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RESEARCH ARTICLE

Phytochemical characterization by GC-MS and *in vitro* evaluation of antioxidant potential of *Walsura piscidia* Roxb. leaves extract

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Abstract

Walsura piscidia Roxb. (Family: Meliaceae) is currently known for rich sources of bioactive compounds with growing multiple therapeutic and medicinal importance. The main objectives of this study were to characterize the phytochemical profile of the leaves of *W. piscidia* by Gas Chromatography-Mass Spectrometry (GC-MS), followed by the evaluation of its antioxidant potential by quantifying the amounts of phenols and flavonoids present within the extracts, through the existing methods of detection. The extractive yield calculated after Soxhlet extraction was seen to be higher for the ethanolic extract with a value of 21.9 %, followed by the methanolic extracts (21.06 %) and the qualitative phytochemical tests gave similar classes of phytochemicals like triterpenoids, phenolic compounds and tannins in the methanolic and ethanolic extracts. The total phenolic content was seen to be higher in the ethanolic extract with a value of 26.192 ± 0.401 mg GAE/g and the total flavonoid content was seen to be higher in the methanolic extract with a value of 42.972 ± 0.214 mg QE/g. The methanolic extract showed promising results in the antioxidant assays with a significantly low IC_{50} value in DPPH assay and high ferric reducing power in ferric reducing antioxidant power (FRAP) assay. The GC-MS chromatograms showed almost similar compounds for both the methanolic and ethanolic leaf extracts, some important ones being n-Hexadecanoic acid, stigmasterol, campesterol, 5-hydroxymethyl furfural, etc, displaying properties of interest like antioxidant, anti-inflammatory, anti-microbial, etc. This work contributes to our better understanding of the medicinal properties of the leaves of *W. piscidia* and has also provided a strong scientific basis to the traditional usage claims of this tree.

Keywords: antioxidant assay; GC-MS analysis; phytochemical analysis; soxhlet extraction; triterpenoids

Introduction

In this contemporary era of science and technology, where curiosity-driven aspects of life are thriving, health-care remains an area that still relies on the traditional conservative treatment methods, primarily utilizing the naturally occurring plant medicinal products, against the synthetic ones. This advantage over the synthetic medications arises mainly from the less harmful or reduced toxic effects of the plant bioproducts. India with its rich heritage of naturally occurring medicinal plant compounds, has witnessed a large population of the rural areas relying on the herbal medicinal systems such as unani, siddha, yoga and ayurveda, for treating various illnesses (1). The antioxidant properties of natural products, medicinal plants mainly, have drawn a lot of interest in their usage as curative agents because they provide a wealth of bioactive compounds such as phenolic acids, flavonoids, tannins, phytosterols, etc. and can be used as a template for synthesizing new drugs with less toxicity (2).

With 16 species having been recognised by the Plant of the World Online (POWO), to date, the genus Walsura has a long-standing history of use in the traditional medicinal practices throughout the tropical regions of various Asian regions including Sri Lanka, the Western Ghats of India, Burma, Thailand, the Philippines and the Andaman islands and has been traditionally used for treating various ailments, but little is known about the secret ingredients behind their therapeutic activities (3). Numerous investigations into this genus have been conducted since the 1970s, leading to the identification of various species and new compounds. Belonging to the Meliaceae family, this genus holds considerable medical significance, due to the presence of some versatile volatile compounds with limonoids and triterpenoids being the two primary metabolites, having about 110 compounds isolated from the former and about 70 compounds isolated from the latter (4). These compounds have demonstrated their therapeutic effectiveness in terms of being anti-inflammatory, anti-diabetic, antioxidant, astringent, cytotoxic against cancer cells, neuroprotective and anti-coagulant agents and they have been widely used to treat skin allergies and diarrhoea.

Walsura piscidia Roxb is a medium-sized tree, the bark of which has been long used as a fish poison and the leaves have been used as traditional medicine but very little is known about its therapeutic effects (5). With the isolation of a novel compound named Piscidinol F, an apotirucallane triterpenoid along with other variants like C, D E and tirucallanes like Piscidinol A and B from *W. piscidia* leaves, using proton nuclear magnetic resonance spectroscopy and carbon-13 nuclear magnetic resonance spectroscopy, Purushothaman et al. and Govindachari et al. laid the blue-print for the investigation of this genus in 1985 and then in 1994, respectively (5, 6). Having very little foundation laid out on this medicinally important genus, this study mainly focusses on screening the important bioactive constituents present within the leaves of *W. piscidia* and evaluating its antioxidant potential.

Materials and Methods

Plant material

The tree specimen (leaves) of *Walsura piscidia* Roxb, intended for the study was sourced from the institute of Foundation for Revitalisation of Local Health Traditions (FRLHT), located in the village of Jarakabande Kaval near Yelahanka in Bengaluru 560064, Karnataka, India. The specimen was critically identified and authenticated by Dr. Noorunnisa Begum, Associate Professor specializing in the conservation of natural resources, The University of Trans-Disciplinary Health Sciences and Technology (TDU) and a voucher specimen was submitted to FRLHT (Accession number: 127195).

Preliminary preparation of sample

The leaf samples were separated from their stems, on the same day of procurement and were washed with tap water to remove all the immediate dust and insects and then with distilled water, after which they were shade-dried at room temperature for three days, followed by drying them in a hot-air oven at 45 °C, to remove any existing moisture. These dried leaves were then pulverized mechanically in a grinder, into coarse powder and were stored in zip-lock bags, in an airtight container, for future experimentation (7).

Extraction and solvent recovery

The leaves extract was prepared in a Soxhlet extractor (LabQuest Borosil, India), using methanol, ethanol, ethyl acetate and hexane as solvents. The leaf powder (20 g) was weighed and inserted into the thimble of the apparatus and the respective solvents for each extract (200 mL) were poured into the boiling flask. The extraction was done at a temperature maintained between 55 and 60 °C for 8 hr. The extraction process was allowed to continue until the extract color disappeared from the syphon, indicating the completion of the extraction process, after which, the flask holding the extract was then allowed to cool down and the solvent was recovered using a rotary evaporator (DLAB Scientific Inc., Beijing, China) (7). The extract was gathered from the rotary flask by gentle scraping through the inner walls and the flask was swirled with the appropriate solvent and finally collected into a petri dish, which was kept for drying. The pure extracts were retrieved back by gentle scraping from the petri dish, followed by their transfer into culture tubes. This was then stored at 4 °C for further usage. With reference to the initial dry powder weights of the methanolic and ethanolic leaf extracts, their percentage yields were calculated.

Phytochemical analysis

Qualitative phytochemical screening

The leaves extract of *W. piscidia* underwent qualitative phytochemical testing for the existence of secondary metabolites, using the standard detection methods, of each phytoconstituent, resulting in various characteristic colours. [alkaloids - Wagner's and picric acid test (8), flavonoids - alkaline reagent and ferric chloride test (9), triterpenoids - Salkowski's test (8), carbohydrates - Barfoed and Molisch's test (8), phenolic compounds and tannins - ferric chloride and lead acetate test (9), terpenoids - horizon test (9)]. Every experiment was carried out in triplicate and the extracts were further chosen for quantitative analyses, based on the results obtained from the extractive yield values and the phytoconstituents obtained after the preliminary screening.

Quantitative estimation of phenols

Incorporating a few minor protocol modifications, the colorimetric method using the most common Folin-Ciocalteu reagent was used to determine the total amount of phenolic compounds in the methanolic and ethanolic leaf extracts of *Walsura piscidia* (10). In this procedure, an aliquot of 0.5 mL of either of the extracts or the standard was diluted with 2.5 mL distilled water, thus bringing the volume to 3 mL. Next, 1 mL of the Folin's reagent (1:9 ratio) was added to this dilution and after a 5- minute standing period, 0.5 mL of 20 % (w/v) Na_2CO_3 was incorporated and the mixture was allowed to rest at room temperature, in a dark environment, for 1-2 hr, after which the wavelength of the above reaction mixture was measured at 765 nm, using a spectrophotometer with ultraviolet-visible wavelength (Model number: UV-1800; Model name: Shimadzu, Kyoto, Japan). The standard curve (20-100 µg/mL) was prepared similarly and gallic acid was the standard utilized. The phenol content was displayed as milligrams of gallic acid equivalents (GAE) per 100 g of dry sample.

Quantitative estimation of flavonoids

The total amount of flavonoids in the methanolic and ethanolic leaf extracts of *Walsura piscidia* was determined through the most common colorimetric method using aluminum chloride (9, 10), incorporating minor modifications to the original method, using quercetin as the standard for analysis. For this purpose, a 10 % aluminum chloride solution was prepared and a 1 M sodium acetate solution was prepared in advance. Briefly, an aliquot of 0.5 mL of either of the extracts or 0.5 mL of the standard were placed in different test tubes and to each tube, the following chemical mix was added: 0.5 mL of the 10 % prepared reagent solution, 0.5 mL of the prepared 1 M sodium acetate solution and 2 mL of 80 % methanol. For half an hour, all the reaction mix tubes were allowed to rest at room temperature. For reference, a quercetin calibration curve ranging from 20 to 100 µg/mL was generated for reference. The wavelength of the mixture was measured at 415 nm, using a spectrophotometer with ultraviolet-visible wavelength (Model number: UV-1800; Model name: Shimadzu, Kyoto, Japan). The flavonoid content was stated as milligrams of quercetin equivalent (QE) per gram of the extract.

Gas chromatography and Mass spectrum analysis

Gas chromatography and Mass spectrum (GC-MS) analysis has become one of the most irreplaceable tools in identifying the trace amounts of plant phytochemical groups, be it from the stem, root or leaf part of plants (13, 14). The chromatographic separation for the methanolic and ethanolic extracts of *Walsura piscidia* leaves was done using the instrument (Company: Shimadzu Corporation, Kyoto, Japan; Model: TQ8040NX). To do this, a tiny amount of the sample, around 1 µL, was fed into the capillary column which has an inner diameter of 0.25 mm and a 30 m length. The mobile phase containing the carrier gas used for this purpose was helium. Its flow rate was set to 1 milliliter per minute. Both the interface and injector temperatures were fixed to 280 °C and 250 °C, respectively. The injector's injections were set to split-less mode with a one-minute holding time. The oven's maximum temperature was set to 320 °C and the detector's scan range was between 50 and 550 atomic mass units. The mass spectral survey was done using the standard mass spectral database of NIST library - National Institute of Standards and Technology, or in other words, for the comparison of the resolved spectra with the standard database spectra of known compounds stored in the library (11).

Non-enzymatic antioxidant assays of the methanolic and ethanolic leaf extracts

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

With only minor modifications in the procedure and optimizing according to the sample concentrations, the radical quenching activity of the methanolic and ethanolic leaf extracts of *W. piscidia* on the stable radical, DPPH, was measured using methanol as a solvent (12). For this purpose, a fresh stock solution of the reagent was prepared which was composed of 0.1 millimolar DPPH reagent, dissolved in methanol and this was stored in the dark, to prevent photodegradation. The reaction mixture consisted of 0.5 mL sample or standard (ascorbic acid) at concentrations ranging from 20 to 100 µg/mL, in combination with 2.5 mL methanol and 1 mL DPPH reagent and the absorbance wavelengths of the methanolic and ethanolic samples and the standard, ascorbic acid, was measured using a spectrophotometer, at 517 nm, with ultraviolet-visible wavelength (Model number: UV-1800; Model name: Shimadzu, Kyoto, Japan). Methanol was taken as blank and DPPH + methanol as the untreated negative control. The formula used to determine the sample's radical scavenging activity is as follows:

$$\% \text{ radical scavenging activity} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

It was noted that a stronger antioxidant activity was correlated with a decrease in the absorbance value.

Ferric reducing antioxidant power (FRAP) assay

Three reagents were mainly used in the estimation of the FRAP of the plant samples, i.e., using potassium ferricyanide, trichloroacetic acid and ferric chloride. These chemicals upon reacting with the antioxidant compounds in the sample form a bluish-green complex which can be measured spectrophotometrically at 700 nm (13). To achieve this, ascorbic acid, being universally antioxidant in nature, was employed as a positive control and the following solutions were prepared for the assay: 1 % potassium ferricyanide, 10 % trichloroacetic acid, sodium phosphate buffer of pH value 6.6 and 0.1 % ferric chloride. One milliliter of either of the methanolic or ethanolic extracts or the standard, in separate tubes, at concentrations from 20 to 100 µg/mL with 2.5 mL of PBS, having a pH value of 7.4 and 2.5 mL of 1 % potassium ferricyanide constituted the reaction mixture. The reaction mix was then left to rest at 50 °C for 20 min, followed by the addition of 2.5 mL of 10 % trichloroacetic acid, before the addition of 2.5 mL distilled water and 0.5 mL $FeCl_3$. Mean and standard deviation were used to show the results and an elevation in the optical density indicated an increase in the reducing power of the extracts.

Statistical analysis

Expression of all the data was in the form of average ± standard deviations, from three separate experiments. Duncan's multiple range tests and post hoc testing of one-way analysis of variance (ANOVA) were used to examine the differences between the means of multiple groups via SPSS Statistics 24.0 (IBM, New York, NY, USA).

Results and Discussion

Extractive value

The leaves of *W. piscidia* was extracted using a Soxhlet extractor, in solvents of accelerating polarity, i.e., hexane, ethyl acetate, ethanol and methanol, being non-polar, moderately polar and highly polar. These extracts were concentrated under low pressure in a rotary evaporator and Table 1 shows the percentage yields of the dried extracts. A visual representation of the extractive values is shown in Fig. 1. Ethanol was seen to be a promising solvent that gave a higher yield compared to the other solvents, followed closely by methanol, giving a very similar extractive yield as ethanol. The extractive values of methanol, ethanol, ethyl acetate and hexane were seen to be 21.06 %, 21.9 %, 7.24 % and 5.72 %, respectively. Prior to being quantitatively assessed, the chosen extracts were first put to the qualitative phytochemical screening process, as a preparatory step to unmask the possible chemical phytoconstituents.

Phytochemical analysis

Qualitative phytochemical screening

Compared to the ten sophisticated quantitative phytochemical screening techniques, such as Gas Chromatography-Mass Spectrometry, Liquid Chromatography, High-Performance Liquid Chromatography, High-Performance Thin Layer Chromatography, etc this type of preliminary phytochemical screening is easy, economical and cheaper and gives us an approximate idea about the different groups of chemical

Table 1. Percentage yields and the physical appearance of *W. piscidia* extracts, after Soxhlet extraction

	Solvent used	Physical appearance	Percentage yield (grams)
W. piscidia	Methanol	Bright green	21.06 ±1.19[a]
	Ethanol	Bright green	21.9 ±1.38[a]
	Ethyl acetate	Light green	7.24 ± 0.35[b]
	Hexane	Moderately green	5.72 ±0.04[c]

Values are mean ± standard deviation (n = 3). Mean values followed by different superscripts in a column are significantly different ($p < 0.05$) according to Duncan's multiple range test.

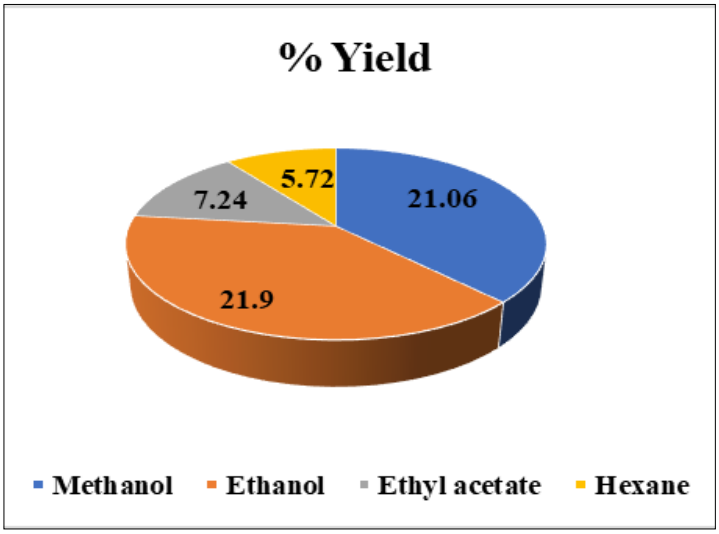

Fig. 1. Percentage yields of the different fractions of the *W. piscidia* leaves.

constituents present in the plant extract, such as alkaloids, flavonoids, phenolic compounds, tannins, terpenoids, triterpenoids, carbohydrates, etc. The presence of these different groups of phytochemicals was tested using three solvents, namely, ethanol, methanol and ethyl acetate. The findings in Table 2 showed that all the three extracts were rich in triterpenoids, thus drawing a match with the previous literature (5). They also tested positive for phenolic compounds, terpenoids, tannins and carbohydrates, thus prompting useful pharmaceutical applications. The presence of triterpenoids itself is an indication of this particular tree leaf to possess various bioactive qualities such as anti-diabetic, anti-inflammatory, antioxidant, hepatoprotective and anti-microbial activities (15), thus opening various opportunities to explore the medicinal potential of W. piscidia. However, this screening of phytochemicals did not prove to be sufficient for us to conclude which extract could be a potential antioxidant agent as all the extracts (0.5 mg/mL) were giving similar compounds. The reason could be attributed to the low concentration of the plant sample taken for the test or maybe due to the low concentrations of phytochemicals, most of them could not be detected. Another reason could be that the lead acetate test, in most cases, might not detect the derivates of flavonoids. Hence, there was a need for quantitative estimation of important phytochemicals like phenolic groups and flavonoids, which are the key players in contributing to the antioxidant properties of the leaves extract.

Table 2. Qualitative phytochemical screening of *W. piscidia* leaves in methanol (WPM), ethanol (WPE) and ethyl acetate (WPEA)

S. No	Phytochemicals	Test	Inference		
	Fractions		WPM	WPE	WPEA
1.	Alkaloids	Wagner's test	–	–	–
		Picric acid test	–	–	–
2.	Flavonoids	Alkaline Reagent test	–	–	–
		Ferric chloride test	–	–	–
3.	Phenolic compounds and tannins	Ferric chloride test	+	–	–
		Lead acetate test	+	+	+
4.	Terpenoids	Horizon test	–	+	+
5.	Triterpenoids	Salkowski's test	+	+	+
6.	Carbohydrates	Barfoed's test	+	+	–
		Molish's test	+	+	–

'+' Present, '–' Absent.

Quantitative estimation of amount of phenolic content

The amount of the phenolic content contained in the selected leaf extracts of *W. piscidia* was evaluated and the results were expressed in terms of the positive control used for this test, i.e., gallic acid equivalents (mg GAE/g) as in Table 3 and the calculations were done on the basis of the standard gallic acid graph plotted and the linear equation for the same is as follows:

$$Y = 0.0114x - 0.0096 \quad \text{(Eqn.1)}$$

$$R^2 = 0.9997$$

The results show a higher phenol content in the ethanolic leaf extracts as compared to the methanolic leaf extracts with a value of 26.192 ± 0.401 mg GAE/g. This may be due to the chemical nature of the phenolic compounds, those which are larger or more hydrophobic like complex tannins, which are easily solubilized by ethanol. Another reason could be the binding patterns of phenolic groups to the cell wall components of plant species, such as lignin and ethanol is an effective candidate to extract such phenolic groups (16).

Quantitative estimation of amount of flavonoid content

The amount of the flavonoid content contained in the selected leaf extracts of *W. piscidia* was evaluated and the results were expressed in terms of the positive control used for this test, i.e., quercetin equivalents (mg QE/g) as in Table 3 and the calculations were done on the basis of the standard quercetin graph plotted and the linear equation for the same is as follows:

$$Y = 0.0097x - 0.0205 \quad \text{(Eqn.2)}$$

$$R^2 = 0.9919$$

Table 3. Total phenolic and flavonoid contents of the methanolic and ethanolic leaf extracts of *W. piscidia*

Solvent/extract	TPC (mg GAE/g)	TFC (mg QE/g)
Methanol	11.573 ± 0.851^{b}	42.972 ± 0.214^{a}
Ethanol	26.192 ± 0.401^{a}	15.343 ± 0.787^{b}

Values are mean ± standard deviation (n = 2). Mean values followed by different superscripts in a column are significantly different ($p < 0.05$) according to Duncan's multiple range test.

The results show a higher flavonoid content in the methanolic leaf extracts as compared to the ethanolic leaf extracts with a value of 42.972 ± 0.214 mg QE/g. The reason can be attributed to the high polar nature of methanol and its effective ability to extract most of the polar compounds, especially the glycosylated flavonoids. Another reason could be the non-selectivity of aluminium chloride and the formation of colours due to the extraction of other compounds like tannins and flavonol derivates, along with flavonoids (17). Since ethanol co-extracts many lipophilic compounds, this can interfere with the TFC results and thus lead to reduced extraction of the target compound (16).

Identification of phytochemicals

W. piscidia's ethanolic and methanolic extracts's GC-MS results showed a number of bioactive compounds with known biological properties, getting eluted as a function of their retention times (RT), as in Fig. 2. Based on this, few compounds with known biological properties were shortlisted and presented as in Table 4 and 5, with their retention times, area %, molecular formula and weight as well as their biological properties. Numerous compounds of interest with antioxidant, anti-inflammatory, anti-microbial, anti-cancer, insecticidal and anti-diabetic properties were obtained, based on the chromatograms obtained.

The main bioactive compounds obtained in the ethanolic extract were n-Hexadecanoic acid (11.66 %), Stigmasta-5,24(28)-dien-3-ol, (3-β,24Z) (20.02 %), γ-Sitosterol (19.85 %), Stigmasterol (19.09 %), Campesterol (18.81 %), Trilinolein (13.84 %), Squalene (15.35 %), 13-Docosenamide, (Z)- (15.21 %), 9,12-Octadecadienoic acid (Z,Z)-Oleic Acid (14.81 %), Hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl) (14.03 %), Eicosanoic acid (13.39 %), cis-13-Eicosanoic acid (13.31 %) and Octadecanoic acid (12.6 %). The methanolic extract on the other hand showed compounds of interest which were mostly similar to the ethanolic extract derived compounds like 5-Hydroxymethylfurfural (6.92 %), Tetradecanoic acid (10.46 %), n-Hexadecanoic acid (11.62 %), Octadecanoic acid (12.02 %), l-(+)-Ascorbic acid 2,6-dihexadecanoate (12.95 %), Eicosanoic acid (13.37 %), cis-13-Eicosanoic acid (13.29 %), 2-Tetradecanone (13.78 %), Hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl) (14.03 %), Docosanoic acid (14.18 %), 9-Octadecenoic acid (Z)-, 2,3 dihydroxypropyl (14.80 %), 1-Hexacosanol (15.81 %), β-Tocopherol (16.93 %), Stigmasta-5,24(28)-dien-3-ol, (3-β,24Z) - (19.98 %), γ-Sitosterol (19.98 %), Stigmasterol (19.04 %) and Campesterol (18.78 %).

Estimation of antioxidant activity by DPPH and FRAP assay

The DPPH assay has been frequently used to test the ability of the test compounds to act as quenchers of free radicals and thus, test their antioxidant potential. Using a slightly modified procedure, the DPPH reagent was utilized to assess the ethanolic and methanolic extract's ability to scavenge the free radicals, where ascorbic acid served as the positive control and DPPH + methanol as the negative control. For this purpose, the percentage radical scavenging activity of the extracts (20-200 µg/mL) was evaluated using the given formula and

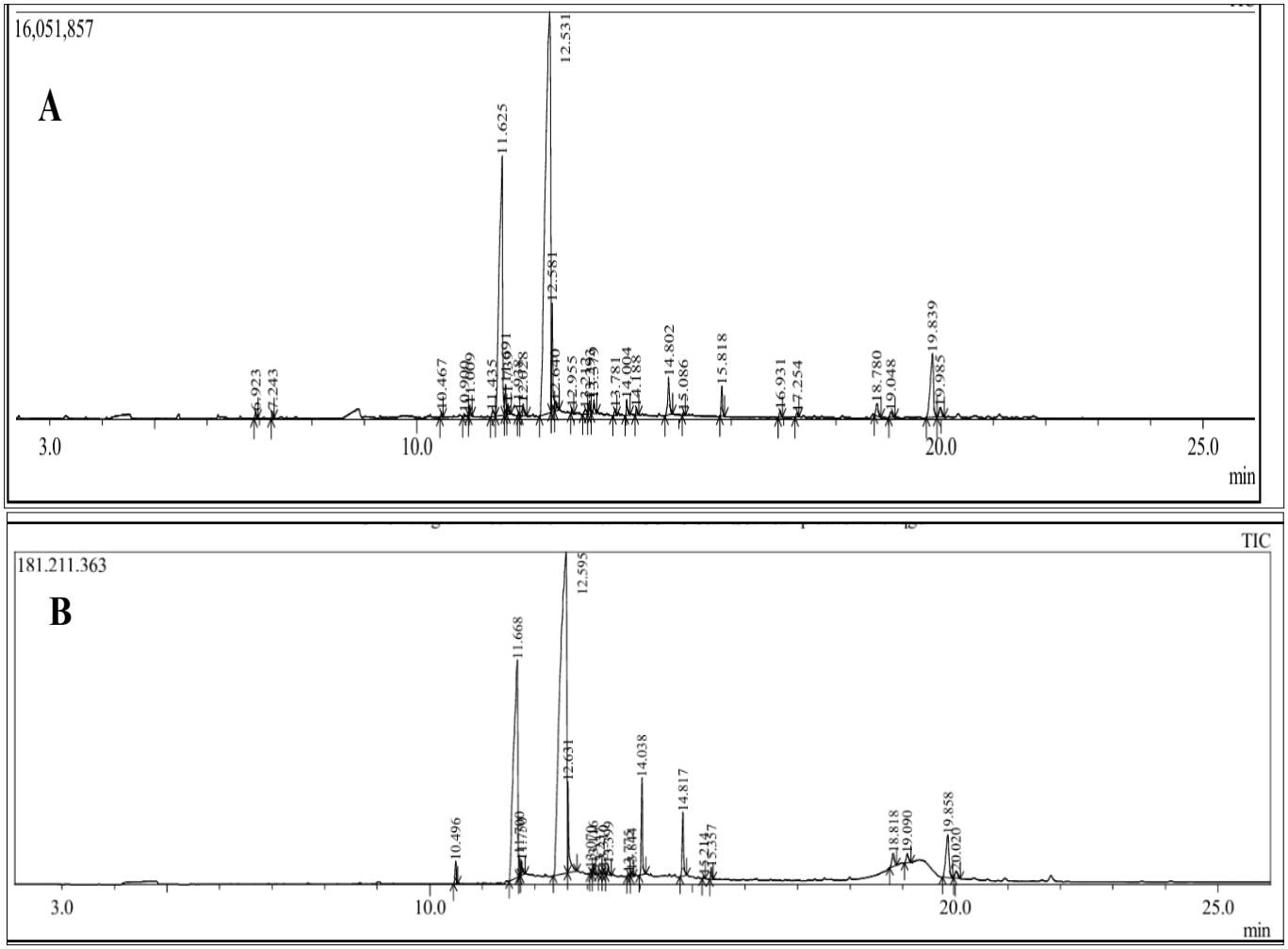

Fig. 2. A typical GC-MS chromatogram of the bioactive compounds present in *W. piscidia* leaves (A) methanolic and (B) ethanolic extracts.

Table 4. Phytochemical profile of the ethanolic extract of *W. piscidia* leaves

S.I. No	Compound Name	Retention Time (min)	Area (%)	Molecular formula	Molecular weight (g/mol)	Biological activity
1.	Tetradecanoic acid	10.496	0.14	$C_{14}H_{28}O_2$	228.3709	Antioxidant, Larvicidal and repellent (18)
2.	n-Hexadecanoic acid	11.668	24.49	$C_{16}H_{32}O_2$	256.4241	Antioxidant, decrease blood cholesterol, Anti-inflammatory (19)
3.	Stigmasta-5,24(28)-dien-3-ol, (3-β,24Z)-	20.020	0.49	$C_{29}H_{48}O$	412.6908	antioxidant, anti-inflammatory (20)
4.	γ-Sitosterol	19.858	3.51	$C_{29}H_{50}O$	414.7067	Antidiabetic, anticancer (21)
5.	Stigmasterol	19.090	0.57	$C_{29}H_{48}O$	412.6908	Anti-inflammatory, anticancer (22)
6.	Campesterol	18.818	0.95	$C_{28}H_{48}O$	400.6801	Antioxidant, anti-inflammatory, anticancer (23)
7.	Trilinolein	13.844	0.15	$C_{57}H_{98}O_6$	879.3844	Antioxidant (24)
8.	Squalene	15.357	0.34	$C_{30}H_{50}$	410.7180	Anti-inflammatory, Antioxidant (25)
9.	13-Docosenamide, (Z)-	15.214	0.08	$C_{22}H_{43}NO$	337.5829	Antimicrobial (26)
10.	9,12-Octadecadienoic acid (Z,Z)- Oleic Acid	14.817	2.54	$C_{18}H_{32}O_2$	280.4455	Anticancer (27)
11.	Hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl)	14.038	3.29	$C_{19}H_{38}O_4$	330.5026	Antioxidant, hypocholesterolemic, antiandrogenic (28)
12.	Eicosanoic acid	13.399	0.34	$C_{20}H_{40}O_2$	312.5304	Anti-fungal, antioxidant (27)
13.	cis-13-Eicosanoic acid	13.310	0.18	$C_{20}H_{38}O_2$	310.5145	Mouse and plant metabolite (28)
14.	Octadecanoic acid	12.631	3.27	$C_{18}H_{36}O_2$	284.4772	Antimicrobial (27)

Table 5. Phytochemical profile of the methanolic extract of *W. piscidia* leaves

S.I. No	Compound Name	Retention Time (min)	Area (%)	Molecular formula	Molecular weight (g/mol)	Biological activity
1.	5-Hydroxymethylfurfural	6.923	0.23	$C_6H_6O_3$	126.1100	Antioxidant, anti-inflammatory (29)
2.	Tetradecanoic acid	10.467	0.14	$C_{14}H_{28}O_2$	228.3709	Antioxidant (18)
3.	n-Hexadecanoic acid	11.625	23.38	$C_{16}H_{32}O_2$	256.4241	Antioxidant, decrease blood cholesterol, Anti-inflammatory (19)
4.	Octadecanoic acid	12.028	0.41	$C_{18}H_{36}O_2$	284.4772	Antimicrobial (27)
5.	l-(+)-Ascorbic acid 2, 6-dihexadecanoate	12.955	0.12	$C_{38}H_{68}O_8$	652.94	Antioxidant, anti-inflammatory, anti-tumor (30)
6.	Eicosanoic acid	13.379	0.50	$C_{20}H_{40}O_2$	312.5304	Anti-fungal, antioxidant (27)
7.	cis-13-Eicosanoic acid	13.293	0.58	$C_{20}H_{38}O_2$	310.5145	Mouse and plant metabolite (28)
8.	2-Tetradecanone	13.781	0.22	$C_{14}H_{28}O$	212.3715	Anti-bacterial, insecticidal (31)
9.	Hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl)	14.038	3.29	$C_{19}H_{38}O_4$	330.5026	Antioxidant, hypocholesterolemic, antiandrogenic (26)
10.	Docosanoic acid	14.188	0.20	$C_{22}H_{44}O_2$	340.5836	Antioxidant, anti-inflammatory, protective against cardiovascular diseases (32)
11.	9-Octadecenoic acid (Z)-2, 3-dihydroxypropyl	14.802	1.84	$C_{21}H_{40}O_4$	356.5399	Acidifier, acidulant, arachidonic acid-inhibitor, increase zinc bioavailability (26)
12.	1-Hexacosanol	15.818	1.04	$C_{26}H_{54}O$	382.7064	Antioxidant (33)
13.	β-Tocopherol	16.931	0.28	$C_{28}H_{48}O_2$	416.6795	Antioxidant (34)
14.	Stigmasta-5,24(28)-dien-3-ol, (3-β,24Z)-	19.985	0.67	$C_{29}H_{48}O$	412.6908	antioxidant, anti-inflammatory (20)
15.	γ-Sitosterol	19.839	5.49	$C_{29}H_{50}O$	414.7067	Antidiabetic, anticancer (21)
16.	Stigmasterol	19.048	0.33	$C_{29}H_{48}O$	412.6908	Anti-inflammatory, anticancer (22)
17.	Campesterol	18.780	0.76	$C_{28}H_{48}O$	400.6801	Antioxidant, anti-inflammatory, anticancer (23)

subsequently their IC_{50} values were also calculated from the ascorbic acid standard graph, through the linear equation generated (y = mx + c). From the IC_{50} values in Table 6 and the percentage radical scavenging activity in Fig. 3, it was seen that the highest percentage radical scavenging activity was recorded in the methanolic extracts with a value of 79.454 ± 0.267 at a concentration of 100 µg/mL and a much lower IC_{50} value of 14.862 ± 0.215 µg/mL, compared to the ethanolic extracts. Hence a conclusion can be drawn that the methanolic extract acts as a potential antioxidant agent compared to the ethanolic extract, as a low IC_{50} value and high radical scavenging activity can be correlated to high antioxidant activity. This can be due to the high amounts of flavonoids that the methanolic extract can solubilize, as flavonoids are potent antioxidant agents.

The FRAP test utilized the fundamental principle of the ability of the plant bioactive compounds to reduce the ferrous ions of the reagent so used to ferric ions and the resultant development of green colour. The intense development of the colour could be correlated to the strong antioxidant potential of the leaves extract (7). From the results as in Fig. 4, it was observed that the methanolic extract had the highest reducing activity at a concentration of 100 µg/mL, as in Fig. 4, with an OD value of 0.8 ± 0.0, compared to the ethanolic extract.

Table 6. Half-maximal inhibitory concentration value (IC-50) of methanolic and ethanolic extracts of *W. piscidia* leaves

Test sample	I C_{50}value (µg/mL)
Methanolic	14.862 ± 0.215^{b}
Ethanolic	23.424 ± 0.061^{a}
Ascorbic acid (Standard)	9.316 ± 1.500^{c}

Values are mean ± standard deviation (n = 3). Mean values followed by different superscripts in a column are significantly different ($p < 0.05$) according to Duncan's multiple range test.

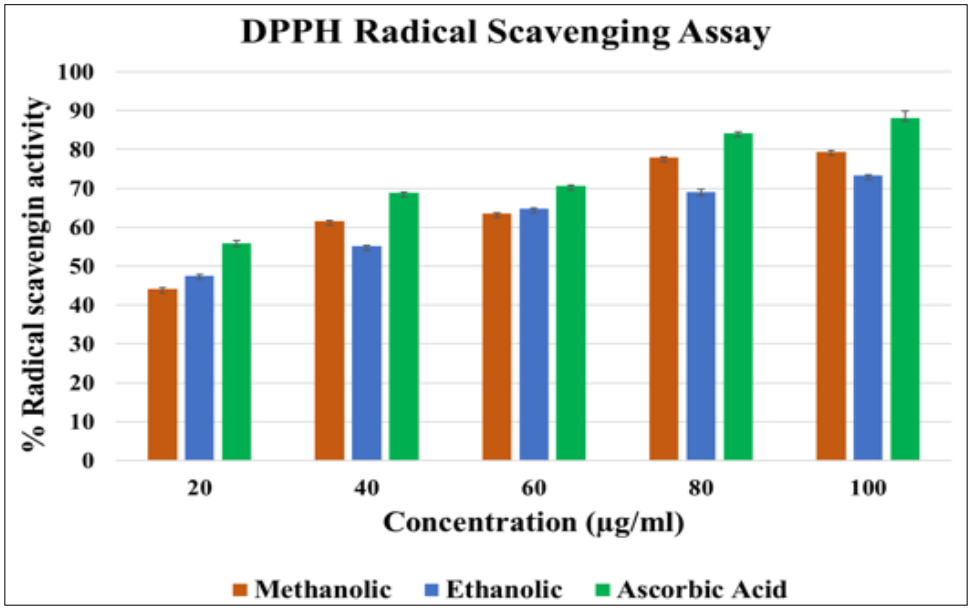

Fig. 3. Percentage radical scavenging activity of the methanolic and ethanolic leaf extracts of *W. piscidia* in comparison to ascorbic acid.

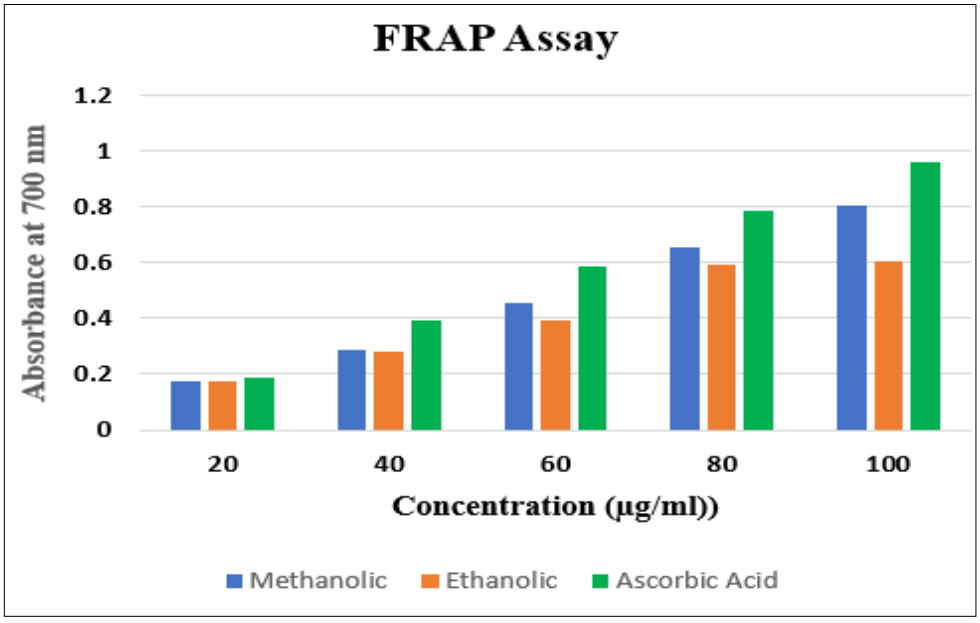

Fig. 4. Reducing activity of the methanolic and ethanolic leaf extracts of *W. piscidia* in comparison to ascorbic acid.

Conclusion

The current study presents the first ever report for the extensive GC-MS phytochemical profiling of the leaves of *W. piscidia.* The main aim of this study was to understand the phytochemical profile of the leaves of *W. piscidia* and evaluate its antioxidant potential and thus get a scientific basis for the traditional medicinal claims of this tree. The extractive yields of both methanolic and ethanolic extracts were almost identical, suggesting that these two solvents were equally effective in extracting the total mass of soluble compounds from the plant material. The qualitative phytochemical tests showed similar classes of phytochemicals for both the extracts at a concentration of 0.5 mg/mL. The TPC and TFC results were very contrasting, as the ethanolic extracts contained the highest phenolic content and the methanolic extract showed the highest flavonoid content. This difference may be due to the presence of maximum amounts of flavonoids in the methanolic extracts and non-flavonoid phenolics like phenolic acids and tannins in the ethanolic extracts. The DPPH assay showed maximum percentage inhibition of the free radical activity for the methanolic extract at a concentration of 100 µg/mL, as seen from its low IC_{50} value, which can be correlated to its high TFC value, as flavonoids are potent antioxidants. The FRAP assay also showed maximum reducing activity for the methanolic extract at a concentration of 100 µg/mL, likely due to the presence of flavonoids. Overall, the methanolic extract was seen to be effective in extracting antioxidant-active flavonoids, making it a potent extract in terms of antioxidant capacity. *W. piscidia* has presented itself as an appropriate candidate for therapeutic applications due to the presence of a vast array of secondary metabolites as seen from the GC-MS profiling of the leaf extracts, some of them being n-Hexadecanoic acid, stigmasterol, campesterol, 5-hydroxymethyl furfural, etc, housing properties like antioxidant, anti-inflammatory, anti-microbial, anti-cancer, insecticidal and anti-diabetic properties. With all the above findings, it has become evident that the leaves of *W. piscidia* can be a potentially useful source of natural antioxidants.

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Authors' contributions

This research has been successfully carried out with the help of an equal contribution from both AN and JX.

Compliance with ethical standards

Conflict of interest: The authors declare that there is no conflict of interest.

Ethical issues: None

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