"PHARMACOGNOSTIC, PHYTOCHEMICAL AND PHARMACOLOGICAL STUDY OF ETHANOL EXTRACT OF AEGLE MARMELOS ROOT BARK"

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Abstract:
The study includes pharmacognostic evaluation, phytochemical analysis, and pharmacological activity of the ethanol extract obtained from the root bark. The pharmacognostic evaluation involved the macroscopic and microscopic examination of the root bark, determining its organoleptic characters, size, shape, surface features, and other parameters. Physico-chemical standards such as loss on drying, total ash, acid-insoluble ash, water-soluble ash, and extractive values were also determined to assess the quality and purity of the root bark. Phytochemical analysis of the ethanolic and aqueous extracts revealed the presence of various phytoconstituents including carbohydrates, reducing sugars, alkaloids, glycosides, cardiac glycosides, flavonoids, triterpenoids, tannins, and steroids. These phytoconstituents are known for their medicinal properties and contribute to the therapeutic potential of the root bark. The pharmacological activity of the ethanol extract was evaluated through anticancer assays using the onion tip root assay and potato disk assay methods. The results showed significant inhibitory effects on tumor growth, indicating potential antitumor activity.Overall, this comprehensive study provides valuable insights into the pharmacognostic evaluation, phytochemical analysis, and pharmacological activity of Aegle marmelos root bark. The findings contribute to the understanding of its medicinal properties and support its potential use in pharmaceutical and natural product research.

Keywords: Aegle marmelos, Pharmacognostic evaluation, Phytochemical analysis, Pharmacological activity, Medicinal plants, Ethanol extract

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INTRODUCTION:

Aegle marmelos, commonly known as Bael or Bengal quince, is a versatile medicinal plant with a rich history in traditional medicine systems, particularly in Ayurveda. It belongs to the family Rutaceae and is native to the Indian subcontinent, although it is also found in other tropical regions of Asia. The various parts of Aegle marmelos, including the leaves, fruits, and roots, have been utilized for their therapeutic properties and have been the subject of extensive research. [1,2]

The root bark of Aegle marmelos has garnered significant attention in recent years due to its diverse range of bioactive compounds and pharmacological activities. It contains several important phytochemical constituents, such as alkaloids, flavonoids, tannins, coumarins, terpenoids, and phenolic compounds, which contribute to its medicinal properties. These bioactive compounds exhibit a wide array of biological activities, including antioxidant, anti inflammatory, antimicrobial, anticancer, antidiabetic, hepatoprotective, immunomodulatory, and neuroprotective effects, among others. [3, 4]

The use of natural products, like Aegle marmelos, as a source of therapeutic agents has gained momentum in recent years. Natural products offer several advantages over synthetic drugs, including a broader spectrum of biological activities and a lower risk of toxicity. The complex mixture of bioactive compounds found in natural products often works synergistically, providing multiple targets for therapeutic intervention and reducing the risk of drug resistance. Moreover, natural products derived from medicinal plants have a long history of traditional use, providing a valuable foundation for their exploration in modern medicine. [5,6]

Ayurveda, the traditional Indian system of medicine, has extensively utilized Aegle marmelos for various therapeutic purposes. In Ayurvedic texts, Aegle marmelos is described as having cooling, digestive, rejuvenating, and antimicrobial properties. It is commonly prescribed for gastrointestinal disorders, respiratory ailments, skin diseases, diabetes, and urinary problems. The leaves, fruits, and root bark of Aegle marmelos are formulated into different Ayurvedic preparations, such as decoctions, powders, and oils, to address specific health conditions. [7,8]

Pharmacognostic evaluation, phytochemical analysis, and assessment of pharmacological activity are crucial steps in validating the traditional claims associated with medicinal plants. Therefore, in this study, we aimed to conduct a comprehensive investigation on the pharmacognostic characteristics, phytochemical constituents, and pharmacological activity of the ethanol extract derived from Aegle marmelos root bark. [9,10]

Pharmacognostic evaluation serves as the initial step in the quality control of herbal drugs and involves the identification and characterization of plant materials based on their macroscopic, microscopic, and organoleptic properties. These parameters provide valuable information about the authenticity, purity, and standardization of the plant material under investigation.[11,12]

Phytochemical analysis plays a vital role in determining the chemical composition of medicinal plants. It involves the identification and quantification of various secondary metabolites, such as alkaloids, flavonoids, terpenoids, phenolics, and glycosides, which are known to possess pharmacological activities. The identification of these bioactive compounds provides insights into the potential therapeutic benefits associated with the plant extract. [13, 14]

Furthermore, the assessment of pharmacological activity aims to explore the therapeutic potential of the Aegle marmelos root bark extract through in vitro and in vivo experiments. Various pharmacological assays will be employed to investigate the extract's potential effects on specific biological targets or disease models. This information is crucial for understanding the mechanisms of action and establishing the therapeutic relevance of the plant extract. [15, 16]
The current study seeks to fill the existing knowledge gaps regarding the pharmacognostic properties, phytochemical constituents, and pharmacological activity of the ethanol extract derived from Aegle marmelos root bark. The findings of this comprehensive study will contribute to the understanding of the medicinal potential of Aegle marmelos and provide a scientific basis for its traditional uses in herbal medicine. Furthermore, the outcomes may pave the way for the development of novel therapeutic agents or natural product-based formulations derived from Aegle marmelos, thus contributing to the advancement of drug discovery and development. [16]

Materials and methods

Plant material and Chemicals

The plant material used in this study was the root bark of Aegle marmelos, a plant belonging to the Rutaceae family. The collection of the root bark was carried out from the local area of Khultabad. To ensure the authenticity of the plant material, a thorough pharmacognostic study and assessment of organoleptic characters were conducted. The authentication process was performed by the Department of Botany and Research Centre at Padmashri Vikhe Patil College of Art, Science, and Commerce, Pravaranagar. The authentication was documented in a letter with the reference number Ref. No. /PVPC/Bot/2022-23/73, confirming the identity and quality of the collected Aegle marmelos root bark.All other chemical were used analytical grade.

Pharmacognostic Study:

Study of macroscopic and microscopic features of crude drug were carried out using various parameters:

Macroscopic features of Root bark:

Macroscopic features were studied by naked eyes and observed. fresh and dried barks were notes: size and shape, surfaces, fracture, texture, colour, odour and taste were done by observing visually.

Microscopic features:

Microscopic features: The root bark of Aegle marmelos was examined using various microscopic techniques. The bark pieces were boiled, sliced into thin sections, washed, cleared with chloral hydrate 30%, stained with phloroglucinol-concentrated hydrochloric acid (1:1), mounted on glass slides with glycerin, and observed under a Motic Electronic Microscope, capturing micrographs at different magnifications for analysis (Khandelwal K. R. 2016).

Powder microscopy: The powdered root bark was analyzed using a mesh size of number 16. Separate specimens were treated with staining solutions like phloroglucinol-hydrochloric acid, ruthenium red, and iodine solution to detect lignin, mucilage, and starch grains, respectively. The stained powder samples were observed under a Motic Electronic Microscope at different magnifications to study the microstructural features (Khandelwal K. R. 2016).

Physico-chemical standards: Loss on drying: Approximately 1.5 grams of powdered drug were dried in an oven and weighed to calculate the percentage of moisture loss using a gravimetric method (Khandelwal K. R. 2016).

Determination of foreign content: Approximately 100 grams of dried bark powder were visually inspected, foreign matter was separated and weighed, and the percentage of foreign content was calculated (Khandelwal K. R. 2016).

Determination of total ash: About 2 grams of dried sample were incinerated, and the remaining ash was weighed to determine the percentage of total ash with respect to the airdried powder (Khandelwal K. R. 2016).

Determination of acid-insoluble ash: The total ash was boiled with dilute hydrochloric acid, filtered, and the residue was incinerated to calculate the percentage of acid-insoluble ash (Khandelwal K. R. 2016).

Determination of water-soluble ash: The total ash was boiled with distilled water, filtered, and the residue was incinerated to calculate the percentage of water-soluble ash (Khandelwal K. R. 2016).

Extractive values: The alcohol-soluble and water-soluble extractives were determined by macerating the powdered drug in ethanol and chloroform water, respectively. The filtrates were evaporated and weighed to calculate the percentages of soluble extractives with reference to the air-dried powder (Khandelwal K. R. 2016).

Extraction:

The root bark of Aegle marmelos was obtained from the department of Botany and Research Centre, PVP College of Arts, Science and Commerce, Pravaranagar, Loni, Ahmednagar, Maharashtra, India 41371, and authenticated. The authenticated root bark was dried in the shade and then coarse powdered using a grinder. The extraction process followed standard procedures using analytical grade solvents. The coarse powder of the root bark was macerated with ethanol at room temperature for 21 days. The resulting extract was concentrated under reduced pressure, resulting in an ethanolic extract. Approximately 15 grams of ethanolic extract was obtained from 250 grams of root bark powder.

Preliminary phytochemical screening

Preliminary phytochemical screening was conducted on ethanolic and aqueous root bark

extracts of Aegle marmelos to identify the presence of various phytoconstituents. Tests were performed for carbohydrates, reducing sugars, alkaloids, glycosides, cardiac glycosides, flavonoids, triterpenoids, saponins, tannins, and steroids. The extracts were subjected to qualitative chemical tests, and observations were recorded. Quantitative estimation was performed on methanolic bark extracts of Zizyphus jujuba to determine the total content of various metabolites. The total alkaloids content was determined by precipitation and filtration, while the total phenolic content was measured using the Folin-Ciocalteu method. The total flavonoid content was determined using the aluminium chloride colorimetric method, and the total terpenoid content was calculated by measuring the weight difference before and after extraction.

Screening of extract for pharmacological actions:

Anticancer Activity

Onion tip root assay methods (Aprem et al).

To prepare the test organism for mitotic studies, the following steps were followed. Five healthy medium-sized onion bulbs weighing 25-28 g were selected, and their outermost brownish scaly skin and dead roots were carefully scraped off near the disc. The prepared bulbs were placed in tubes filled with tap water, ensuring that the discs were submerged, and allowed to grow for three days. The growth process took place at room temperature (average temperature of 24-25°C) with an average humidity of 46.6% and partial exposure to sunlight until the roots reached a length of approximately 1 cm. The zero hour or stat Mitotic Index was then determined for each bulb, serving as the starting point for the subsequent mitotic studies.

Squash Preparation:

To prepare slides for mitotic studies, the following steps were carried out. Initially, the terminal 2-3 mm of the root meristems was cut and heated in a mixture of Acetocarmine and N/10 HCl in a 9:1 ratio. The watch glass containing the root tips was then heated until they became soft and darkly stained. Subsequently, a tip was selected and gently squashed in a drop of fresh acetocarmine on a clean slide, which was then covered with a cover slip. The slide was carefully wrapped with two layers of filter paper and pressed down vertically using the thumb to create a squashed sample. These prepared slides were then observed under a microscope at 40X magnification, examining various fields. Both cells undergoing different stages of mitosis and non-dividing cells were identified and counted. A range of 500-800 cells per onion bulb were counted to ensure accurate representation and statistical significance for the study.

Estimation of Mitotic Index (MI):

1) The bulbs were then put in 3 containers containing control, standard and extract at concentrations 1mg/ml and readings of MI were taken after 48 hours of exposure.
2) Each test was run in triplicate.
3) The data of MI was recorded.
4) The various stages of cell division were noted down separately.
5) The Mean and Standard Deviation (SD) were calculated.
6) The significance of the difference in MI at various times of exposure was calculated by Student‘t’ test.

Mitotic index % = (Total number of dividing cells/Total number of cells examined) × 100

Potato disk assay methods

(Turker and Camper, 2002; Coker et al., 2003; Hussain et al., 2007)

Antitumor activity of twig ethanol extract of was assessed using the potato disc bioassay.

Following design was followed:

- 600 µl test extract + 150 µl Double Distilled Water (DDW) + 750 µl A. tumefaciens in PBS.
- Camptothecin was used as positive control replacing test extract.
- Potato discs (5 mm×8 mm in size) were collected from red-skinned potatoes (Solanum tuberosum L., Solanaceae) using sterilize cork borer.
- Each potato disc was overlaid with 50 µl of appropriate inoculums with particular concentrations (10 ppm, 100 ppm and 1000 ppm) of test extract.
- Petri dishes were sealed by parafilm and incubated at room temperature (27-30 °C) for 3 weeks.
- After 3 weeks, potato discs were stained with Lugol's iodine (10% KI and 5% I2) for 30 minutes and tumors were observed under stereo microscope, where the tumor cells lack starch (look like orange color).
- Each experiment was done in triplicate.

Percentage of tumor inhibition was calculated using standard formula. (Hossain et al. (2007), (Russell D. Freed, Crop and Soil Sciences Department of Michigan State University, USA).

$$\text{Percentage Inhibition} = 100 - \frac{\text{Numbers of tumor with sample}}{\text{Numbers of tumor with control}} \times 100$$

RESULTS AND DISCUSSION:

Pharmacognostical Study:

Macroscopy:

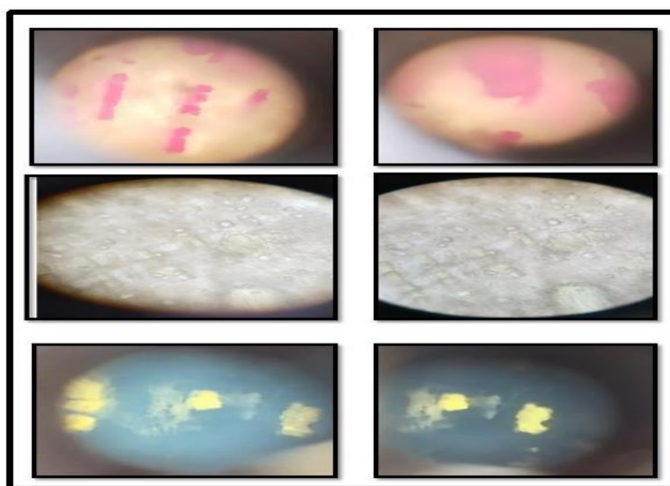
The macroscopical study of Aegle marmelos root bark revealed that the bark has a gray or brownish color with a pleasant odor. It has a slightly sweet taste and is flat in shape, measuring approximately 15-20 cm in length and 1.5 to 2 cm in thickness. The outer surface appears rough due to the presence of tubercules, longitudinal, and transverse cracks. The bark has a hard and splintery fracture, and the inner surface exhibits a similar gray or brownish color as the outer surface.

Table No 01: Macroscopical Characters

Sr. no.	Macroscopical characters	Observation
1.	Color	Bark grey or brownish in colour
2.	Odor	Pleasant
3.	Taste	Slightly sweet in taste
4.	Size	Flat 15-20 cm in length and 1.5 to 2 cm in thickness
5.	Shape	Outer surface rough due to presence of tubercules, longitudinal and transverse cracks
6.	Fracture & Fissure	Hard and Splintery
7.	Inner surface	Bark grey or brownish in colour

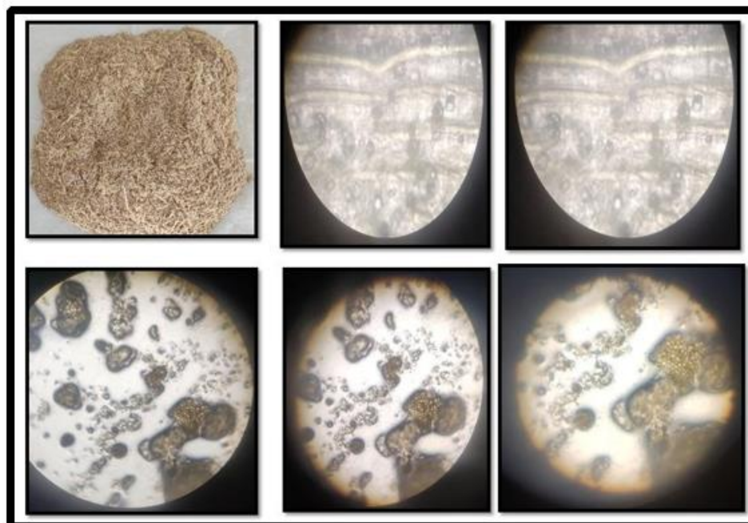
Microscopy:

T. S. of Aegle marmelos root bark :



Microscopic Characters	Observations
Cork Cells	Polygonal-shaped cells, impregnated with a layer of suberin
Phellodermal Cells	Mostly parenchymatous in nature
Lenticels	Present in the periderm, similar in function to stomata, open pores with no guard cells
Sclerenchyma	Hardened, dead tissue with thickened and lignified cells, providing mechanical strength
Phloem	Living tissue conducting food from leaves to other parts of the plant
Xylem	Dead tissue conducting water from roots to leaves
Sclereids or Stone Cells	Isodiametric or irregular, lignified cells with U-shaped thickening and varying lumen
Sclerenchymatous Fibers	Narrow, elongated lignified fibers with pointed ends
Medullary Rays	Parenchymatous cells extending from pith to cortex, conducting food and water laterally
Calcium Oxalate Crystals	Single prismatic crystals occurring in groups, surrounded by parenchymatous cells
Photomicrograph (TS) Observations	The bark exhibits a brown-colored cork tissue with phloroglucinol and hydrochloric acid staining. It consists of layers of phellogen and phelloderm, lenticels in the periderm, a cortex layer containing phloem elements, secondary phloem with stone cells and sclereids, medullary rays, polygonal cork cells, lignified sclereids and stone cells, prismatic calcium oxalate crystals with a crystal sheath, and narrow, elongated lignified fibers and xylem vessels with pitted walls.

Powder Characteristics:



Micro chemical Test:

Sr. No.	Reagent	Observation	Characteristics
1	Phloroglucinol + Conc. HCl (1:1)	Pinkish	Lignified cells: fibre's, stone cells, Sclereids, cork cells
2	Iodine	Blue	Starch
3	Ruthenium red	No Pink Colour	No Mucilage cells
4	Acetic acid	Insoluble	Calcium oxalate crystals
	Dil. Hydrochloric acid	Soluble	
5	Alcoholic Picric acid	No Yellow Colour	No Aleurone grains found
6	Sudan red III	No Red colour	Oil globules absent

Table No 03: Physico-chemical standards''

Physico-chemical standards:
The provided physico-chemical standards include parameters such as Loss on Drying, Total Ash, Water Soluble Ash, Acid Insoluble Ash, Water Soluble Extractive, and Alcohol Soluble Extractive. These parameters measure the moisture content, inorganic residue, solubility in water and alcohol, and other characteristics of the sample. These results are important for assessing the quality and purity of the sample, and they can be used for quality control purposes in various industries including pharmaceuticals, herbal medicine, and food production.

Parameter	**Result (% w/w)**
Loss on Drying	2.4 gm
Total Ash	4 gm
Water soluble ash	2.16 gm
Acid Insoluble ash	0.67 gm
Water soluble extractive	33 % w/w
Alcohol soluble extractive	11.2 gm

Table No 04: Physico-chemical standards

Phytochemical Screening:
The phytochemical screening of the sample using ethanolic and aqueous extracts revealed the presence of various phytoconstituents. Both extracts showed high levels of carbohydrates, reducing sugars, alkaloids, glycosides, cardiac glycosides, flavonoids, triterpenoids, tannins, and steroids. These phytoconstituents are known for their potential medicinal properties and may contribute to the therapeutic value of the sample. However, saponins were only detected in the ethanolic extract, while they were absent in the aqueous extract. These findings provide valuable information about the chemical composition of the sample and suggest its potential use in pharmaceutical or natural product research.

Sr. No	Type of Phytoconstituent	Ethanolic extract	Aqueous extract
1	Carbohydrates	+++	+++
2	Reducing sugars	+++	+++
3	Alkaloids	+++	+++
4	Glycosides	+++	+++
5	Cardiac glycosides	+++	+++
6	Flavonoids	+++	+++
7	Triterpenoids	+++	+++
8	Saponins	+++	---
9	Tannins	+++	+++
10	Steroids	+++	+++

Table No 05: Preliminary Phytochemical Screening

Quantitative Estimation

Sr. No	Phytochemical Content	Content Amount	Method	Calculated using
1	Total Content Alkaloid	22 mg/gm	Standard Method using Ammonium Hydroxide	Formula
2	Total Content Phenolic	123.7 mg/gm	Folin-Ciocalteu method	Calibration Curve
3	Total Content Flavonoid	83.29 mg/gm	Aluminium chloride colorimetric method	Calibration Curve
4	Total Content Terpenoid	19.21 mg/gm	Standard Method Using PET ether	Formula

Table No 06: Quantitative Estimation of Extract

Thin layer Chromatography

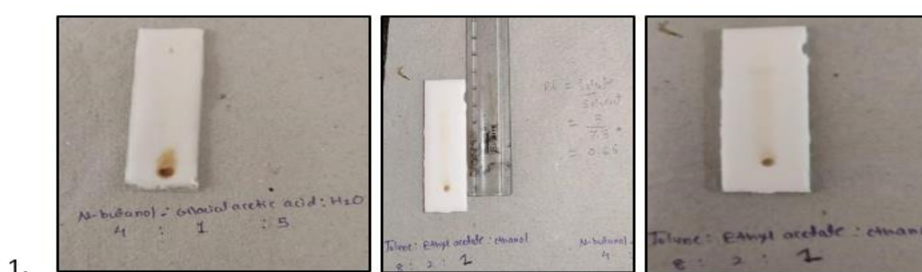
In TLC, the mixture to be analyzed is spotted near the bottom of the TLC plate. The plate is then placed in a developing chamber containing a solvent system, which consists of two or more solvents that are mixed in specific ratios. The solvent system moves up the TLC plate through capillary action, carrying the components of the mixture along with it. As the solvent system moves, the components of the mixture separate based on their different affinities for the stationary and mobile phases.

The separation of components on a TLC plate is visualized by applying a suitable visualization technique, such as UV light, iodine vapor, or a chemical staining reagent. The components appear as individual spots or bands on the plate, and their relative positions and distances traveled provide information about their relative polarities and interactions with the stationary phase.

TLC systems:

1. **N-butanol : Glacial acetic acid : H2O (0.5 : 4 : 1 : 5):** This solvent system consists of n-butanol, glacial acetic acid, and water in a ratio of 0.5 : 4 : 1 : 5. It means that for every 0.5 part of n-butanol, there are 4 parts of glacial acetic acid and 5 parts of water. This system is polar due to the presence of water and acetic acid. It can be useful for separating polar compounds or those with moderate polarity.
2. **Toluene : Ethyl acetate : Ethanol (0.66 : 8 : 2 : 2):** The second solvent system consists of toluene, ethyl acetate, and ethanol in a ratio of 0.66 : 8 : 2 : 2. Toluene is a nonpolar solvent, while ethyl acetate and ethanol are polar. This system is relatively nonpolar due to the higher proportion of toluene. It is commonly used for separating nonpolar or weakly polar compounds.
3. **Toluene : Ethyl acetate : Ethanol (0.64 : 8 : 2 : 1):** The third solvent system also consists of toluene, ethyl acetate, and ethanol. In this case, the ratio is 0.64 : 8 : 2 : 1. Similar to the previous system, it is relatively nonpolar but with a slightly higher proportion of ethanol. This system may provide a slightly more polar environment compared to the previous one, making it suitable for separating compounds with moderate polarity.

1.



Coloumn Chromatography:

The column chromatography setup consists of a glass or plastic column packed with a stationary phase material, such as silica gel or alumina. The stationary phase is often chosen based on its ability to adsorb and separate the components of the mixture effectively. The column is typically equipped with a stopcock or a valve at the bottom to control the flow of the mobile phase.

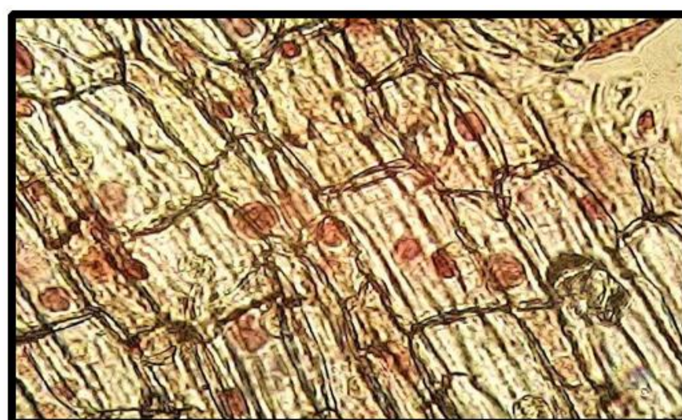
The mixture to be separated is dissolved or suspended in a suitable solvent known as the mobile phase. The mobile phase is carefully chosen based on its ability to dissolve the sample components and elute them through the column at different rates. It is important to select a solvent system that provides good separation of the components while maintaining their stability.

In vitro antimitotic activity Onion root tip method:

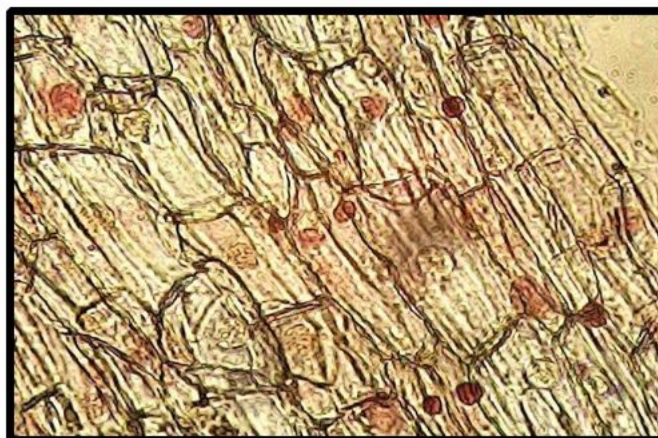
Extract	Concentration in mg/mL	Total No. of Cells	Cell in Division	Mean	Mitotic Index %
Control	0.1mg/ml	200	57	57.33	28.66
	0.1mg/ml	200	61		
	0.1mg/ml	200	54		
Standard	0.1mg/ml	200	162	168.66	84.33
	0.1mg/ml	200	169		
	0.1mg/ml	200	175		
Extract	0.1mg/ml	200	105	114.33	57.16
	0.1mg/ml	200	121		
	0.1mg/ml	200	117		

Treatment not only brought down the frequency of dividing cells but also produced a good number of anomalies in the mitotic cells when you compared with standard reports.

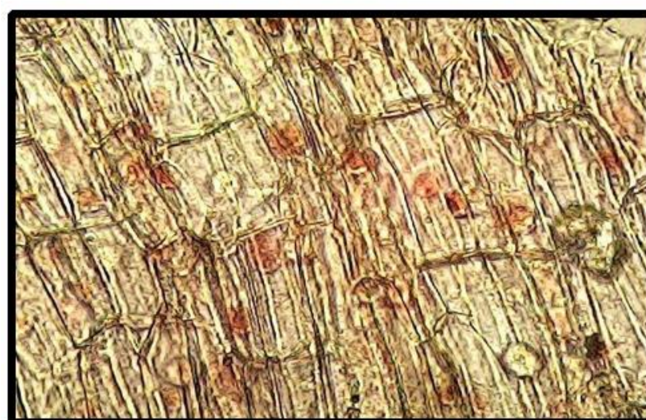
The extracts also caused chromosomal and mitotic aberrations including accumulation of prophases, sticky chromosomes at metaphase, spindle disturbance at prophase and anaphase bridges.



Control



Standard



Extracts

Antitumor potato disc assay Statistical analysis showed that the methanol extract inhibit tumor growth on potato disc significantly in a concentration dependent manner across the strains (Table 1). Highly significant difference was observed among three Agrobacterium strains suggests their differential sensitivity (Table 1). Maximum tumor inhibition was observed at 1000 ppm plant extract against the strain AtSl0105 (39.16%). No significant tumor inhibition was observed at 10 ppm concentration (Fig. 2). Inhibition percentage was calculated to compare with the control. On the basis of tumor forming ability, it was observed that A. tumefaciens AtSl0105 (8.0±0.20) was more effective strain than AtAc0114 (7.0±0.41) and AtTa0112 (6.2±0.17) (Fig. 2 & Fig. 3).

Table-1: Statistical analysis of tumor inhibition by the extract and tumor induction by the three strains of A. tumefaciens on potato discs

Source of variations	Degree of freedom	Sum of squares	Mean square	F value	Prob.
Strains(S)	2	11.49	5.74	21.36	0.000
Concentrations(C)	3	33.04	11.01	40.95	0.000
Interaction between strains and concentration(S×C)	6	0.58	0.09	0.36	
Erorr	24	6.45	0.26		
Total	35	51.57			

Least Significant Differences Test (Strains)
LSD value = 0.7569 at alpha = 0.050

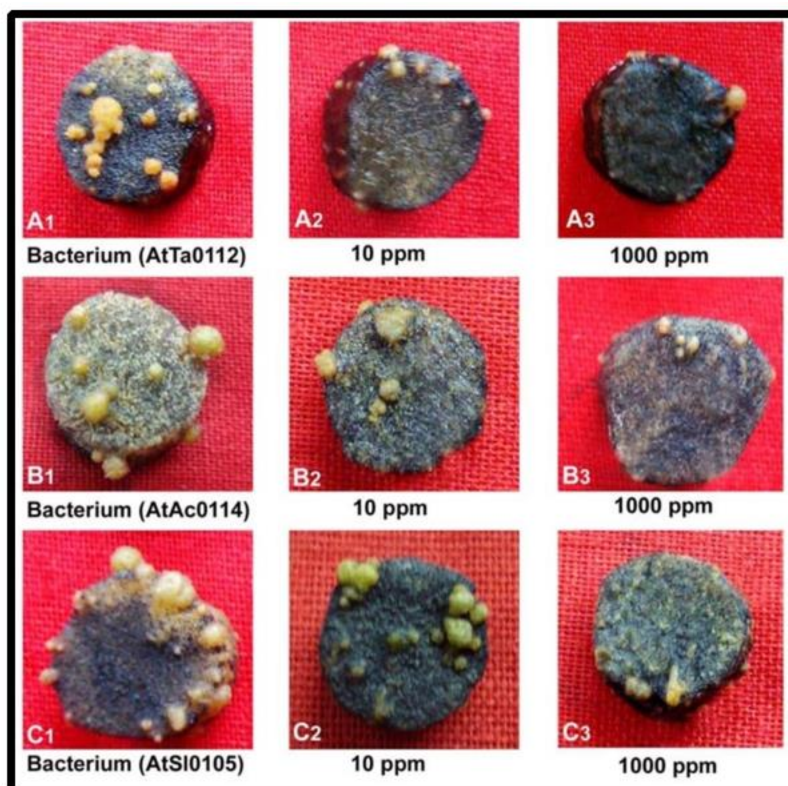


Fig. 3: Photographs show tumor inhibition by the twig extract on potato discs, galls are produced by Agrobacterium strains. A1, B1, C1 used as experimental control, A2, B2, C2 & A3, B3, C3 as 10 ppm & 1000 ppm treatment concentration, respectively.

CONCLUSION:

In conclusion, the comprehensive study conducted on the ethanol extract from Aegle marmelos root bark involved pharmacognostic evaluation, phytochemical analysis, and pharmacological activity assessment. The pharmacognostic study revealed the macroscopic and microscopic features of the root bark, providing valuable information about its physical characteristics and internal structure. The physico-chemical standards determined important parameters such as moisture content, inorganic residue, and extractive values, which are crucial for assessing the quality and purity of the sample.The phytochemical screening of the ethanolic and aqueous extracts demonstrated the presence of various phytoconstituents, including carbohydrates, reducing sugars, alkaloids, glycosides, cardiac glycosides, flavonoids, triterpenoids, tannins, and steroids. These phytoconstituents contribute to the potential medicinal properties of Aegle marmelos root bark and suggest its suitability for further pharmaceutical and natural product research.The pharmacological activity assessment focused on the anticancer activity of the extract using the onion tip root assay and the potato disk assay. The results indicated significant inhibition of tumor growth, suggesting the potential of the extract as an anticancer agent. These findings provide preliminary evidence of the pharmacological activity of Aegle marmelos root bark and warrant further investigation to elucidate the underlying mechanisms and evaluate its therapeutic potential.Overall, this comprehensive study contributes to the understanding of Aegle marmelos root bark as a valuable medicinal plant, highlighting its pharmacognostic features, phytochemical composition, and pharmacological activities. Further research and exploration of its bioactive constituents and their mechanisms of action are essential for harnessing the therapeutic potential of this plant in drug development and natural medicine.

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