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Phytochemical Investigation and Cytotoxic Activity Studies of Extracts of Aegle Marmelos Linn

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We chose the root bark of the Rutaceae plant Aegle marmelos L. for this study in order to determine its phytochemical composition, antioxidant capacity, and cytotoxic potential. The complete methanolic extract and the resulting aqueous extract were evaluated phytochemically, revealing the presence of terpenoids, amino acids, alkaloids, glycosides, phenolic, and flavonoids. The antioxidant investigations were carried out on the methanolic extract and aqueous root bark of A. marmelos L. using the DPPH and ABTS techniques. For cytotoxic tests on HL60 (Human promyelocytic leukaemia) cell lines using the MTT technique, the extract with the most antioxidant properties (methanolic extract) was chosen. The findings of the cytotoxic and antioxidant activity investigations were encouraging.

Keywords: Medicinal Plant, Aegle marmelos Antioxidant activity, HL60 Cell line.

1. Introduction

In both traditional and modern medicine, the use of medications produced from plants is common and steadily growing. [1] The World Health Organisation reports that over 80% of people in impoverished nations rely mostly on plant-based medications to meet their fundamental medical needs. Some of these genera—Morus, Ficus, and Aegle—are inexpensive food sources and have numerous use in industry, agriculture, and traditional medicine. [2, 3] Because these genera include secondary metabolites with valuable biological functions and therapeutic significance, they have attracted a lot of scientific attention. [4] The "back-to-nature" trend in medication selection has contributed to the rise in popularity of traditional medicine in recent decades. Traditional medicine made from a variety of medicinal plants is used in many nations worldwide to treat a range of illnesses and ailments [5, 6]. According to a WHO estimate, around 80% of people worldwide utilise nutritional supplements and nutraceutical traditional medicine, primarily in underdeveloped nations due to its exceptional pharmacological potential, low toxicity, and uncommon adverse effects. [7] Traditional medicine has so far yielded a large number of pharmacological compounds; this could be a good place to start when looking for new treatments [8, 9, 10]. However, little attention has been paid to the potential of plants as raw materials for medicinal drugs.

Known as the bael fruit tree [11], Aegle marmelos (L.) Corr. (Rutaceae) has long been used to treat a wide range of illnesses [12]. Skimmianine, aegeline, lupeol, cineol, citral, citronella, cumin aldehyde, eugenol, and marmesinine are among the bioactive compounds that have been extracted from this amazing plant [13]. These compounds are being used to treat a variety of conditions, including asthma, anaemia, fractures, wound healing, swollen joints, high blood pressure, jaundice, and diarrhoea [14, 15]. Although aqueous and ethanolic extracts of Aegle marmelos leaves, seeds, and fruit have been shown in previous studies to affect male fertility by reducing sperm motility [16,17] and antitesticular [18] activity in a reversible manner, their recovery was found to be prolonged [19]. However, there are currently no specific reports on the potential anticancer properties of Aegle marmelos bark extract. In our study, we selected the root bark of the plant Aegle marmelos, the total phenolics, and flavonoids of the root barks were evaluated. The aim of this study was to establish the antioxidant property and cytotoxic activity of the extracts of root bark of the plant, which was not done before.

2. Materials and Methods

Collection and Extraction of Plant Sample: The Aegle marmelos L. root bark utilised in this study was gathered in March 2024 from the Meerut district in Uttar Pradesh, India. After 5.0 kg of bark was shade-dried and ground into a powder using an electric grinder, 2.0 kg of the drug powder was extracted using 6.0 L of methanol and aqueous solution over the course of 48 hours, stirring occasionally. A brown soluble residue weighing 60 g (6.0% w/w) and a yellowish-brown residue weighing 35 g (3.5% w/w) were produced by concentrating the filtrate (2.0 L) at 40°C under decreased pressure. [20] To get rid of any remaining solvent, the residue was further dried in an oven at 37°C. It was then kept in a dark, airtight plastic container at 4–8°C until it was needed.

Preliminary Phytochemical Evaluation: To identify the different phytoconstituents, a

qualitative chemical analysis was performed on the methanolic and aqueous extract. Standard protocols were used to evaluate the drug's phytochemical properties. [21, 22]

Antioxidant Assay:

DPPH Assay (2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Assay): Ascorbic acid served as the standard. A 100 mg standard flask was filled with 100 mg of ascorbic acid to make up the volume. To make up the volume (stock solution), pipette 1 ml from this into a 10 mL standard flask. The concentrations of 100, 200, 400, 600, and 800 µg/mL were obtained by pipetting 1, 2, 4, 6, and 8 mL into separate 10 mL standard flasks and adjusting the volume accordingly. [23, 24, 25]

Percentage inhibition = (absorbance of blank – sample/absorbance of blank) × 100

Every sample was examined three times before being averaged. A graph showing the percentage of inhibition vs concentration was used to determine the extract concentration needed to scavenge 50% of the radicles.

ABTS Assay: In 50 millilitres of distilled water, ABTS 2 mM (0.0548g) was made. In distilled water, 70 mM potassium per sulphate (0.0189 g in 1 mL) was made. After two hours, 50 mL of ABTS and 200 µL of potassium per sulphate were combined and utilised. This solution, known as the ABTS radical cation, was employed in the test. 0.3 mL of ABTS radical cation and 1.7 mL of phosphate buffer with a pH of 7.4 were added to 0.5 mL of extract at different doses. Ethanol was used as a control instead of extract. At 734 nm, the absorbance was measured. [26, 27] Three duplicates of the experiment were conducted. The following formula was used to get the percentage inhibition:

% Inhibition = (Avg. OD of control - Avg. OD of Test)/ Avg. OD of control × 100

In-vitro Cytotoxic Assay:

MTT Assay: The reduction of yellow 3-(4, 5- dimethythiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase is measured by this colorimetric test. After entering the cells, the MTT is converted to a dark purple, insoluble formazan product in the mitochondria. Following the solubilisation of the cells with an organic solvent (such as isopropanol), the solubilised formazan reagent that is liberated is quantified using spectrophotometry. [28] The degree of activity is a gauge of the cells' viability because only metabolically active cells can reduce MTT. The National Centre for Cell Sciences (NCCS), located in Pune, India, was the original source of the HL60 (Human promyelocytic leukaemia) cell line, which was kept in Dulbecos modified Eagles media (Gibco, Invitrogen). DMEM supplemented with 10% FBS, L-glutamate, sodium bicarbonate, and an antibiotic solution containing Amphotericin B (2.5µg/mL), Streptomycin (100µg/mL), and Penicillin (100U/mL) was used to cultivate the cell line in a 25 cm2 tissue culture flask. Using a humidified 5% CO_2 incubator (NBS Eppendorf, Germany), cultured cell lines were maintained at 37ºC. [29, 30] Direct cell observation using an inverted phase contrast microscope and the MTT assay method were used to assess the vitality of the cells.

Cells Seeding in 96 Well Plate: Trypsinization was performed on a confluent monolayer of cells that were two days old. The cells were then suspended in 10% growth media, planted in 96-well tissue culture plates using 100µL of the cell suspension (5×104 cells/well), and

cultured at 37 °C in a humidified 5% CO_2 incubator. [31]

Preparation of Compound Stock: To guarantee sterility, the chemical solution was passed through a 0.22 μm Millipore syringe filter. A freshly made compound in 5% DMEM was extracted after 24 hours, and 6.25μg/mL, 12.5μg/mL, 25μg/mL, 50μg/mL, and 100μg/mL were taken and made up to 250μg/mL using 5% MEM. These were then added in triplicate to the corresponding wells and incubated at 37 oC in a humidified 5% CO2 incubator. Following a 24-hour incubation period, the entire plate was examined under an inverted phase-contrast tissue culture microscope, and microscopic observations were captured as pictures. [32] Any discernible alterations in the cells' shape, such as rounding or shrinking, granulation, or vacuolization in the cytoplasm, were regarded as markers of cytotoxicity.

Anti-proliferative Assay by MTT Method: After being thoroughly diluted in 3 millilitres of PBS, 15 milligrammes of MTT (Sigma, M-5655) was reconstituted and sterilised using a filter. The sample content in the wells was removed after the incubation period of 24 hours, and 30μL of reconstituted MTT solution was added to each test and cell control well. The plate was then gently shaken thoroughly and incubated for 4 hours at 37 oC in a humidified 5% CO2 incubator. In order to solubilise the formazan crystals, the supernatant was discarded after the incubation period, and 100μL of MTT Solubilisation Solution DMSO was added. The wells were then gently pipetted up and down. The absorbance values were measured by using a microplate reader at a wavelength of 540 nm. [33, 34, 35] The percentage of growth inhibition was calculated using the formula:

% viability = (OD of Test/ OD of Control) × 100

3. Results and Discussion:

Alkaloids, glycosides, phenolic, flavonoids, carbohydrates, proteins, amino acids, terpenoids, sterols, and saponins were among the phyto-elements identified by qualitative chemical analysis of the methanolic and aqueous extract's preliminary phytochemical evaluation.

Table 1: Phytochemical test

S. No.	Name of phytoconstituent	Name of chemical test	Plant extract	
			Methanol	Aqueous
1.	Alkaloids	Picric acid test	+	+
		Iodine test		
2.	Carbohydrates	Starch test	+	+
		Molish test	_	_
3.	Reducing sugars	Bontrager's test	+	+
		Aqueous NaOH test		
4.	Flavonoids	Zinc hydrochloride reduction test	+	+
5.	Phenolic compounds	Iodine test	+	+
		Ferric chloride test		
6.	Glycosides	-	+	+

The percentage inhibition achieved with the standard, as shown in Table 2, was contrasted with the percentage inhibition obtained for the various concentrations of sample extracts in the DPPH experiment. When the methanolic and aqueous extracts' DPPH scavenging properties were examined in the concentration range of 100-800 (µg/mL) and standard, the extract displayed a dose-dependent pattern in DPPH radical scavenging, as evidenced by a decrease in the production of purple colour. The methanolic extract's IC50 value was 112.6 µg/mL, whereas the aqueous extract's was 117.4 µg/mL. Ascorbic acid's IC50 value was determined to be 66.33 µg/mL. The ability of Aegle marmelos' methanolic and aqueous extracts to scavenge DPPH+ radicals is contrasted with that of regular ascorbic acid. Both samples demonstrated strong DPPH+ radical scavenging capabilities. The methanolic and aqueous extracts demonstrated good antioxidant capacity, according to the DPPH radical scavenging method's estimation of antioxidant activity.

Table 2: % Inhibition by Standard Ascorbic Acid And sample of Aegle marmelos For DPPH Assay

Sample	Concentration (µg/mL)	Absorbance*	% Inhibition	IC_{50} (µg/mL)
Methanolic extract	100	1.621 ± 0.05	1.33	112.6
	200	1.431 ± 0.04	14.65	
	400	1.256 ± 0.21	24.42	
	600	0.893 ± 0.13	49.83	
	800	0.809 ± 0.01	56.11	
Aqueous extract	100	1.865 ± 0.05	0.97	117.4
	200	1.611 ± 0.17	12.65	
	400	1.366 ± 0.18	21.64	
	600	1.123 ± 0.19	39.76	
	800	1.002 ± 0.13	49.76	
Standard (Ascorbic acid)	100	0.324 ± 0.004	14.65	66.33
	200	0.211 ± 0.003	40.25	
	400	0.187 ± 0.002	45.56	
	600	0.158 ± 0.003	53.35	
	800	0.096 ± 0.002	75.45	

*Values are expressed as mean ± SD, n = 3.

Table 3 lists the percentage inhibition obtained in the ABTS antioxidant assay for various sample extract concentrations in comparison to the percentage inhibition obtained using the standard. A protonated radical called ABTS+ has a distinctive absorption maximum around 734 nm, which reduces proton radical scavenging. The evaluated extracts' activity in the ABTS+ cation radical scavenging method was expressed as a micromolar equivalent of an ascorbic acid solution with an antioxidant equivalent to 1g dry matter of the experimentally investigated sample.

Table 3:% Inhibition by Standard Ascorbic Acid and sample of A. marmelos For ABTS Assay

Sample	Concentration (µg/mL)	Absorbance*	% Inhibition	IC_{50} (µg/mL)
Methanolic extract	125	0.079 ± 0.0002	15.76	896.23
	250	0.061 ± 0.0003	38.27	
	500	0.052 ± 0.0003	43.86	
	1000	0.035 ± 0.0002	65.72	
	2000	0.029 ± 0.0003	79.77	
Aqueous extract	100	0.059 ± 0.0001	39.68	345.17
	200	0.045 ± 0.0003	46.34	
	400	0.038 ± 0.0002	57.11	
	600	0.031 ± 0.0002	62.65	
	800	0.015 ± 0.0002	81.43	
Standard (Ascorbic acid)	100	0.095 ± 0.0005	8.43	952.56
	200	0.082 ± 0.0003	21.74	
	400	0.055 ± 0.0006	44.74	
	600	0.032 ± 0.0005	63.75	
	800	0.019 ± 0.0006	87.87	

*Values are expressed as mean ± SD, n = 3

Table 3 lists the methanolic extract and aqueous extract of A. marmelos ABTS+ radical scavenging activity. Both samples demonstrated strong ABTS+ radical scavenging capabilities. In comparison to ascorbic acid (952.56µg/mL), the methanolic extract (896.23µg/mL) demonstrated favourable outcomes.

Cytotoxicity Activity: The MTT technique was used to determine the methanolic extract of A. marmelos's anti-proliferative activity on the HL60 cell line. Following incubation with the sample methanolic extract, cell viability was assessed using the MTT reduction test. The IC50 values were graphically extracted from the dose-response curve and converted to the percentage of control.

The methanolic extract fraction was chosen to test its cytotoxic potential on the HL60 cell line using the MTT assay technique since it demonstrated higher antioxidant potential than the aqueous fraction. Using the MTT assay method, the methanolic extract's IC50 value on the HL60 cell line was determined to be 35.524 µg/mL. As a result, A. marmelos' methanolic extract exhibits great promise as a cytotoxic agent against the HL60 cell line.

Table 4: % Inhibition by methanolic of A. marmelos L. On HL60 Cell Line

Sample	Concentration (µg/mL)	Absorbance*	% Inhibition	IC_{50} (µg/mL)
Methanolic extract	6.25	0.639 ± 0.0046	75.61	36.42

	12.5	0.468 ± 0.0069	55.57	
	25	0.403 ± 0.0025	51.32	
	50	0.291 ± 0.0011	35.66	
	100	0.244 ± 0.0052	28.43	
Control	-	0.811 ± 0.0012	-	-

*Values are expressed as mean ± SD, n = 3

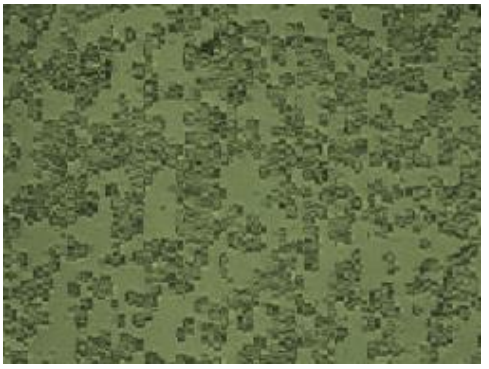

Fig: 1. Effect of methanolic extract On HL60 Cells Using MTT Assay. Phase Contrast Analysis of Cell Morphology (Control Cells)

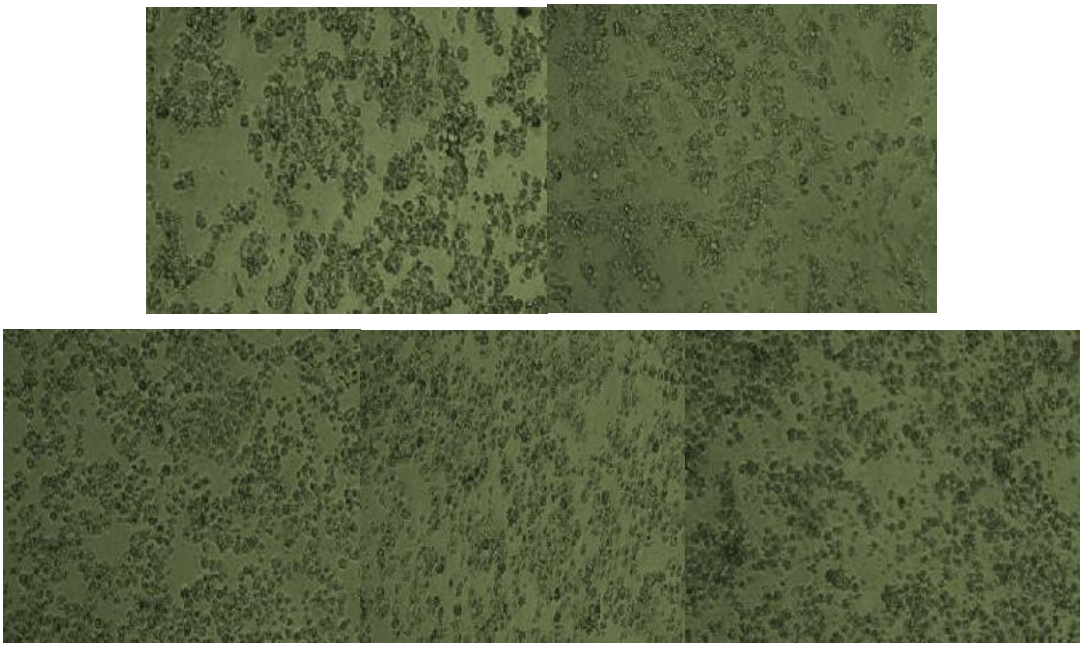

Figure 2. Effect of methanolic extract On HL60 Cells Using MTT Assay with different concentrations

4. CONCLUSION

The dried root bark of A. marmelos L. was extracted using methanol and water in the current investigation. When compared to the standard ascorbic acid, antioxidant activity experiments using the DPPH and ABTS radical scavenging methods revealed that the methanolic extract had excellent antioxidant activity and the aqueous extract had good antioxidant activity. With an IC50 value of 36.42 µg/mL, the methanolic extract's cytotoxic investigation demonstrated encouraging activity with the HL60 cell line using the MTT assay. Therefore, A. marmelos's methanolic extract exhibits extremely promising potential as a cytotoxic agent against the human promyelocytic leukaemia (HL60) cell line. Future research will focus on identifying the chemical ingredient that gives Aegle marmelos Lin root bark extracts their cytotoxic properties.

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