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Original Research Article

GC-MS profiling, anticancer and antioxidant evaluation of *Millettia pinnata* (L.) Panigrahi (Fabaceae family) seed extract

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ABSTRACT

Cancer remains the leading cause of mortality worldwide. Phytochemicals are increasingly recognized as a valuable source of effective and safer agents against various types of cancer. In this study, defatted seed powder was subjected to extraction by Soxhlet apparatus. Qualitative analysis was conducted through biochemical tests, high-performance thin-layer chromatography (HPTLC), Fourier transform infrared (FTIR) spectroscopy, and gas chromatography-mass spectrometry (GC-MS). The quantitative analysis of phenols, flavonoids, and tannins was estimated using standard curves of gallic acid, quercetin and tannic acid, respectively. The antioxidant activity of the extract was assessed using four different free radical scavenging assays. The lethality and anticancer activity of the extract were evaluated using the brine shrimp lethality assay (BSLA) and the MTT assay, respectively. The seed extract was found to be non-toxic, biologically active, and rich in various phytocomponents with potent antioxidant activity. The sample exhibited cytotoxicity towards the MCF-7 breast cancer cell line and the HCT116 colorectal cancer cell line.

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1. Introduction

Since ancient times, medicinal plants have been widely used to cure diseases. Medicinal plants are rich in potentially valuable secondary metabolites such as phenols, flavonoids, aromatic components, terpenoids, sterols, essential oils, alkaloids, tannins, and anthocyanins (Moncayo et al., 2021; Monika and Gupta, 2023; Singh et al., 2023; Zare et al., 2024). Approximately, 30% of plant species are utilized for therapeutic purposes depending on the type and amount of secondary metabolites they contained (Ashu Agbor and Naidoo, 2015). According to the World Health Organization (WHO), nearly 80% of the global population uses medicinal products to address their healthcare needs (Khan et al., 2020). As a result, a significant number of medicinal plants with therapeutic potential are now used to treat a variety of ailments (Hashemi-Moghaddam et al., 2018; Mohammadhosseini et al., 2021). Pharmaceuticals, nutraceuticals, cosmetics, and food supplements are all utilizing medicinal plants (Gezici and Şekeroğlu, 2019; Twilley et al., 2020). It has been well-documented that in a wide range of plant materials, secondary metabolites play a crucial role in therapeutic and biological activities, including hypoglycemic, antidiabetic, antioxidant, antibacterial, anti-inflammatory, antimalarial, anticholinergic, anti-leprosy and anti-carcinogenic properties (Verpoorte, 1998; Negi et al., 2011).

Cancer is one of the most significant and life-threatening global health issues. The rising incidence of mortality and morbidity reflects the inadequacy of current clinical cancer therapies, which include chemotherapy, radiation, surgery, and immunotherapy (Twilley et al., 2020). Mainstream medications, when used to treat cancer are frequently associated with harmful side effects. Plant-based medicines are easy

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to obtain, inexpensive, and effective with minimum harmful effects (Thite et al., 2013). As a result, the use of easily available and inexpensive medicinal plants as an alternative to synthetic pharmaceuticals is the remedy for the toxic side effects of synthetic drugs (Rasheed et al., 2010).

Millettia pinnata (L.) Panigrahi (commonly known as Karanja) is a drought resistance evergreen tree belonging to the family of Leguminaceae (Bobade and Khyade, 2012) widely distributed in India, China and Bangladesh (Baswa et al., 2001). In the Ayurveda and Siddha systems of medicine, *M. pinnata* has been extensively used in the treatment of various ailments, including wounds, ulcers, painful rheumatic joints, tumors, hemorrhoids, skin diseases, itching, and diarrhea (Jena et al., 2020). *M. pinnata* seeds contains various bioactive compound, *e.g.*, karanjin, pongamol, pongagalabrone, pongapin, pinnatin, kanjone, glabrin, and karanjachromene (Al Muqarrabun et al., 2013). Phytocomponents like karanjin and pongapin which are abundantly present in seeds exhibit anticancer activity by inhibiting cervical cancer cells HeLa (Roy et al., 2019). The methanolic extract of *M. pinnata* seeds inhibits β-carotene degradation, superoxide levels, possesses inhibitory effect against α-amylase as well as α-glucosidase enzyme and Type II diabetes (Vadivel and Biesalski, 2011). Furthermore, the methanolic and hyrdro-methanolic seed extract has shown antimicrobial activities towards various strains of bacteria (Sajid et al., 2012). Wood and root extract are reported to have ulcer protective and healing effects (Prabha et al., 2003).

There is insubstantial data available regarding the anticancer potential of *M. pinnata* hydro- methanolic seed extract (MPHME). Due to the importance given in the above context, the preliminary screening of phytochemicals in plants is vital to discover and develop new therapeutic agents that can benefit humankind. Therefore, the present study focuses on evaluating MPHME based on qualitative and quantitative phytochemical analysis, *in vitro* antioxidant and anticancer potentials.

2. Experimental

2.1. Collection and authentication of *Millettia pinnata*

Fresh seeds of the fully mature *Millettia pinnata* (Family: Fabaceae) plants were collected from Gujarat University campus (23.0364° N, 72.5467° E). The plant was authenticated by a taxonomist (Dr. Hitesh Solanki), from the Department of Botany, Gujarat University, Ahmedabad. Following that, plant samples were authenticated at Gujarat Biotechnology Research Centre, Department of Science and Technology, Government of Gujarat using DNA barcoding. The seed sample was lysed using Preman Ultra (Thermofisher) sample lysis buffer. For amplification of specific region of DNA, forward and reverse *rbcl* primers along with PCR Master mix Takara (EmeraldAmp^{R} GT PCR Mater mix) and ready to use Taq DNApolymerase were utilized. The PCR was carried out in Thermal cycler Veriti (Applied Bio System). *De novo* sequencing was carried out utilizing a fluorescence-based cycle sequencing approach with a Thermo Fisher Bid Dye Terminator v3.1 cycle sequencing and purification kit using Applied Bio System 3500XL Genetic Analyzer. The electropherogram generated by the 3500XL Genetic Analyzer was analyzed using Codon code aligner and was compared with against BLASTn standard (nr/nt).

2.2. Defattation of *M. pinnata* seed

The seeds of *M. pinnata* were thoroughly washed twice with distilled water and shade dried at room temperature (RT). It was pulverized to fine powder using a mechanical grinder. 10-g portions of seed powder was defatted using 100 mL of petroleum ether with continuous stirring at 37 °C for 24 hours on a magnetic stirrer. After 24 hours, the material was filtered using Whatman filter paper no. I and air dried to evaporate any remaining solvent. The defatted powder was then stored in an airtight container.

2.3. Preparation of *M. pinnata* seed extract

Soxhlet extraction was performed on 5 g of seed powder using a 7:3 methanol:distilled water (DW) solvent solution. The extraction was carried out at 65 °C for 24 hours. The *M. pinnata* hydromethanolic seed extract (MPHME) was filtered using Whatman filter paper no. I and the filtrate was concentrated at 37 °C. The extract was finally stored at 4 °C until further use.

2.4. Qualitative analysis of MPHME

2.4.1. Determination of pH, percentage Yield and Preliminary Phytochemical Analysis

The pH of MPHME was measured using pHScan WP2. The extract was subjected to preliminary phytochemical analysis to determine the presence of several secondary metabolites such as phenol, flavonoid, tannin, saponins, and alkaloids using standard techniques described by Harborne (1973). The percent yield of the MPHME was calculated using the formula given below (Eqn. 1).

Percentage yield = ((Weight of the extract after solvent evaporation (g)/(Weight of the powder used in Soxhlet system))×100 (Eqn. 1)

2.4.2. High performance thin layer chromatography (HPTLC)

HPTLC was performed on a 10 × 10 cm TLC plate silica gel 60F_{254} (Merck, Germany) by semi-automated using CAMAG Linomat5 applicator. The MPHME (1 mg/mL) was prepared in DW, filtered and sterilized using 0.22 µm syringe filter. The sample was applied with Hamilton microsyringe (Switzerland) arranged on a Linomat 5 applicator under nitrogen flow (150 nl/s). The bands were spotted with a width of 8 mm. The syringe was programmed by VisionCATS software version 2.5. The plate was kept in CAMAG twin chamber

in a toluene:ethyl acetate (7:3 *v/v*) saturated solvent system. The plate was air dried and scanned using TLC Scanner 4 at 254 nm. The densitogram and R_f value were assessed using the same software.

2.4.3. Fourier transform infrared (FTIR)spectroscopy

FTIR analysis of MPHME for the detection of various functional groups was conducted using a BRUKER Alpha Platinum attenuated total reflectance (ATR) spectrometer. The spectral range was 500 to 4000 cm^{-1}, with a resolution of 8 cm^{-1}. On the prism plane, approximately 5 mg of extract was placed and acquired for analysis and the obtained results were analyzed using OPUS V 7.5 software.

2.4.4. Gas chromatography-mass spectrometry (GC-MS) profiling

The GC-MS profiling was carried out using the Clarus® 680 gas chromatography instrument, coupled with the Clarus® SQ 8 C Mass Spectrometer and the TurboMatrix 16 headspace sampler from PerkinElmer® (Ghada et al., 2017; Purkait et al., 2019). The results were analyzed using the TurboMass GC/MS Software provided by PerkinElmer® and compared with the NIST (National Institute of Standards and Technology) 14 library. Helium served as the carrier gas, with a solvent delay of 3.00 min. The GC temperature program started at an initial temperature of 70 °C for 1 min, then increased at a rate of 25 °C/min to reach 150 °C, with no holding time. It was then ramped at 5 °C/min to 200 °C, where it was held for 10 min, followed by a ramp of 12 °C/min to 280 °C, where it was held for an additional 6 min. The injector temperature was set to 280 °C, with a volume of 0 µL and a split ratio of 10:1. The transfer line temperature was also set to 280 °C, as was the source temperature. The scan range was from 50 to 600 Da, and the column dimensions were 30 m by 250 µm.

2.5. Quantitative analysis of few secondary metabolites in MPHME

The quantitative analysis of MPHME was performed by estimating total phenol (Singleton and Rossi, 1965), flavonoid (Dewanto et al., 2002), and tannin contents (Ojha et al., 2018). The total phenolic, flavonoid and tannin contents were calculated by applying regression formula on gallic acid, quercetin and tannic acid standard curve, respectively. The results were expressed as milligram of gallic acid equivalent (mg GAE/g) for phenolic content, milligram of quercetin equivalent (mg QE/g) for flavonoid content and milligram of tannic acid equivalent (mg TAE/g) tannin content. The absorbance was recorded using Epoch microplate UV-Vis spectrophotometer (Agilent Instruments, USA) equipped with Gen5 software (V 3.04). All the groups were analyzed in triplicate.

2.6. *In vitro* antioxidant assays of MPHME

The antioxidant potential of the MPHME (3-1000 µg/mL) was determined by four different free radical scavenging assays viz. 1,1-diphenyl-2-picrylhydrazyl activity (DPPH) assay (Shimamura et al., 2014), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Zheleva-Dimitrova et al., 2010), nitric oxide (NO) (Ebrahimzadeh et al., 2008) and superoxide anion (SO) scavenging assays (Amir et al., 2011) while using ascorbic acid as standard. The absorbance was recorded using Epoch microplate UV-Vis spectrophotometer (Agilent Instruments, USA) equipped with Gen5 software (V 3.04). All groups were examined in triplicate. The percentage inhibition of the above-mentioned assay was calculated by the given formula (Eqn. 2):

Percentage inhibition = $((A_0-A_1)/A_0) \times 100$ (Eqn. 2)

Where, A_0 and A_1 respectively account for the absorbance of control and the absorbance of the test sample.

2.7. Brine shrimp lethality assay (BSLA)

The brine shrimp lethality assay (BSLA) was conducted using the method described by Meyer et al. (1982). Approximately, 5-mg portions of *Artemia salina* eggs (brine shrimp eggs) were allowed to hatch in a shallow Petri dish filled with artificial saline water for 24 hours. In each well, 10 nauplii and 200 µL of artificial saline water were added. The nauplii were then exposed to different concentrations of MPHME (ranging from 3.9 to 1000 µg/mL) and incubated for 24 hours at 37 °C. After the incubation period, the percentage mortality was recorded for each well in comparison to the control. The LC_{50} was calculated using simple linear regression on the concentration versus percent mortality curve. Potassium dichromate ($K_2Cr_2O_7$) was used as a test control.

2.8. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay

The *in vitro* anticancer activity of MPHME was determined by performing MTT assay (Mosmann, 1983) on human breast cancer cell line MCF-7 and colorectal cancer cell line HCT 116 procured from NCCS Pune, India. MCF-7 cells were grown in RPMI 1640 media, and HCT 116 cells in DMEM, both contained fetal bovine serum (FBS, 10%) as well as penicillin (100 U/mL) and streptomycin (100 g/mL). The cells were incubated with 5.0% CO_2 at 37 °C. Briefly, MCF-7 and HCT 116 cells in their exponential phase were seeded in 96 well plate (10^4 cells/well in 200 µL of media) and allowed to adhere for 24 hours. The extract was dissolved in distilled water, filtered, sterilized and diluted to obtain various concentrations (10 to 120 µg/mL). Cells were exposed to various concentrations of extract and incubated for 24 hours. The control group contains only cells and media. After 24 hours, the media was removed from each well followed by 100 µL PBS wash. After PBS wash, 20 µL of MTT (5 mg/mL in PBS) was added in each well and incubated for 4 hours for formation of crystals. Furthermore, MTT was removed followed by addition of 100 µL DMSO to dissolve the formazan crystals. The

plate was then incubated for 30 min. The optical density (OD) was measured at 570 nm by Epoch microplate UV-Vis spectrophotometer (Agilent Instruments USA) equipped with Gen5 software (V 3.04) and percent cell viability was calculated using the formula below (Eqn. 3):

Percentage cell viability = $(A_t/A_c) \times 100$ (Eqn. 3)

Where, A_t and A_c respectively account for the absorbance of the treated cells and the absorbance of the control.

2.9. Statistical analysis

Data were statistically analyzed using GraphPad Prism (V 9.4.0) and expressed as mean ± standard error (SE).

3. Result and Discussion

Research on medicinal plants has demonstrated a significant impact on health and a remedial effect on various disease conditions. They are also referred to as traditional, herbal, supplementary, and alternative medicines. Due to fierce competition in the marketplace, they are frequently offered without being tested for quality (Ekor, 2014). To determine the medicinal value of plants, phytochemical analysis and characterization of their bioactive compounds is an initial step. Knowledge of the phytochemical components of plants is useful because such components are responsible for many biological activities and are valuable for the synthesis of complex drugs (Abubakar et al., 2020). Therefore, the hydromethanolic extracts of *M. pinnata* seeds were examined using various parameters to ascertain their medicinal value.

3.1. Authentication of plant species

Nucleotide FASTA Sequence of rbcL region of *M. pinnata* after sequencing:

AAGATTATAAATTGACTTATTATACTCCTGACTATGAA
ACCAAAGATACTGATATCTTGGCAG-CATTCCGAGTAA
CTCCTCAACCTGGAGTTCCGCCTGAAGAAGCAGGTG
CTGCGG-TAGCTGCCGAATCTTCTACTGGTACATGGAC-
AACTGTGTGGACCGATGGGCTTACCAGTCTT-GATCGTTA
CAAAGGACGATGCTACCACATCGAACCCGTTGCTGGAG
AA-GAAAATCAATATATTGCTTATGTAGCTTATCCCTTAGAC
CTTTTTGAAGAAGGTTCTGTTACT-AATATGTTTACTTCCAT
TGTAGGTAATGÏATTTGGGTTCAAAGCCCTGCGTGCTCTAC
GTTT-GGAGGATTTGCGAATCCCTAATTCTTATATTAAAAC
TTTCCAAGGTCCACCTCATGG-TATCCAAGTTGAGAGAGAT
AAATTGAACAAGTATGGACGTCCCTTATTGG-GATGTACTA
TTAAACCTAAATTGGGGTTATCCGCTAAGAATTACGGTAG
AGCGGTTTAIGAATGICTACGTGGTGGA

With a 99.81% identification rate and an E-value of 0.0, the BLASTn analysis of a 536bp sequenced region identified the matched species as *M. pinnata* (Table 1). These findings indicate that the sample is *M. pinnata*.

3.2. Qualitative analysis of MPHME

3.2.1. Determination of pH and percentage yield

For therapeutic purposes, yielding is important to estimate the composition of active constituents of raw materials of the extract. The percentage yield and the pH of the MPHME obtained was 17.8% and 7.0 ± 0.05, respectively.

3.2.2. Preliminary phytochemical analysis

The first and most significant phase in the process of drug development from plant sources is the selection of plant candidates for extraction/isolation of active principle components and screening for biological activities (Najmi et al., 2022). The phytochemical components of medicinal plants are primarily responsible for their wide range and diversity of pharmacological effects. They are lead chemicals which contribute to the development of drugs used in modern medicine to treat conditions ranging from cancer to migraines (Hussein and El-Anssary, 2019). The preliminary phytochemical analysis of the MPHME confirmed the presence of many phytocomponents such as amino acids, carbohydrates, glycosides, phenol, flavonoids, alkaloids and tannins. Similar results were observed by Kumar et al. (2013) during qualitative phytochemical screening of petroleum ether, chloroform, and methanol extract of *M. pinnata* seeds.

3.2.3. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra (Fig. 1) were analyzed to detect various functional groups present in the extract. The spectra indicated the presence of functional groups, as summarized in Table 2. The FTIR spectrum of MPHME exhibited a strong peak at 1710-1680 cm^{-1}, which corresponds to conjugate acids and aldehydes (C=O stretching). Additionally, two significant peaks were observed at 3000-2840 cm^{-1}, corresponding to the alkane functional group (C-H stretching) (Table 2). C-H (carbon-hydrogen) stretching is commonly observed in plant extracts, as many organic molecules in plants contain carbon-hydrogen bonds in various forms. Other minor peaks were also detected, corresponding to amines, halo compounds, sulfoxides, and sulfonyl chlorides, among others (Table 2).

3.2.4. High performance thin layer chromatography (HPTLC)

Botanical extracts and herbal products are complicated because they contain several different components. The development of the contemporary HPTLC method has made it an effective instrumental tool for evaluating herbal medicines and extracts both qualitatively and quantitatively. It has been used to analyze flavones, flavonols, and flavonoids in various complicated combinations (Vyas et al., 2023). The HPTLC was carried out to detect the presence of the principle components present in the MPHME using the solvent system consisting of toluene:ethyl acetate (7:3 *v/v*) which gave high separation of the components present

Table 1
Result of BLAST analysis. BLAST against NCBI standard database (nr/nt).

Sample ID	Identified species	Query length (bp)	Total score	Query cover	E- value	Per Ident[1]	Acc. Len.[2]	Subject Accession
S1	Pongamia pinnata	536	985	100%	0	99.81%	601	MK309383.1

[1]Per. Ident. - Percent Identity, [2]Acc. Len. - Accession Length.

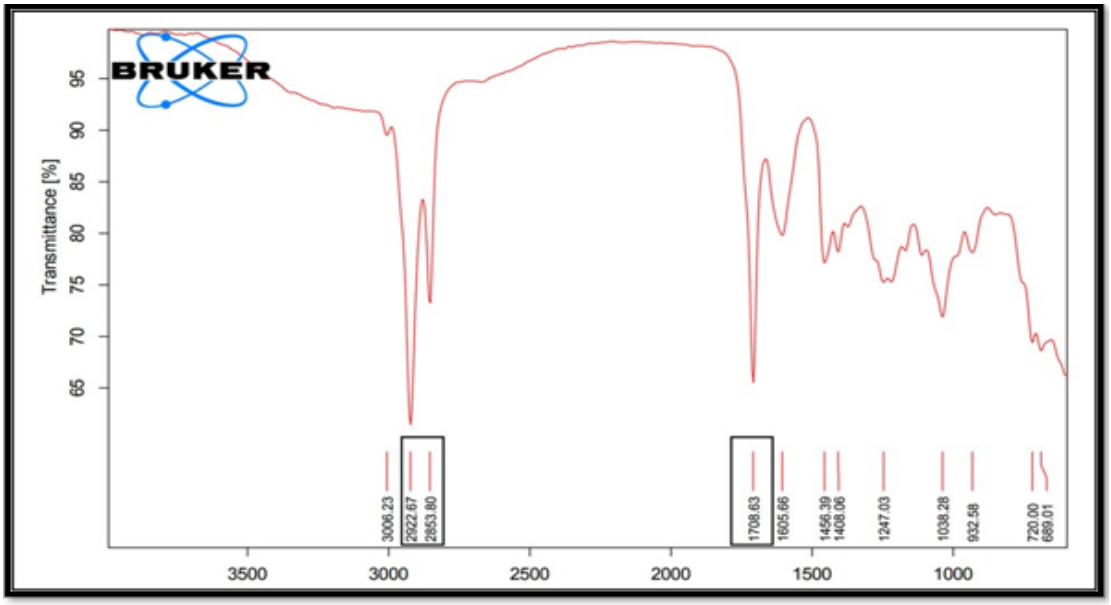

Fig. 1. FTIR spectrum of MPHME (X axis represents wavenumber in cm^{-1}; Y axis represents percent transmittance).

Table 2
Functional group detected from the MPHME on the basis of FTIR spectra.

Absorption frequency (cm^{-1})	Characteristic bond	Compound class
3100-3000	C=H stretching	Alkene
3000-2840	C-H stretching	Alkane
1710-1680	C=O stretching	Conjugated acid and aldehyde
1250-1020	C-N stretching	Amine
995-700	Bending and stretching	Mono, di and tri-substituted
690-515	C-Br stretching	Halo compound
1070-1030	S=O stretching	Sulfoxide
1410-1380	S=O stretching	Sulfonyl chloride
1650-1600	C=C stretching	Conjugated alkene

in the extract with well-defined peaks as observed in Fig. 2. Plates were observed at UV 254 nm, a total of 10 peaks were observed in the sample. This suggests that MPHME is rich in various phytocomponents. Ravikanth et al. (2009) observed a total of 8 peaks in methanolic extract of *M. pinnata* seeds by using the same solvent system.

3.2.5. GC-MS profiling of MPHME

GC-MS analysis was conducted to detect and identify phytocomponents present in the sample. Gas chromatography-mass spectrometry (GC-MS) is a powerful analytical technique widely used for the identification and quantification of phytocomponents found in plants. GC-MS combines the features of gas chromatography and mass spectrometry, offering high sensitivity and selectivity (Gomathi et al., 2015). Important components were detected in the

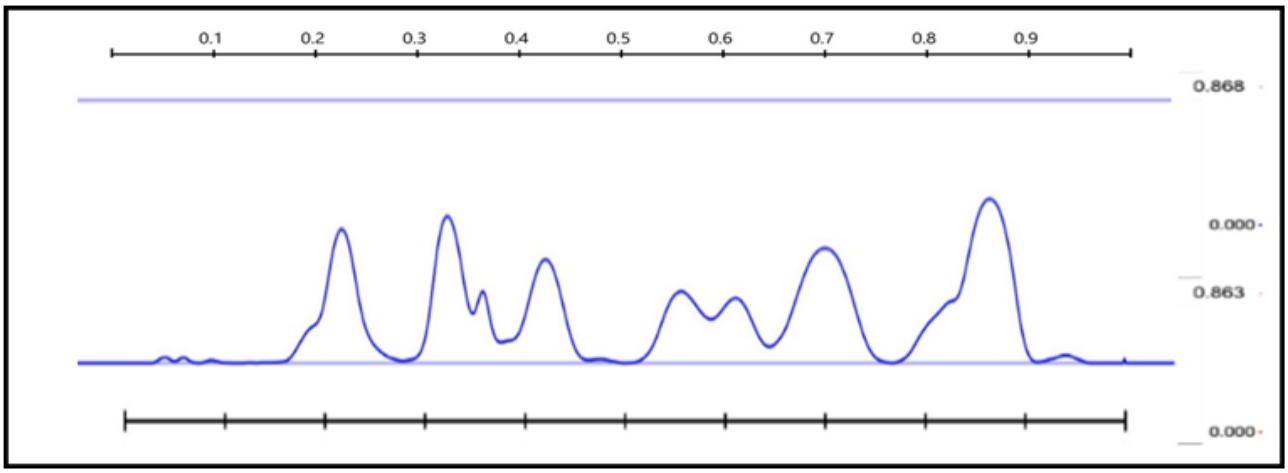

Fig. 2. HPTLC densitogram of MPHME at 254 nm.

MPHME (Fig. 3), *i.e.*, n-hexadecanoic acid, palmitic acid, 9,12-octadecadienoic acid, *cis*-vaccenic acid, 13-tetradecynoic acid which are known to possess various biological activities (Table 3). For instance, *cis*-vaccenic acid is a type of *trans*-fatty acid (omega-7 fatty acid) found in human milk, known for its various biological effects, including antibacterial and hypolipidemic effects in rats (Qadir et al., 2020). 13-Tetradecynoic acid, methyl ester exhibits pharmacological activities such as anti-inflammatory and antioxidant effects (Alatee et al., 2017). *n*-Hexadecanoic acid may function as an anti-inflammatory agent (Aparna et al., 2012). Palmitic acid inhibits the growth and metastasis of gastric cancer by blocking the STAT3 signaling pathway (Yu et al., 2023). These compounds altogether may enhance the medicinal properties of MPHME.

3.3. Quantitative analysis of few secondary metabolites in the MPHME

The most prevalent molecules in plants are phenolic compounds, which include various subclasses such as phenolic acids, flavonoids, and tannins. Due to their chemical functional groups, these substances can target the various signaling pathways that are either directly or indirectly involved in cell transformation (Mitra et al., 2022). Flavonoids have demonstrated anti-inflammatory, immunomodulatory (Yahfoufi et al., 2018), and potent anticancer effects (Abotaleb et al., 2018) in both *vitro* and *in vivo* studies. Similarly, phenolic compounds are also involved in induction of apoptosis, autophagy, and cell cycle arrest with high specificity (Islam et al., 2021). The MPHME was found to be rich in phenols, flavonoids, and tannin. Tannin was present in the highest amount in MPHME followed by phenol and flavonoid (Table 4). A quantitative analysis of MPHME (Table 4) revealed that the extract contains high levels of phenols (65.88 ± 2.98 mg GAE/g), followed by tannins (48.22 ± 0.98 mg TAE/g) and flavonoids (11.18 ± 0.43 mg QE/g). A study conducted by Sajid et al. (2012) found phenols (71 ± 0.05 mg GAE/g DW) and flavonoids (2.1 ± 0.03 mg CE/g DW) in the aqueous-methanolic (20:80) extract of *M. pinnata* seeds. In addition to the extraction method employed, the quantity of phytoconstituents was influenced by various factors, including environmental conditions, type of solvent, temperature, etc. (Venkatesan et al., 2019). Therefore, the variation in results may be attributed to multiple reasons.

3.4. *In vitro* antioxidant assay of MPHME

Natural antioxidants are preferred over synthetic ones because they are safer for consumption and less harmful to the environment (Al-Dabbagh et al., 2018). Researchers have indicated that estimating the antioxidant properties of a sample using a single assay is inadequate, as these properties are influenced by numerous factors (Číž et al., 2010; Gan et al., 2010). Therefore, the current study employed four different assays to evaluate the antioxidant potential of MPHME. The findings demonstrated that the extract exhibits a high antioxidant response in a dose-dependent manner; specifically, the total antioxidant capacity increases with the concentration of the extract (Fig. 4). For instance, in different free radical scavenging assays, *M. pinnata* seed HME scavenged 77.53% of ABTS radicals, 73.62% of DPPH radicals, 69.18% of nitric oxide free radicals, and 68.1% of superoxide free radicals at a concentration of 1 mg/mL (Fig. 4). In comparison, the standard solution of ascorbic acid scavenged 92.9% of ABTS radicals, 85.9% of DPPH radicals, 78.26% of nitric oxide free radicals, and 81.7% of superoxide free radicals at the same concentration (Fig. 4). The seed extract exhibited the highest inhibition in the DPPH assay (IC_{50} 19.26 ± 2.5 µg/mL), followed by ABTS (IC_{50} 21.48 ± 0.66 µg/mL), NO (IC_{50} 80.20 ± 2.2 µg/mL) and SO (IC_{50} 479.38 ± 4.1 µg/mL) scavenging assays (Table 5). Moreover, the standard ascorbic acid demonstrated the highest inhibition in the ABTS assay (IC_{50} 2.57 ± 0.17 µg/mL), followed by

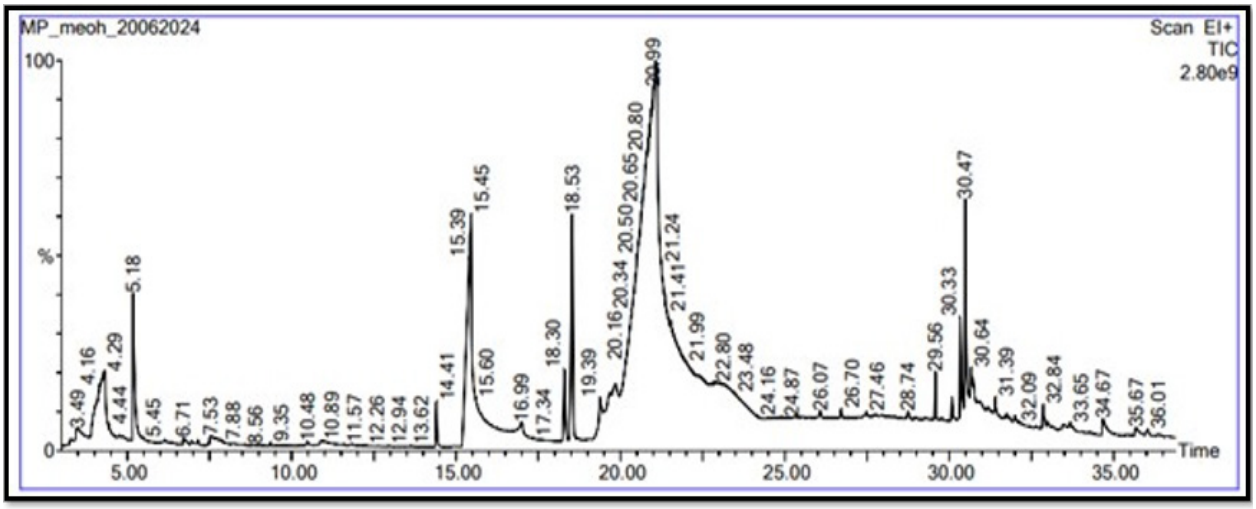

Fig. 3. GC-MS chromatogram of MPHME.

Table 3
Chemical compounds identified in the MPHME using GC-MS.

Sr. No.	Compound name	RT	Area%
1	Propane, 2-fluoro-2-methyl-	4.307	4.071
2	4-Piperidinamine, N,1-dimethyl-	5.183	1.703
3	*n*-Hexadecanoic acid	15.448	12.45
4	Palmitic Acid, TMS derivative	16.99	1.487
5	9,12-Octadecadienoic acid, methyl ester	18.298	0.929
6	*trans*-13-Octadecenoic acid, methyl ester	18.532	3.006
7	13-Tetradecynoic acid, methyl ester	19.84	3.624
8	*cis*-Vaccenic acid	21.067	69.231
9	1-(3a-Hydroxy-1-methyl-2-thioxo-2,3,3a,8a-tetrahydro-1H-1,3,8-triaza-cyclopenta[a]inden-8- yl)-ethanone	30.327	0.9
10	2-[5-(2-Methyl-benzooxazol-7-yl)-1H-pyrazol-3-yl]-phenol	30.469	1.787

Table 4
Quantitative analysis of few secondary metabolites in the MP HME.

Sr. No.	Phytochemical	Content
1	Total phenol content	65.88 ± 2.98 mg GAE/g
2	Total flavonoid content	11.18 ± 0.43 mg QE/g
3	Total tannin content	48.22 ± 0.98 mg TAE/g

GAE = Gallic Acid Equivalent; QE = Quercetin Equivalent; TAE = Tannic Acid Equivalent

DPPH (IC_{50} 4.13 ± 0.87 µg/mL), NO (IC_{50} 36.66 ± 2.5 µg/mL), and SO (IC_{50} 343.00 ± 6.5 µg/mL) scavenging assays (Table 5). Sajid et al. (2012) reported similar results in the DPPH assay using the aqueous methanol extract of *M. pinnata* seeds. Higher concentrations of phenolic compounds lead to more potent free radical scavenging effects. Additionally, researchers have shown that plants rich in phytochemicals, such as phenols and flavonoids, are also excellent sources of antioxidants (Pourmorad et al., 2006; Yu et al., 2021). Therefore, the antioxidant potential of MPHME can be attributed to its high phenolic and flavonoid content.

3.5. Brine shrimp lethality assay (BSLA)

The brine shrimp lethality assay (BSLA) serves as a rapid, high-throughput method for assessing the cytotoxicity of bioactive substances, particularly for evaluating the toxicity of phytochemicals found in plant extracts. In fact, BSLA is often employed as an initial

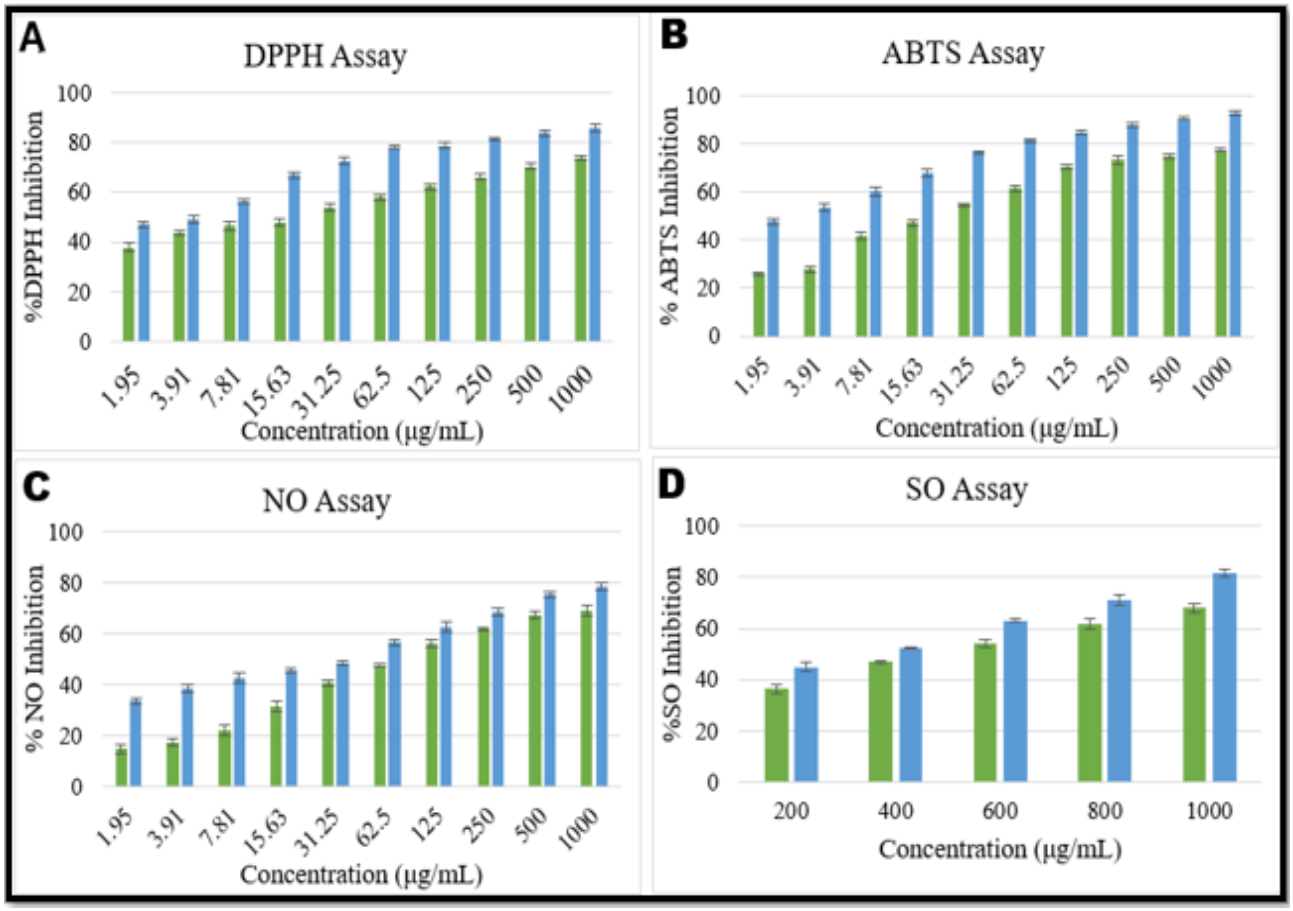

Fig. 4. Antioxidant scavenging assays of the MPHME. (A) DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay; (B) ABTS (2,2′-Azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid) assay; (C) Nitric oxide (NO) scavenging assay; (D) Superoxide anion (SO) scavenging assay; Ascorbic Acid was used asa standard.

Table 5
IC_{50} (µg/mL) values of MPHME and Ascorbic acid for DPPH, ABTS, NO and SO radical scavenging assays.

Sr. No.	Antioxidant assay	MPHME IC_{50} (µg/mL)	Ascorbic acid IC_{50} (µg/mL)
1	DPPH	19.26 ± 2.5	4.13 ± 0.87
2	ABTS	21.48 ± 0.66	2.57 ± 0.17
3	NO	80.20 ± 2.2	36.66 ± 2.5
4	SO	479.38 ± 4.1	343.00 ± 6.5

Values are expressed as mean ± standard error (SE).

toxicity screening tool for further research involving animal models (Wu, 2014). In this study, the toxicity of MPHME was assessed using BSLA and compared to Meyer's toxicity index. According to Meyer's toxicity index, extracts with an LC_{50} value of less than 1000 µg/mL in BSLA are considered toxic, while those with an LC_{50} greater than 1000 µg/mL are classified as non-toxic (Meyer et al., 1982). The present study demonstrated a direct correlation between the concentration of the extract and the lethality of brine shrimp. The extract began to exhibit lethality at a concentration of 400 µg/mL, whereas the test control, potassium dichromate ($K_2Cr_2O_7$), initiated lethality at a concentration lower than 100 µg/mL (Fig. 5). The LC_{50} of MPHME was determined to be 1512.8 ± 28.99 µg/mL, while the LC_{50} of the test control (potassium dichromate) was found to be 32.553 ± 8.54 µg/mL. Therefore, MPHME can be classified as non-toxic to zoological systems.

3.6. Checking *in vitro* anticancer activity of MPHME by MTT assay

The results of the MTT assay showed anticancer activity of the MPHME against the breast cancer cell line MCF-7 and the colorectal cancer cell line HCT 116. The MPHME exhibited significant cytotoxicity towards both cell lines

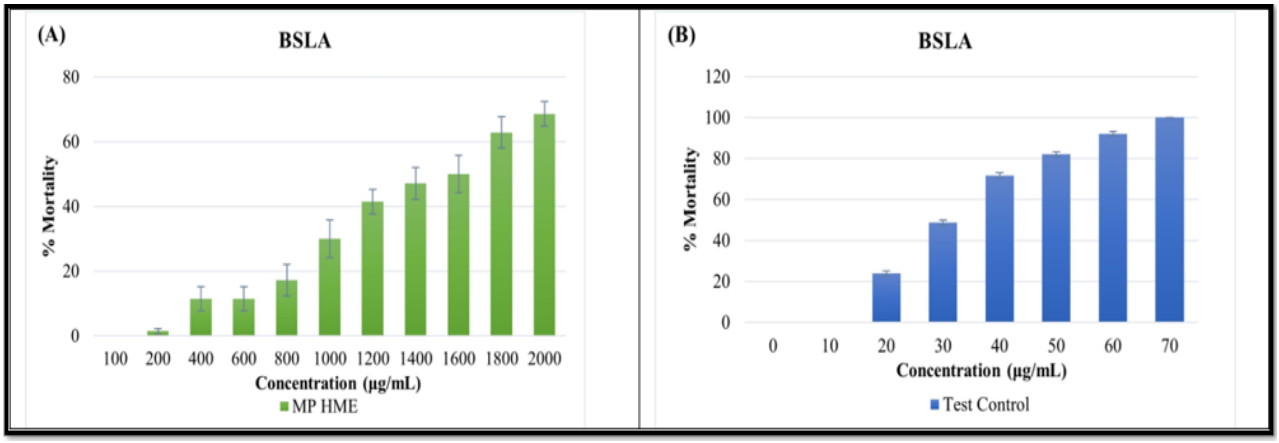

Fig. 5. Brine shrimp lethality assay (BSLA) of **(A)** MPHME and **(B)** Test control ($K_2Cr_2O_7$) (x axis represents percentage mortality; y axis represents concentration in µg/mL).

in a dose-dependent manner. The extract showed 68.81% cell viability at a concentration of 10 µg/mL, while cell viability decreased to 26.81% at the highest concentration of 120 µg/mL in the MCF-7 cell line (Fig. 6). On the other hand, MPHME showed 66.54% cell viability at concentration of 10 µg/mL, with cell viability dropping to 37.4% at the highest concentration of 120 µg/mL in the HCT 116 cell line (Fig. 6). The IC_{50} values of MPHME for the MCF-7 and HCT 116 cell lines after 24 hours were found to be 28.16 ± 1.85 and 30.34 ± 2.63 µg/mL, respectively (Table 6). An IC_{50} value of less than 30 µg/mL in preliminary assays is the criterion for cytotoxic activity established by the American National Cancer Institute (NCI) for crude extracts (Suffness, 1990). The investigation of plant-derived anticancer compounds is being regarded as a promising avenue for the development of new chemotherapeutic agents and for enhancing the effectiveness of conventional treatments (Iqbal et al., 2017). The results of the MTT assay indicate that MPHME is a valuable source of phytochemicals with potent anticancer properties against both cell lines. Traditional plants have historically been considered an inexhaustible source of new compounds for the development of pharmaceuticals and drugs (Garcia-Oliveira et al., 2021).

4. Concluding remarks

The results of this study after preliminary phytochemical analysis of MPHME showed the presence of carbohydrates, flavonoids, phenols, tannins etc. along with various other phytocomponents identified by GC-MS including *n*-hexadecanoic acid, palmitic acid, 9,12-octadecadienoic acid, *cis*-vaccenic acid, and 13-tetradecynoic acid. Quantitative analysis suggested the presence of high phenolic and flavonoid contents. All this together are responsible for its potential antioxidant and anticancer activity. In BSLA, the extract was found to be non-toxic according to Meyer's toxicity index and biologically active. The extract inhibited the growth of human breast cancer cell line MCF-7 and colorectal cancer cell line HCT 116 and hence proved its anticancer activity effectively. Based on the findings of this study, it was concluded that MPHME is a rich source of naturally occurring antioxidants and anticancer agents that could be used as potential therapeutic agents. This study also considers as torchbearer for identification and isolation of active components from *M. pinnata* and investigation of their mode of action against tumors using *in silico, in vitro* as well as *in vivo* experimental models to combat against cancer and reduced harmful side effects of the conventional medicine.

Author contribution statement

Conceptualization and literature search were performed by Khairah Ansari, Priyesh Kumar, Vaibhavi Srivastava, and Ann Maria Joseph. The first draft of the manuscript was prepared by Khairah Ansari. Nilam Parmar, Krupali Trivedi and Devendrasinh Jhala critically analyzed and gave suggestions to finalize the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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Table 6
IC_{50} values of MPHME obtained in MTT assay.

Cell line	IC_{50} (µg/mL)
MCF-7	28.16 ± 1.85
HCT116	30.34 ± 2.63

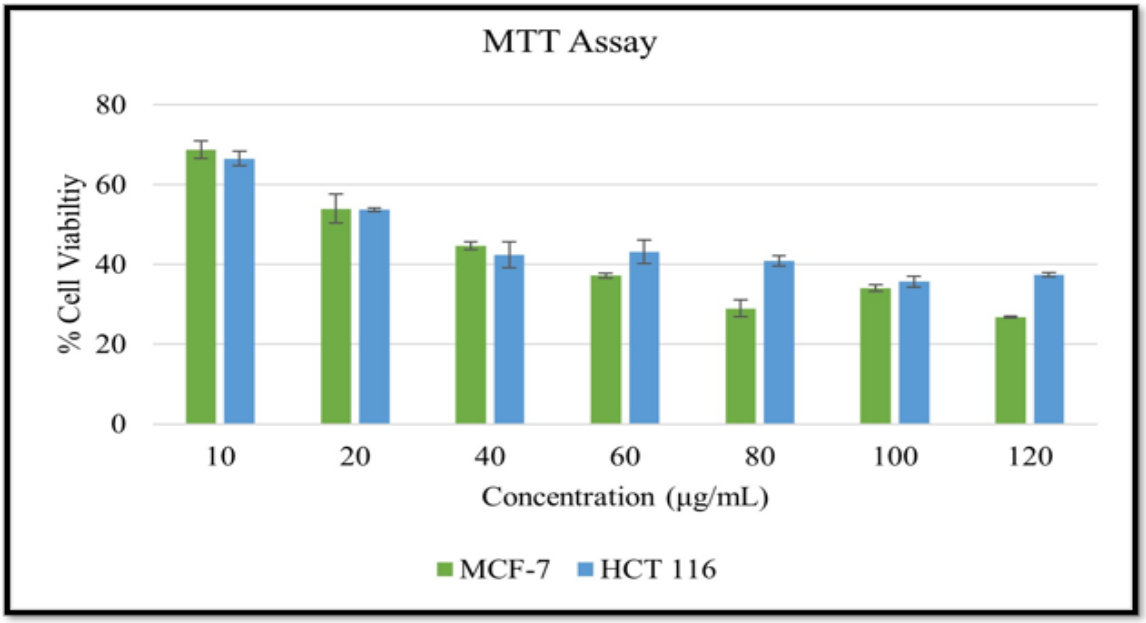

Fig. 6. Percentage cell viability of MCF-7 and HCT116 after 24 h treatment MPHME at various concentrations (x axis represents concentration in µg/mL; y axis represents percentage cell viability).

References

Abotaleb, M. Samuel, S.M., Varghese, E., Varghese, S., Kubatka, P., Liskova, A., Busselberg, D., 2018. Flavonoids in cancer and apoptosis. Cancers 11(1), 28.

Abubakar, A.R., and Haque, M., 2020. Preparation of medicinal plants: Basic extraction and fractionation procedures for experimental purposes. J. Pharm. Bioallied Sci. 12(1), 1-10.

Al Muqarrabun, L.M.R., Ahmat, N., Ruzaina, S.A.S., Ismail, N.H., Sahidin, I., 2013. Medicinal uses, phytochemistry and pharmacology of *Pongamia pinnata* (L.) Pierre. J. Ethnopharmacol. 150(2), 395-420.

Al-Dabbagh, B., Elhaty, I.A., Al Hrout, A., Al Sakkaf, R., El-Awady, R., Ashraf, S.S., Amin, A., 2018. Antioxidant and anticancer activities of *Trigonella foenum-graecum*, *Cassia acutifolia* and *Rhazya stricta*. BMC Complement. Altern. Med. 18(1), 1-12.

Altaee, N., Kadhim, M. J., Hameed, I. H., 2017. Characterization of metabolites produced by *E. coli* and analysis of its chemical compounds using GC-MS. Int. J. Current Pharm. Rev. Res. 7(6), 13-19.

Amir, M., Khan, A., Mujeeb, M., Ahmad, A., Usmani, S., Akhtar, M., 2011. Phytochemical analysis and *in vitro* antioxidant activity of *Zingiber officinale*. Free Radic. Antioxid. 1(4), 75-81.

Aparna, V., Dileep, K.V., Mandal, P.K., Karthe, P., Sadasivan, C., Haridas, M., 2012. Anti-inflammatory property of *n*-hexadecanoic acid: Structural evidence and kinetic assessment. Chem. Biol. Drug Des. 80(3), 434-439.

Ashu Agbor, M., Naidoo, S., 2015. Ethnomedicinal plants used by traditional healers to treat oral health problems in Cameroon. eCAM 2015, 649832.

Baswa, M., Rath, C.C., Dash, S.K., Mishra, R.K., 2001. Antibacterial activity of Karanj (*Pongamia pinnata*) and Neem (*Azadirachta indica*) seed oil. Microbios 105(412), 183-189.

Bobade, S., Khyade, V., 2012. Detail study on the properties of *Pongamia pinnata* (Karanja) for the production of biofuel. Res. J. Chem Sci. 2(7), 16-20.

Číž, M., Čížová, H., Denev, P., Kratchanova, M., Slavov, A., Lojek, A., 2010. Different methods for control and comparison of the antioxidant properties of vegetables. Food Control 21(4), 518-523.

Dewanto, V., Wu, X., Adom, K.K., Liu, R.H., 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agric. Food Chem. 50(10), 3010-3014.

Ebrahimzadeh, M.A., Pourmorad, F., Hafezi, S., 2008. Antioxidant activities of Iranian corn silk. Turkish J. Biol. 32, 43-49.

Ekor M., 2014. The growing use of herbal medicines: Issues relating to adverse reactions and challenges in monitoring safety. Front Pharmacol. 4, 177.

Gan, R.Y., Xu, X.R., Song, F.L., Kuang, L., Li, H.B., 2010. Antioxidant activity and total phenolic content of medicinal plants associated with prevention and treatment of cardiovascular and cerebrovascular diseases. J. Med. Plants Res. 4(22), 2438-2444.

Garcia-Oliveira, P., Otero, P., Pereira, A.G., Chamorro, F., Carpena, M. Echave, J., Fraga-Corral, M., Simal-Gandara, J., Prieto, M.A., 2021. Status and challenges of plant-anticancer compounds in cancer treatment. Pharmaceuticals 14(2), 157.

Gezici, S., Şekeroğlu, N., 2019. Current perspectives in the application of medicinal plants against cancer: Novel therapeutic agents. Anti-Cancer Agents Med. Chem. 19(1), 101-111.

Ghada, E.A., Manal, E.A.E., Amal, E.M., Hala, E.M., 2017.

Application of tomato leaves extract as pesticide *Againstaphis gossypii* Glover (Hemiptera: Aphididae). Int. J. Adv. Res. 5(4), 286-290.
Gomathi, D., Kalaiselvi, M., Ravikumar, G., Devaki, K., Uma, C., 2015. GC-MS analysis of bioactive compounds from the whole plant ethanolic extract of *Evolvulus alsinoides* (L.) L. J. Food Sci. Tech. 52(2), 1212-1217.
Harborne, I.B., 1973. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. 2nd Ed. Chapman and Hall, New York.
Hashemi-Moghaddam, H., Mohammadhosseini, M., Azizi, Z., 2018. Impact of amine- and phenyl-functionalized magnetic nanoparticles impacts on microwave-assisted extraction of essential oils from root of *Berberis integerrima* Bunge. J. Appl. Res. Med. Aromat. Plants 10, 1-8.
Hussein, R.A., and El-Anssary, A.A., 2019. Plants secondary metabolites: The key drivers of the pharmacological actions of medicinal plants. Herb. Med. 1(3) 11-30.
Iqbal, J., Abbasi, B.A., Mahmood, T., Kanwal, S., Ali, B., Shah, S.A., Khalil, A.T., 2017. Plant-derived anticancer agents: A green anticancer approach. Asian Pac. J. Trop. Biomed. 7(12), 1129-1150.
Islam, B.U., Suhail, M., Khan, M.K., Zughaibi, T.A., Alserihi, R.F., Zaidi, S.K., Tabrez, S., 2021. Polyphenols as anticancer agents: Toxicological concern to healthy cells. Phytother. Res. 35, 6063-6079.
Jena, R., Rath, D., Rout, S.S., Kar, D. M., 2020. A review on genus *Millettia*: Traditional uses, phytochemicals and pharmacological activities. Saudi Pharm. J. 28(12), 1686-1703.
Khan, M.F., Kader, F.B., Arman, M., Ahmed, S., Lyzu, C., Sakib, S.A., Tanzil, S.M., Zim, A.I.U., Imran, M.A.S., Venneri, T. and Romano, B., Haque M.A., Capasso R., 2020. Pharmacological insights and prediction of lead bioactive isolates of Dita bark through experimental and computer-aided mechanism. Biomed. Pharmacother. 131, 110774.
Kumar, P., Kumar, M., Teixeira da Silva, J. A., 2013. Pharmacognostic and phytochemical investigation of *Pongamia pinnata*. Open Access Sci. Rep. 2(2), 634.
Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.J., McLaughlin, J.L., 1982. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med. 45(5), 31-34.
Mitra, S., Tareq, A.M., Das, R., Emran, T.B., Nainu, F., Chakraborty, A.J., Ahmad, I., Tallei, T.E., Idris, A.M., Simal-Gandara, J., 2022. Polyphenols: A first evidence in the synergism and bioactivities. Food Rev. Int. 39(7), 4419-4441.
Mohammadhosseini, M., Frezza, C., Venditti, A., Sarker, S., 2021. A systematic review on phytochemistry, ethnobotany and biological activities of the genus *Bunium* L. Chem. Biodivers. 18(11), e2100317.
Moncayo, S., Cornejo, X., Castillo, J., Valdez, V., 2021. Preliminary phytochemical screening for antioxidant activity and content of phenols and flavonoids of 18 species of plants native to western Ecuador. Trends Phytochem. Res. 5(2), 93-104
Monika, M., Gupta, S., 2023. Unleashing the power of garlic polyphenols: Insights into extraction, identification, structural characteristics and bioactivities. Trends Phytochem. Res. 7(4), 262-278.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65(1-2), 55-63.
Najmi, A., Javed, S.A., Al Bratty, M., Alhazmi, H.A., 2022. Modern approaches in the discovery and development of plant-based natural products and their analogues as potential therapeutic agents. Molecules 27(2), 349.
Negi, J.S., Bisht, V.K., Bhandari, A.K., Singh, P., Sundriyal, R.C., 2011. Chemical constituents and biological activities of the genus *Zanthoxylum*: A review. Afr. J. Pure Appl. Chem. 5(12), 412-416.
Ojha, S., Raj, A., Roy, A., Roy, S., 2018. Extraction of total phenolics, flavonoids and tannins from *Paederia foetida* L. leaves and their relation with antioxidant activity. Pharmacog. J. 10(3), 541-547.
Pourmorad, F., Hosseinimehr, S.J., Shahabimajd, N., 2006. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. Afr. J. Biotechnol. 5(11), 1142-1145.
Prabha, T., Babu, M.D., Priyambada, S., Agrawal, V.K., Goel, R.K., 2003. Evaluation of *Pongamia pinnata* root extract on gastric ulcers and mucosal offensive and defensive factors in rats. Indian J. Exp. Biol. 41(4), 304-310.
Purkait, A., Biswas, S., Saha, S., Hazra, D.K., Roy, K., Biswas, P.K., Ghosh, S.K., Kole, R.K., 2019. Formulation of plant based insecticides, their bio-efficacy evaluation and chemical characterization. Crop Protect. 125, 104907.
Qadir, A., Aqil, M., Ali, A., Ahmad, F.J., Ahmad, S., Arif, M., Khan, N., 2020. GC-MS analysis of the methanolic extracts of *Smilax china* and *Salix alba* and their antioxidant activity. Turk. J. Chem. 44(2), 352-363.
Rasheed, Z., Akhtar, N., Khan, A., Khan, K.A., Haqqi, T.M., 2010. Butrin, isobutrin, and butein from medicinal plant *Butea monosperma* selectively inhibit nuclear factor-κB in activated human mast cells: Suppression of tumor necrosis factor-α, interleukin (IL)-6, and IL-8. J. Pharmacol. Exp. Ther. 333(2), 354-363.
Ravikanth, K., Thakur, M., Singh, B., Saxena, M., 2009. TLC based method for standardization of *Pongamia pinnata* (Karanj) using karanjin as marker. Chromatographia 69(5), 597-599.
Roy, R., Pal, D., Sur, S., Mandal, S., Saha, P., Panda, C.K., 2019. Pongapin and Karanjin, furanoflavanoids of *Pongamia pinnata*, induce G2/M arrest and apoptosis in cervical cancer cells by differential reactive oxygen species modulation, DNA damage, and nuclear factor kappa-light-chain-enhancer of activated B cell signaling. Phytother. Res. 33(4), 1084-1094.
Sajid, Z.I., Anwar, F., Shabir, G., Rasul, G., Alkharfy, K.M., Gilani, A.H., 2012. Antioxidant, antimicrobial properties and phenolics of different solvent extracts from bark, leaves and seeds of *Pongamia pinnata* (L.) Pierre. Molecules 17(4), 3917-3932.
Shimamura, T., Sumikura, Y., Yamazaki, T., Tada, A., Kashiwagi, T., Ishikawa, H., Ukeda, H., 2014. Applicability of the DPPH assay for evaluating the antioxidant capacity of food additives-inter-laboratory evaluation study. Anal. Sci. 30(7), 717-721.

Singh, A., Singh, D., Sharma, S., Mittal, N., 2023. A review on biosynthesis, regulation, and applications of terpenes and terpenoids. Trends Phytochem. Res. 7(4), 228-245.
Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16(3), 144-158.
Suffness, M., 1990. Assays Related to Cancer Drug Discovery. Methods in Plant Biochemistry: Assays for Bioactivity. 6, pp, 71-133.
Thite, S.V., Chavan, Y.R., Aparadh, V.T., Kore, B.A., 2013. Preliminary phytochemical screening of some medicinal plants. Int. J. Pharm. Chem. Biol. Sci. 3(1), 87-90.
Twilley, D., Rademan, S., Lall, N., 2020. A review on traditionally used South African medicinal plants, their secondary metabolites and their potential development into anticancer agents. J. Ethnopharmacol. 261, 113101.
Vadivel, V., Biesalski, H.K., 2011. Contribution of phenolic compounds to the antioxidant potential and type II diabetes related enzyme inhibition properties of *Pongamia pinnata* L. Pierre seeds. Process Biochem. 46(10), 1973-1980.
Venkatesan, T., Choi, Y.W., Kim, Y.K., 2019. Impact of different extraction solvents on phenolic content and antioxidant potential of *Pinus densiflora* bark extract. Biomed. Res. Int. 2019, 3520675.
Verpoorte, R., 1998. Exploration of nature's chemodiversity: The role of secondary metabolites as leads in drug development. Drug Discov. Today 3(5), 232-238.
Vyas, A., Jain, V., Sahu, U., Kumar, N., Joshi, N., 2023. HPTLC method development of herbal drugs and its validation: An overview. Res. J. Pharm. Technol. 16(8), 3964-3976.
Wu, C., 2014. An important player in brine shrimp lethality bioassay: The solvent. J. Adv. Pharm. Technol. Res. 5(1), 57-58.
Yahfoufi, N., Alsadi, N., Jambi, M., Matar, C., 2018. The immunomodulatory and anti-inflammatory role of polyphenols. Nutrients 10(11), 1618.
Yu, M., Gouvinhas, I., Rocha, J., Barros A.I., 2021. Phytochemical and antioxidant analysis of medicinal and food plants towards bioactive food and pharmaceutical resources. Sci. Rep. 11(1), 10041.
Yu, X., Peng, W., Wang, Y., Xu, W., Chen, W., Huang, L., Xu, H., He, X., Wang, S., Sun, Q., Lu, W., Xu, Y., 2023. Palmitic acid inhibits the growth and metastasis of gastric cancer by blocking the STAT3 signaling pathway. Cancers 15(2), 388.
Zare, M., Barzegari, A.A., Parvizpour, S., 2024. *In silico* targeting cysteine protease 2 of *Giardia lamblia* by *Origanum vulgare* L. flavonoids as potential inhibitors. Trends Phytochem. Res. 8(4), 213-225.
Zheleva-Dimitrova, D., Nedialkov, P., Kitanov, G., 2010. Radical scavenging and antioxidant activities of methanolic extracts from *Hypericum* species growing in Bulgaria. Pharmacogn. Mag. 6(22), 74-78.