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Investigating the antioxidant and antiproliferative activity of *Mimusops elengi* Linn Extract against Hepatocellular Carcinoma

Research Article

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Abstract

The antioxidant profile of the methanolic extract of *Mimusops elengi* was measured using various antioxidant assays, and the cytotoxicity was evaluated using MTT and Alamar blue assay against a hepatocellular carcinoma cell line (Hep-G2). In the DPPH radical scavenging assay, IC50 values were 91.55±0.02 µg/ml. Superoxide Radical Scavenging IC50 Value is 57.14 ±0.088 µg/ml, Nitrogen Scavenging IC50 Value is 15.88 ±0.028 µg/ml, CUPRAC IC50 Value is 7.731±0.5 µg/ml, FRAP IC50 Value is 14.32 ±0.046 µg/ml, Hydroxyl radical scavenging IC50 value is 22.46 ±1.02 µg/ml, and ABTS IC50 value is 4.00±0.08 µg/ml. the cytotoxicity study by MTT Assay the IC50 Values 34.98± 0.24, Alamar Blue Assay, it was showed that when the HepG2 cell line was exposed to various concentrations of the sample on different treatment doses, MEME Mean Fluorescence intensity (MFI%) of Alamar blue was found increasing with decrease of Treatment Dose of sample MEME. The highest MFI was estimated at 105.20 % at a 3.601 µg/ml dose of sample MEME, while the Lowest MFI% % (99.24%) was observed at a higher treatment dose of 14.405 µg/ml for Sample MEME concerning the control.

Keywords: *Mimusops elengi,* Antioxidant, Antiproliferative, Hep G2 Cell lines.

Introduction

Mimusops elengi belongs to the Sapotaceae family and is commonly referred to as Bakula in Ayurvedic medicine. Our study examined the possible presence of proteolytic activity in an aqueous Mimusops elengi leaf extract about the traditional and Ayurvedic medicinal usage of *M. elengi* leaves, particularly in wound healing and dental care (1). It is a well-known plant in Indian traditional medicine (2). An indigenous herb from India, *M. elengi,* has long been utilized in medicine. The plant parts strong potential therapeutic usefulness led to extensive research on it in most parts of the world. All of this plant's parts, such as root, fruit, seed, leaf, flower, and bark, have traditionally been used to treat a variety of illnesses. The information gathered here will help advance the current examination of various medical studies on *M. elengi.* (3) The tropical region of Aceh is frequently covered in a variety of medicinal plants, including *M. elengi*. Triterpene and phenolics, the primary constituents of *M. elengi* flower extract, possess antibacterial, antifungal, antioxidant, and antineoplastic properties. Maceration typically extracts chemical compounds, including essential oils, yielding modest amounts of chemical compounds (4). To varying degrees, the *M. elengi* extracts demonstrated cytotoxicity towards CCRF-CEM leukemia cells (5). The study aimed to assess the phytochemical makeup, antioxidant capacity, and cytotoxic effects of *M. elengi* Linn extract (ME) compared to normal human cultured adult gingival fibroblasts (HGFs) (6). Traditionally used to treat anxiety and panic attacks and as a brain tonic in several nations. In Wistar rats, the impact of a standardized hydroalcoholic extract of *M. elengi* flowers (ME) against excitotoxicity and oxidative stress caused by MSG was assessed (7). The identification, measurement, antioxidant, and possible biofunctional characteristics of lesser-known (8). In recent years, there has been increasing awareness of the potential anticancer, antioxidant, and apoptosis-inducing properties of some polyphenolic compounds derived from plants. Therefore, the role of plant-produced polyphenols in cancer chemoprevention has emerged as an intriguing area of research. (9).

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Materials and Methods

Plant Material

The plant *mimusops elengi* L. (Leaves) was obtained from the Botanical Garden of Nagpur (Maharashtra) and authenticated by the botanist, and the herbarium sheet was deposited in the Botany Department, voucher specimen no. 102. The second part of July 2022 was the time for plant collection.

Extraction of Plant Material

After thoroughly rinsing the Leaves in distilled water, they were allowed to air dry. Dried Leaves were

pulverized to a coarse powder (100 g) and macerated with methanol for 7 days. After the maceration of the extract was concentrated by a rotary evaporator and allowed to dry in a Lyophilizer, 20 g (20%) of *mimusops elengi* L. methanolic extract was obtained.

Phytochemical study

The Phytochemical screening was carried out on methanolic extracts of *Mimusops elengi* L. leaves. identify the active phytoconstituents, including terpenoids, alkaloids, glycosides, flavonoids, steroids, saponins, etc., present in the ethanolic extract, using the following standard phytochemical tests.

Test for alkaloids

1. Dragendroff's reagent test: 2 mL of extract was heated with 2% H 2 SO 4. A small amount of Dragendroff's reagent was added, and orange-red precipitate was observed.
2. Mayer's test: 1-2 mL of ethanolic extract was taken into a test tube, then 1-2 drops of Mayer's reagent were added. The result was positive; a creamy white precipitate formed.
3 Wagner's tests: 1-2 mL of extract solution was taken, and 1- 2 drops of Wagner's reagent were added. then, a brown precipitate formed.

Test for glycoside

Borntrager test: 1- 2 mL of extract solution was taken, 2-3 mL of Chloroform was added, then shaken, and the chloroform layer was separated. 10% ammonia solution added. The result was positive, a red coloured solution formed.

Test for flavonoids

Shinoda test: 1- 2 mL of extract was taken, and 5 mL of ethanol added, then added few grains of magnesium, turnings, with a few drops of conc. HCL, the sample was positive for flavonoids, red to pink coloured

Test for Phenolic compounds

Gelatin test: The ethanolic extract was dissolved in 5 mL of distilled water, followed by the addition of a 1% gelatin solution and a 10% sodium chloride solution, resulting in the formation of a white precipitate.

Test for terpenoids

Salkowski's test: The Alcoholic extract solution was taken, and a few drops of concentrated Sulphuric acid was added to the sample was positive for steroids when a red colour formed in the lower layer (29), (30).

Test for tannins

Nitric acid test: 2- 3 mL of the ethanolic extract was taken in a test tube, a few drops of dil. nitric acid was added, then a reddish yellow colour formed (29).

Test for fixed fats and oils

Spot test/stain test: a small quantity of plant extract is pressed between to filter papers, then an oil stain appears on the paper.

Test for saponin

Foam formation test: 2 mL of aq. the solution was taken into a test tube and shaken vigorously. If foam was formed and did not disappear for 5 min. (29)

Antioxidant assays

DPPH Assay

Various stock solutions of the test sample, each measuring 5 µl, were combined with 0.1 ml of 0.1 mM DPPH (2,2-diphenyl-1- 1picrylhydrazyl) solution in 96-well plates. The solution mixture was prepared in triplicate, and two blank samples were created using 0.2 ml of DMSO (Dimethyl sulfoxide) and 5 ml of the sample at different concentrations. Wells without any treatment served as controls. The plate was kept in the dark for 30 minutes. Following the incubation period, a microplate reader (iMark, BioRad) was used to measure the decolorization at 517 nm. A control reaction mixture containing 20 microliters of deionized water was employed. The scavenging activity was expressed as '% inhibition' relative to the control. Software Graph Pad Prism 6 was utilized to calculate the IC50. A graph was constructed with the sample concentration on the X-axis and the % inhibition compared to the control on the Y-axis. (10), (11), (12).

CUPRAC Assay

In designated wells of 96-well plates, 10µl of various test sample concentrations were deposited. Following this, 200µl of a reagent mixture was added to each well. The solution was prepared in triplicate for samples and duplicate for blanks, consisting of 200µl Methanol and 10µl of a compound at different concentrations for both sample and standard (Trolox – Ottokemi). This mixture underwent a 30-minute incubation period in dark conditions. Untreated wells served as controls. After incubation, a microplate reader (iMark, BioRad) was used to measure the absorbance and evaluate decolorization at 490 nm. The control reaction mixture replaced the sample or standard with 20µl of deionized water. Scavenging activity was reported as "% inhibition" about the control. IC50 was calculated using GraphPad Prism 6 software. A graph was plotted with the percentage of inhibition compared to the control on the Y-axis and sample concentration on the X-axis. (13), (14).

FRAP Assay

The reducing power of the test samples was determined using absorbance values obtained from a microplate reader. A higher absorbance value indicated greater reducing power, with ascorbic acid used as the positive control. GraphPad Prism 6 software was employed to calculate IC50 values, which represent the concentration needed for 50% reduction, allowing for comparison across various samples. The experimental procedure involved adding 10 microliters of different test sample stocks and ascorbic acid standard (SRL) to a mixture containing 0.04 ml of 0.2 M sodium phosphate (Rankem) buffer (pH 6.6) and 0.05 ml of 1% potassium ferricyanide (SRL) solution. After thorough vortexing, the reaction mixture was incubated at 50°C for 20

minutes. Wells without treatment served as controls. Following incubation, 0.5 ml of 10% trichloroacetic acid (SRL) was introduced to the mixture. Subsequently, 50µl of deionized water and 50 microliters of 0.1% ferric chloride solution (Fischer Scientific) were added. The resulting colored reaction solution was measured at 700 nm against a blank using a microplate reader (iMark, BioRad). IC50 values were calculated using GraphPad Prism 6 software. (13), (14), (15).

Hydroxyl free radical scavenging Assay

A mixture comprising 66µl Reagent Mixture, 10µl EDTA 0.5 M (HiMedia), 24.14 mg Deoxyribose (SRL), 88µl Ferric chloride (Fischer Scientific), 28 µl H 2 O 2 of 6 % (Neurochem Laboratories), and water was prepared. To this, 10µl of plant extract, 24 µl of phosphate buffer (pH 7.4), and 10 µl of ascorbic acid (SD Fine) were sequentially added to the wells of a 96-well plate. The mixture was then incubated at 37°C for 1 hour. Wells without treatment served as controls, and Gallic Acid (SRL) was utilized as the standard. Post-incubation, 50µl of 10% TCA (Fischer Scientific) and 50µl of 1% TBA (HiMedia) were introduced to each well, resulting in the formation of a pink chromogen. Subsequently, absorbance was measured at 540 nm using a microplate reader (iMark, BioRad). IC50 was determined using GraphPad Prism software, with a graph plotted between Sample Concentration (X-axis) and % inhibition relative to control (Y-axis). (14), (15), (16), (17), (18). The IC50 value denotes the sample concentration needed to inhibit 50% of the enzymatic activity. This metric is frequently employed to assess the efficacy of potential inhibitors or drug candidates. The graphical representation of sample concentration versus percentage inhibition offers a visual insight into the dose-response relationship, facilitating easy interpretation of the compound's inhibitory effects.

Super Oxide Anion Radical Scavenging Assay

Different concentrations of extract and standard were combined with riboflavin solution and incubated for 30 minutes in 96-well plates under ambient light conditions. The reaction mixture was subsequently added to the pre-incubated solution and thoroughly mixed. Wells without treatment served as controls. Following this, absorbance measurements were taken at 560 nm using an ELISA plate reader (iMark, BioRad). The IC50 was determined using GraphPad Prism 6 software. A graph was constructed with sample concentration on the X-axis and percentage inhibition relative to the control on the Y-axis. (11), (19), (20). The absorbance data were utilized to calculate the half-maximal inhibitory concentration (IC50) of the sample, which denotes the sample concentration needed to inhibit 50% of the measured biological or biochemical function. The resulting graph offers a visual depiction of the relationship between sample concentration and inhibition percentage, facilitating easy interpretation of the sample's efficacy.

Antioxidant assay by ABTS methods

The preparation of ABTS (2,2′-casino-bis (3-ethylbenzothiazoline-6-sulfonic) acid) (SRL-Chemicals) radicals involved combining APS (2.45 mM) and ABTS (7 mM) solutions, followed by a 100-fold dilution to create the ABTS free radical reagent. In 96-well plates, 10 microliters of various Ascorbic Acid (SD Fine) stocks, serving as standards and samples, were added to 200 microliters of the ABTS free radical reagent. The mixture was then incubated at room temperature for 10 minutes in the dark. Wells without treatment served as controls. Following incubation, a microplate reader (iMark, BioRad) was used to measure the absorbance of the decolorization at 750 nm. Results were compared to the negative control. The IC50 was determined using the Software GraphPad Prism 9.5.1. A graph was constructed with the sample concentration on the X-axis and the percentage inhibition relative to the control on the Y-axis. (9) (21), (22).

Antioxidant assay by Reactive Nitrogen Oxide Scavenging methods

A reaction solution was formulated by combining 50 microliters of 10 mM sodium nitroprusside (Fisher Scientific), 40 microliters of distilled water, and 10 microliters of gallic acid (SRL). The untreated reaction mixture served as a control. This solution underwent pre-incubation for 15 minutes at ambient temperature in the presence of light. Subsequently, 100 microliters of Griess reagent were introduced to both the test and control wells, followed by an additional 5-10 min incubation at room temperature to allow for chromophore formation and stabilization. A microplate reader (iMark, BioRad) was employed to measure absorbance at 540 nm and 660 nm. The IC 50 value was determined using Software GraphPad Prism 6. (10), (23), (24).

Antiproliferative assay

In Vitro Cytotoxicity assessment of the compound by MTT

The cytotoxicity of MEME was evaluated on the HepG2 cell line using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells (10000 cells/well) were cultivated in 96-well plates for 24 hours at room temperature with 5% carbon dioxide in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic solution. The following day, cells were exposed to various concentrations of the compound, with untreated cells serving as controls. After a 24-hour incubation period, the MTT solution was introduced to the cell culture and incubated for an additional 2 hours. Upon completion of the experiment, the culture supernatant was removed, and 100 microliters of DMSO (dimethyl sulfoxide) were used to dissolve the cell layer matrix. Data was measured using an ELISA plate reader (iMark, Biorad) at 540 and 660 nm. The IC50 was determined using GraphPad Prism-6 software. An inverted microscope (Olympus eK2) and an Amscope digital camera (10 MP Aptima CMOS) were employed

to capture images. Cell Proliferation Studies with Alamar Blue Assay on HepG2 (25), (26).

Alamar Blue Assay

To assess cell proliferation in HepG2 cell line samples, the Alamar blue test was utilized. A 96-well plate was prepared with 5000-8000 cells per well and incubated for 24 hours at 37 °C with 5% carbon dioxide in DMEM (Dulbecco Modified Eagle Medium) enriched with 10% FBS (Flavin Bovine Solution) and 1% antibiotic solution. The cells were then subjected to final concentrations of 1, 10, and 25µM for a full day. Following these 24 hours, 10 microliters of Alamar Blue reagent were introduced to every 100 microliters of media, and the plate was incubated for an additional 3-4 hours. The final measurement was conducted using an Agilent BioTek Epoch 2 fluorescence ELISA plate reader at a wavelength of 540 nm.(28)

Results

Phytochemical screening (Table 1) revealed that the leaves of the methanolic extract of *M. elengi* L. are rich in secondary metabolites, particularly flavonoids, alkaloids, glycosides, tannins, terpenoids, saponins, fixed oils, and phenolic compounds.

Table 1: Phytochemical screening of the leaf extract of *M. elengi*

Sr. No	Test	Observation	Results
1	**Alkaloids** Dragendroff's test Mayer's test Wagner's test	 A reddish-brown precipitate White precipitated Brown precipitated	 + ve + ve + ve
2	**Glycosides** Borntrager test	 The red colour solution formed	 + ve
3	**Tannins** Ferric chloride test	 The greenish blue colour formed	 + ve
4	**Phenolic compound** Gelatin test	 White precipitated	 + ve
5	**Flavonoids** Shinoda test	 Red to pink precipitated	 + ve
6	**Terpenoids** Salkowski's test	 Red colour formed at a lower layer	 + ve
7	**Saponin** Foam test	 Foam formed	 + ve
8	**Fixed Fats and oils** Spot test	 Oil spot observed	 + ve

+ve = Positive test, - ve = Negative

The methanolic extracts of *M. elengi* L. leaves were subjected to various antioxidant assays, with the results displayed in Table 2. The DPPH assay revealed an IC50 value of 110.53±1.20 µg/ml, while the Superoxide Radical Scavenging assay showed 64.32±0.10 µg/ml. Nitrogen Scavenging and CUPRAC assays yielded IC50 values of 37.10±0.5 µg/ml and 7.002±0.5 µg/ml, respectively. The FRAP assay demonstrated an IC50 value of 9.585±0.097 µg/ml, Hydroxyl radical scavenging registered 23.16±1.29 µg/ml, and the ABTS assay recorded 4.00±0.10 µg/ml. Table 3 further illustrates the efficacy of these extracts, presenting their antioxidant activity and inhibition percentages across different assays.

Table 2: In vitro antioxidant activity and IC50 Values

Sr. No	Antioxidant Assay	Standard	IC50 Values
1	DPPH	Ascorbic acid	91.55 ±0.02 µg/ml
2	ABTS	Ascorbic acid	4.62±0.08 µg/ml
3	CUPRAC	Trolox	7.731 ±0.5 µg/ml
4	FRAP	Ascorbic acid	14.32±0.046 µg/ml
5	NOSA	Gallic acid	15.88 ±0.028 µg/ml
6	SOARSA	Gallic acid	57.14±0.10 µg/ml
7	HFRSA	Gallic acid	22.46 ±1.29 µg/ml

Table 3: In vitro antioxidant activity and % inhibition Values

Sr. No	DPPH	ABTS	CUPRAC	FRAP	NOSA	SOARSA	HFRSA
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	-1.05	14.66	-2.78	-1.92	20.14	24.52	20.51
3	5.48	72.79	56.67	19.27	42.57	44.03	32.69
4	27.14	84.81	118.33	57.44	64.32	47.74	51.28
5	51.78	95.76	337.22	102.16	73.32	53.23	86.22

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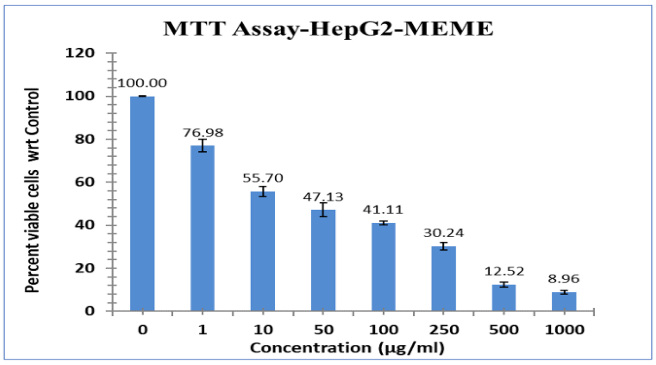
6	88.50	97.00	588.33	274.45	81.31	59.35	102.88
7	90.67	96.82	786.67	390.36	85.64	64.84	120.19
8	93.51	107.95	1320.00	446.14	92.52	63.39	145.19

Table 4: The antiproliferative capacity of MEME

Antiproliferative assay	Celline	IC50 Values	MFI %
1. MTT Assay	Hep-G2	34.98 ± 0.25 µg/ml	----
2. Alamar Blue Assay	Hep-G2	-------	
a. Dose 3.61 µg/ml			105.20
b. Dose 14.40 µg/ml			99.24

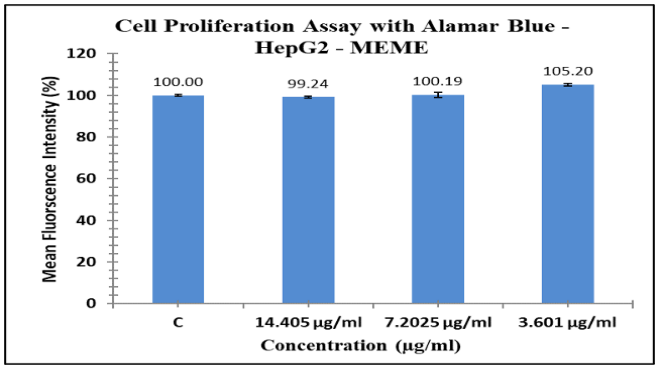
The cytotoxic activity(Table 4) of the sample MEME was demonstrated through MTT test results, which revealed an IC50 value of 34.98 ± 0.25 µg/ml when the HepG2 cell line was exposed to various sample concentrations. The IC50, as illustrated in Figure 1, denotes the sample concentration that reduces cell viability by 50%. Figure 1 presents the MTT assay findings for the methanolic extract of *Mimusops elengi* L. on Hepatocellular carcinoma cell lines (Hep-G2), confirming the cytotoxic nature of the sample MEME.

Figure 1: MTT Assay of MEME against HepG2 Celline



The Alamar Blue Assay results revealed that the Mean Fluorescence Intensity (MFI%) of Alamar blue in the HepG2 cell line exhibited an inverse relationship with the treatment dose of sample MEME. As the concentration of the sample MEME decreased, the MEME MFI% increased. The peak MFI was recorded at 105.20% when the sample MEME dose was 3.61 µg/ml, while the lowest MFI% (99.24%) was observed at a higher treatment dose of 14.60 µg/ml for Sample MEME, in comparison to the control, as depicted in Figure 2.

Figure 2: Cell proliferation assay with Alamar blue assay of MEME on HepG2 Cell line



Discussion

Coarse powder (100 g) of *M. elengi* leaves was macerated with methanol for 7 days. After the maceration of the extract was concentrated and dried by a rotary evaporator, 20 g (20%) of methanolic extract was obtained. The Phytochemical screening (Table 1) shows that the leaves of the methanolic extract of *M. elengi* L. are rich in secondary metabolites, such as flavonoids, glycosides, tannins, terpenoids, saponins, fixed oils, alkaloids, and phenolic compounds. Methanol was utilized as an extractant due to its established efficacy in obtaining extracts for antioxidant and antiproliferative activity studies of M. elengi L. plants. This study demonstrated that extracts from M. elengi L. leaves exhibited antioxidant properties through various assays to determine IC50 values. A comparative analysis based on different antioxidant assays revealed that the ABTS method displayed potential antioxidant scavenging activity, with an IC50 of 4.62±0.08 µg/ml compared to ascorbic acid, as shown in Table 1. Additionally, in vitro antioxidant activity and % inhibition values were assessed, with the DPPH assay demonstrating better scavenging activity, as presented in Table 2. Cytotoxic properties against hepatocellular carcinoma (Hep-G2) cell lines were evaluated using the MTT test. Results indicated that the MEME sample (IC 50 = 34.98 ± 0.25 µg/ml) showed cytotoxic activity when HepG2 cells were exposed to varying doses, as illustrated in Table 3. The IC50, defined as the concentration at which the number of viable cells is reduced by half, is depicted in Figure 1. Furthermore, the Mean Fluorescence Intensity (MFI%) of Alamar blue for MEME was observed to increase as the treatment dose decreased. The highest MFI was recorded at 105.20% with a 3.61 µg/ml dose of MEME, while the lowest MFI% (99.24%) was noted at a higher treatment dose of 14.60 µg/ml, as shown in Table 3 and Figure 2, respectively. The MEME extracts demonstrated antiproliferative qualities. In conclusion, the investigated extracts of M. elengi L. exhibited both antiproliferative and antioxidant effects, attributed to the combined action of their phytoconstituents.

Conclusion

Research findings demonstrated that extracts from *M. elengi* L. leaves exhibited cytotoxicity towards Hep-G2 hepatocellular cancer cell lines, inducing apoptosis. The antiproliferative and antioxidant effects observed were likely attributed to the phytoconstituents

present in the examined extracts, particularly flavonoids, alkaloids, glycosides, tannins, terpenoids, saponins, fixed oils, and phenolic compounds. These results lend credence to the traditional belief that *M. elengi* (L.), a perennial herb belonging to the Sapotaceae family, possesses cancer-preventive properties.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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