	4.92	$C_{20}H_{29}O_2$	Antioxidant	(35)
12	Stigmasterol	19.156	13.05	$C_{29}H_{48}O$	Anticancer, Antimicrobial	(36,37)
13	gamma. -Sitosterol	19.891	10.98	$C_{29}H_{50}O$	Anticancer, Anti-hyperglycaemic	(38,39)
14	Tetradecanoic acid	10.492	2.15	$C_{14}H_{28}O_2$	Antimicrobial	(40)
15	beta. -Amyrin	20.569	5.21	$C_{30}H_{50}O$	Antimicrobial, Anti-inflammatory, Anticancer	(41,42)
16	Neophytadiene	10.88	18.7	$C_{20}H_{38}$	Anti-inflammatory, Antioxidant, Antidepressant, Anticonvulsant	(43,44)
17	Phytol	12.255	3.09	$C_{20}H_{40}O$	Antimicrobial, Anti-inflammatory, Antioxidant, Anticancer	(45,46)
18	Squalene	15.324	2.41	$C_{30}H_{50}$	Antioxidant, Anti-inflammatory	(47,48)
19	(E)-Atlantone	16.942	5.81	$C_{15}H_{22}O$	Antimicrobial, Anticancer, Anti-inflammatory	(49,50)
20	alpha. -Tocopheryl acetate	17.543	3.84	$C_{26}H_{42}O_2$	Antioxidant, Antitumor	(34)

after performing the GC-MS analysis. A total of 25 compounds were identified in methanolic extract followed by 20 in aqueous extract and 11 in chloroform extract, out of which 20 were found to be biologically relevant.

In vitro antioxidant assay

The DPPH antioxidant assay was employed to assess the ability of the leaf extracts to scavenge free radicals. IC_{50} value (µg/mL) was determined to quantify the ability of the extracts to quench free radicals. Methanolic extract showed the lowest IC_{50} value of 32.45 ± 0.22 µg/mL, indicating its higher effectiveness in scavenging free radicals. Chloroform leaf extract exhibited the highest IC_{50} value of 61.55 ± 0.39 µg/mL which indicates a lower antioxidant activity. The IC_{50} values of the plant extracts in different solvents are given Fig. 3.

Ferric reducing antioxidant power (FRAP) assay was conducted to determine the capacity of antioxidants present in the plant extracts to convert Fe^{3+} ions to Fe^{2+} ions using potassium ferricyanide method. Plant extracts with higher levels of antioxidants exhibited a significant increase in absorbance value. A higher OD value indicates higher antioxidant activity. The antioxidant activity of the leaf extracts in different solvents is given in Fig. 4.

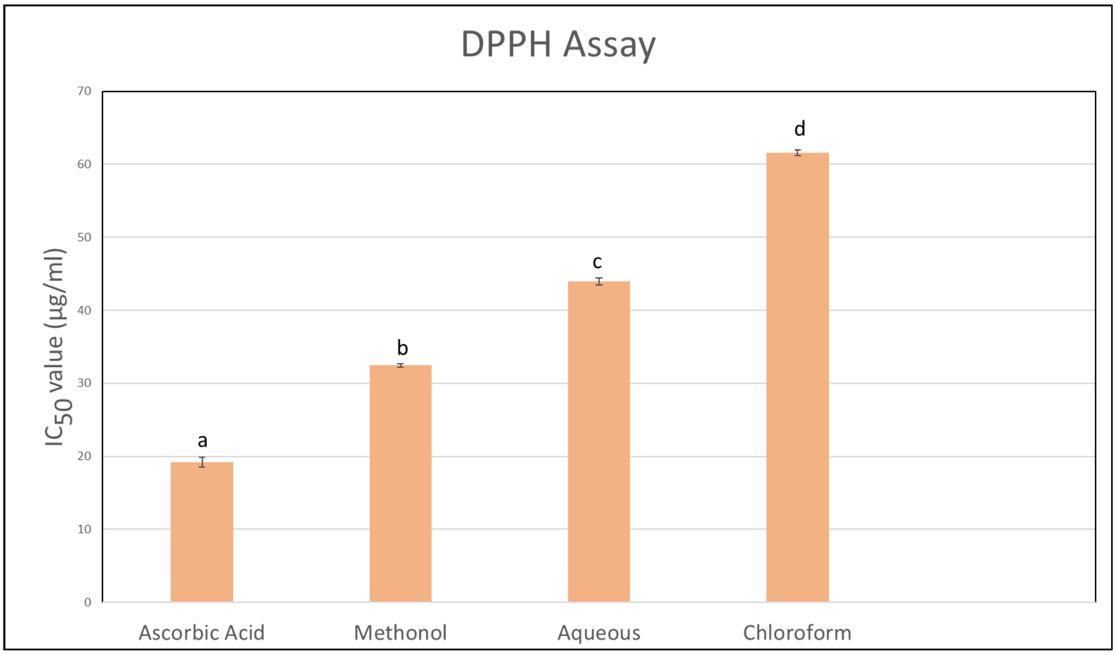

Fig. 3. DPPH analysis of leaf extract of *D. malabaricum* in different solvents.

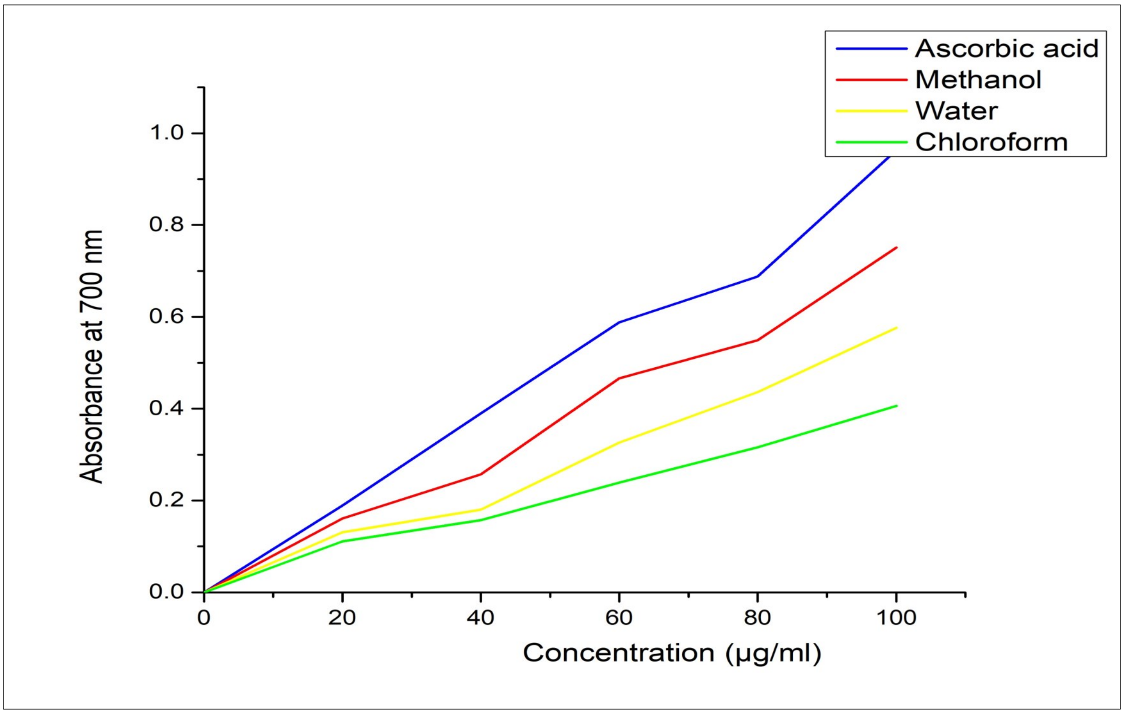

Fig. 4. FRAP analysis of leaf extract of *D. malabaricum* in different solvents.

Discussion

The study explored the phytochemical composition, antioxidant activity and identification of the phytocompounds present in the leaf extracts of *D. malabaricum* through GC-MS profiling.

The yield percentage of crude extracts of the plant material after soxhlet extraction was found to be the highest in methanol (18 %), suggesting that methanol is an efficient solvent for extracting the target compounds from the leaf extracts. Methanol being a polar solvent can dissolve a broad range of phytocompounds like phenolics, alkaloids and flavonoids potentially contributing to its higher extraction efficiency. Chloroform exhibited the lowest yield (10 %) as it is less polar and typically favours the extraction of non-polar lipids and certain alkaloids which might reflect the lower yield percentage (51) .

The preliminary phytochemical screening unveiled the presence of important bioactive phytocompounds like alkaloids, phenolics, flavonoids, tannins, saponins, terpenoids and steroids in methanolic leaf extract. Aqueous extract showed positive results for alkaloids, flavonoids, tannins, saponins, terpenoids and phenolics. Chloroform extract tested positive for all phytocompounds except saponins and tannins. The quantitative phytochemical composition (total concentration of phenolics and flavonoids) of the leaf extracts of *D. malabaricum* in different solvents were determined using standard protocols like FC method and aluminium chloride method respectively. The phenolic concentration was found to be greater in methanolic extract followed by aqueous extract and the least concentration was found in chloroform extract. This variation in the content of phenolic compounds can be linked to the solubility of the compounds in different solvents which can further depend on the polarity of the solvent (52). Similarly, the flavonoid composition was also found to be highest in methanolic extract and least in chloroform extract. Thus, methanol can be considered a better solvent for the extraction of both phenolic compounds and flavonoids which is in accordance with the results from previous literature (18).

GC-MS profiling of the leaf extract of *D. malabaricum* revealed the presence of a wide range of bioactive metabolites like n-hexadecenoic acid, Oxazole, 4,5-dihydro-2-pentadecyl, Methyl eicosa-5,8,11,14,17-pentanoate, Docosanoic acid, delta. -Tocopherol, Stigmasterol, gamma. -Sitosterol, beta. -Amyrin, Neophytadiene, Phytol, Squalene, (E)-Atlantone, etc. Previous reports reveal the biological activities of these compounds. n-hexadecanoic acid was found to demonstrate excellent anti-inflammatory, antimicrobial and antioxidant properties (24, 25). A study conducted by Kakkar and Narasimhan (27) showed the antibacterial, antifungal and antitumor activities of Oxazole, 4,5-dihydro-2-pentadecyl. Previous studies have shown that methyl eicosa-5,8,11,14,17-pentanoate have anti-inflammatory properties (32). Compounds like beta. -Amyrin, Phytol and (E)-Atlantone were found to have remarkable antimicrobial, anti-inflammatory and anticancer properties (41, 46, 50). Studies conducted on Neophytadiene and squalene have demonstrated the antioxidant and anti-inflammatory properties of the compounds (43, 47). Docosanoic acid, delta. -Tocopherol and Stigmasterol were found to exhibit anticancer properties (31, 34, 36).

The radical scavenging potential of the leaf extract in different solvents was assessed using DPPH assay. Ascorbic acid exhibited an IC_{50} value of 19.2± 0.65 µg/mL which was comparable to the IC_{50} value of methanolic extract (32.45 ± 0.22 µg/mL) which indicates a high antioxidant activity. The higher antioxidant activity of methanolic extract followed by the aqueous extract can be attributed to the higher concentration of phenols and flavonoids as they are known to be effective antioxidants (53). Chloroform extract exhibited a higher IC_{50} value of 61.55 ± 0.39 µg/mL which can be correlated to the lower phenolic and flavonoid concentrations. The ferric reducing antioxidant power of the leaf extract was also found to be highest for the methanolic extract with the highest OD values at all concentrations (10-200 µg/mL). Higher the absorbance value higher is the antioxidant potential and vice versa. The least OD values were recorded for the chloroform extract. The results from previous studies also confirm that antioxidant activity and the total concentration of flavonoids and phenols are positively correlated (54, 55).

Conclusion

To the best of our knowledge, the current study, is the first comprehensive report on the phytochemical screening, GC-MS profiling and assessment of antioxidant potential of the leaf extract of *D. malabaricum.* The quantitative phytochemical composition (phenolic and flavonoid content) of the leaf extracts in three different solvents (methanol, water and chloroform) was carried out and the methanolic extract was found to have the highest phenolic and flavonoid content and lowest in chloroform. This can be attributed to the polarity of the solvents and the solubility of the phytocompounds in different solvents. GC-MS profiling revealed the presence of a wide range of secondary bioactive constituents with notable biological activities. *In vitro* antioxidant activity was found to be maximum in methanolic extract as it exhibited the highest radical scavenging activity which can be directly correlated to the higher concentration of phenolics and flavonoids. From the study it can be concluded that the leaves of *D. malabaricum* share similar medicinal properties like other parts of the plant as well as other members of the genus.

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Authors' contributions

ASM and JX equally contributed to this work.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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