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Phytochemical profiling and biological evaluation of *Annona muricata* L. root extract: A comprehensive assessment

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Abstract

Annona muricata Linn., a component of the Annonaceae family, is a fruit-bearing tree endowed with a rich tradition of utilization. Recognized by various colloquial names such as soursop, graviola, and guanabana, *Annona muricata* is an evergreen botanical species predominantly found in tropical and subtropical zones globally. The current study was designed to evaluate the antioxidant and antibacterial properties while simultaneously elucidating the quantitative composition of phytochemical constituents within the root extract responsible for the pharmacological activities of the investigated subject. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used to assess antioxidant activities, while the well diffusion method evaluated antibacterial activity against common pathogens. Researchers have reported various bioactive compounds alkaloids, phenols, etc., that exhibit antibacterial, antifungal, antitubercular, anticancer, antioxidant, and other prophylactic activities. The chloroform extract displayed notable inhibitory efficacy against *E. coli* MTCC 443, indicating increased susceptibility with a minimum inhibitory concentration (MIC) of 0.3 mg/mL. The sample exhibited a comparatively elevated alkaloid content compared to its phenolic content. Bioactive phytoconstituents with diverse biological activities, justifying the rationale for their therapeutic use by local inhabitants.

Keywords

Annona muricata; antibacterial; antioxidant; DPPH method; MIC

Introduction

Flora assumes crucial roles in the human ecosystem, serving as the foundational cornerstone of traditional medicinal practices (1). In contrast to synthetic drugs employed in treating diverse infections, plants exhibit efficacy, safety, affordability, and minimal side effects (2). A substantial proportion of these plants possess the capability to synthesize various classes of secondary metabolites, constituting a primary factor underlying their therapeutic efficacy since ancient times (3). The numerous plant compounds were utilized as pharmaceutical agents in their natural and chemically modified forms (4). These secondary metabolites, characterized by structural diversity, manifest effectiveness against pathogens and environmental challenges (5). Moreover, these bioactive compounds demonstrate significant medicinal activities encompassing antibacterial, antioxidant, antifungal, anti-allergic, anti-inflammatory, antiparasitic, anticancer, and antihypertensive properties (6). These compounds have been used to treat mild to severe therapeutic conditions, including swelling, hyperglycemia, cancer, and peptic ulcers (7-9). It is reported that a quarter

of artificial drugs are derived from plants initially employed in traditional medicine (10). Moreover, an upward trend in using herbal medicines as complementary or alternative treatments was noted, with numerous medications experiencing significant benefits from natural products (11).

Graviola (*Annona muricata*) belongs to the family Annonaceae (12, 13) and is distributed across numerous tropics and subtropics regions worldwide, encompassing parts of the Americas, Asia, and Africa (14). This tropical, evergreen, and perennial flowering tree can reach heights of up to 8 meters, yielding consumable fruits (15). Various plant components are integral to traditional medicinal practices for treating diverse diseases and ailments. These applications include addressing inflammation (16), rheumatism (17), diabetes (12), hypertension (18) and parasitic infestation (19). The seeds are extracted and utilized for anthelmintic purposes, while the fruits have traditional applications in treating arthritis and fever. Both seeds and fruits are employed in the traditional management of parasitic infections. The leaves are a conventional remedy against crumples (20), hypoglycemia, and swelling and are used as an alleviation agent against cramps (12). Notably, the plant's leaves have acquired the moniker "the cancer killer" and are employed in traditional medicine for cancer treatment (21, 22). Widely recognized as a source of bioactive metabolites due to their diverse therapeutic properties (21).

Annona muricata is considered a promising candidate for integrative medicine. Phytochemical profiling revealed that extracts from the plant exhibit a high concentration of various secondary metabolite compounds, including alkaloids, saponins, terpenoids, flavonoids, coumarins, lactones, anthraquinones, tannins, cardiac glycosides, phenols, and phytosterols (23). These compounds, collectively called Annonaceous acetogenins (AGEs), have been demonstrated to induce cell cytotoxicity by inhibiting mitochondrial complex I (24). Other studies also suggest the presence of compounds such as megastigmanes (25), cyclopeptides, essential oils (26, 27), and essential minerals, including potassium (K), calcium (Ca), sodium (Na), copper (Cu), iron (Fe,) and magnesium (Mg) (28). Nevertheless, limited information is available about the bioactive compounds found in the chloroform extract of *Annona muricata* and its antioxidant and antibacterial properties. Considering the increased emphasis on exploring novel drugs derived from natural products through advanced technologies like high-throughput screening (29), this study aimed to investigate the antibacterial, antioxidant, and phytochemicals present in the chloroform extract of *Annona muricata* roots.

Materials and Methods

Sample collection and morphological identification

The root of the plant specimen (*Annona muricata*) was procured from Inchivila, Trivandrum District, and Kerala State. The herbarium specimen was prepared according to standard procedure (30). The plants were identified (31) and deposited in Jawaharlal Nehru Tropical Botanic Garden and Research Institute Herbarium (TBGT) with voucher number 45131.

Sample preparation

The plants were washed and chopped, and the roots were air-dried in shaded conditions for 4 weeks. Following the drying period, the plant samples were ground into a uniform powder using a grinder, serving as the substrate for subsequent investigations.

Solvent extraction for phytoconstituents

The chloroform extract of the sample was prepared by immersing 10 grams of dried powder in 150 mL of chloroform for 12 hours. Subsequently, the extract was filtered using Whatman filter paper (110mm) and utilized for subsequent investigations. Five grams of *Annona muricata* root powder were introduced into the Soxhlet apparatus for extraction. In the round-bottom flask attached to this setup, 100 mL of chloroform was added. The entire assembly was positioned on a heating mantle with a temperature of 65-80°C. As the chloroform vaporized, it ascended to the condenser, where it condensed back into a liquid and descended into the plant sample in the cone. Alkaloids, phenols were extracted and collected in the round-bottom flask (32).

Test organism

The bacterial isolates employed in this investigation were *Escherichia coli* MTCC 443, a Gram-negative bacterium, and *Bacillus subtilis* MTCC 441, a Gram-positive bacterium procured from the Institute of Microbial Technology in Chandigarh, India. For resuscitation, these microbial strains were introduced aseptically into nutrient broth and incubated at 37°C for 24 hours before conducting the antibacterial assay.

Preparation and standardization [calibration] of microbial inoculum

The bacterial strains were inoculated in a broth for 24 hours at the optimal temperature of 37°C. A suspension, equivalent to a cell density of 1 × 10^8 CFU/mL, was prepared following the McFarland standard for each isolate. Subsequent dilutions were carried out until the cell density reached 1 × 10^6 CFU/mL a confirmation achieved using a UV-visible spectrophotometer (Thermo Electron Corporation, USA) at an absorbance of 625 nm. Standardization was meticulously maintained throughout the experiment (33).

Well diffusion method

The susceptibility of bacteria to solvent extracts of *A. Muricata* root was assessed using the agar well diffusion method following the protocol (34). Muller Hinton Agar was sterilized at 15 psi for 15 minutes in an autoclave, and after cooling below 45°C, 20 µL of test microbes from 24-hour-old slant cultures were introduced sequentially. Prepared media were aseptically poured into sterilized petri plates and solidified under aseptic conditions. Using an autoclave cork borer, 6 mm-diameter agar wells were created. Subsequently, 20 µL of the extract solution, prepared with dimethyl sulfoxide, was added to the wells

using a micropipette. 20 µL of dimethyl sulfoxide (DMSO) was added to a well, which served as a negative control. Ciprofloxacin, an antibacterial agent, was included as a positive control at a concentration of 5 µg/mL. Petri dishes were incubated for 24 hours at 37°C. Inhibitory zones around the agar wells indicated antimicrobial activity, assessed through zone measurement or an antibacterial scale, following the methodology outlined (35).

Determination of MIC and MBC

The Minimum Inhibitory Concentration (MIC) is the minimum concentration of antibiotics that hinders observable bacterial growth. The microtiter plate dilution method assessed MIC values against diverse human pathogens. In this approach, overnight cultures of the test organisms were introduced into flat-bottomed sterile Tarson 96-well plates at a volume of 10 µL per well, followed by the addition of antibiotics at varying concentrations. The plates were subsequently incubated at 37°C for 24 hours to facilitate growth and observation. As established in prior studies, Sterile Mueller-Hinton (MH) broth was the control in this experimental setup (36, 37).

Following the determination of MIC for the chloroform extract, the colorimetric 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as outlined (38), was conducted. Specifically, 10 µL of MTT solution (5 mg/mL) was introduced into each well of a 96-well microplate, followed by a 4-hour incubation period at 37°C. Subsequently, acid isopropanol was administered to all wells to facilitate the dissolution of the dark blue crystals.

Determination of antioxidant activity-2,2-Diphenyl-1-picrylhyd-azyl (DPPH) radical scavenging method

A modified technique was employed to assess the scavenging potential of the extracts through the DPPH free radical scavenging assay (39). After dissolving 1.1829 g of DPPH in methanol and making the solution up to 30 ml, a 0.1 mM DPPH solution was created. For 30 minutes, the solution was incubated in the dark to finish the process. After mixing 22 µL of the DPPH solution with different concentrations of extracts (0.2, 0.4, 0.6, 0.8, and 1 mg/mL), the mixture was incubated for 30 minutes at room temperature. A spectrophotometric analysis of the resulting combination was conducted at 517 nm. Using the formula, the free radical scavenging activity was measured.

$$\% \text{ Inhibition} = (Ac - At) / Ac \times 100 \qquad \text{(Eqn.1)}$$

Where AC represents the absorbance of the test sample, and At represents the absorbance of the control. Ascorbic acid served as a reference in the assessment, utilizing extract sample concentrations as the standard (40). The Antioxidant activity of the sample was expressed by the IC50 value, denoting the concentration that inhibits DPPH radical generation by 50%, as defined (41, 42).

Quantification of total phenolics

The determination of Total Phenol content in methanolic extract was carried out spectrophotometrically using the Folin–Ciocalteu reagent with certain modifications (43). A methanolic solution of gallic acid (1 mL; 50, 100, 150, 200, and 250 µg/mL) was combined with 5 ml of tenfold diluted Folin-Ciocalteu reagent and sodium carbonate solution in distilled water (4 ml, 0.7 M) to formulate a calibration curve. The absorbance was measured at 765 nm using a UV -Vis spectrophotometer. The same reagents replaced 1 mL of gallic acid with 1 mL of plant extracts in three test tubes. After an hour, the absorbance was measured to determine the total phenolic contents. To quantify the absorbance, the test extract was excluded from the reagent blank, which consisted of the same reagents. Gallic acid equivalents (GAE), or mg of GAE/g sample, were used to express and determine the total concentration of phenolic compounds in the plant extracts. The formula was as follows:

$$T = (C \times V)/M \qquad \text{(Eqn.2)}$$

Where,

T = total content of phenolic compounds (mg of GAE/g sample)

C = the concentration of gallic acid derived from the calibration curve (mg/ml)

V = volume of extract (ml)

M = weight of the methanolic or ethyl acetate plant extract (gram)

Quantification of total alkaloid

The quantification of alkaloid content was conducted gravimetrically (44). In a succinct summary, 100mg of each sample (methanolic and ethanolic) was precisely weighed using an analytical balance and dispersed into 1 mL of a 10% acetic acid solution in ethanol. The mixture was thoroughly shaken and left undisturbed for about four hours before filtering. The filtrate was reduced to a quarter of its initial volume on a heated plate. Consistently adding concentrated ammonium hydroxide allowed the alkaloids to precipitate. After that, the precipitate was filtered through pre-weighed filter paper and cleaned with a 1% ammonium hydroxide solution. After the precipitate-containing filter paper was dried for 30 minutes at 60°C in the oven, it was placed in desiccators to cool, and it was then weighed again until a steady weight was reached and noted down. The total weight of the alkaloid was calculated as a percentage of the tested sample weight using the filter paper's weight difference. For every sample, the experimental protocol was repeated three times, and the results were reported as the mean of the three replicates.

Statistical Analysis

Every antioxidant analysis was carried out three times, including evaluations of the overall phenolic and alkaloid content. The average values and their standard deviations were then presented. The interrelationships among the

DPPH assay, total phenolic content (TPC), and total alkaloid content (TAC) were assessed through correlation analysis using the software MS Excel (version 13).

Results

Well diffusion method

Annona muricata root extracts dissolved in DMSO were tested in vitro against Gram-positive and Gram-negative bacterial species, such as *B. subtilis*, using the agar well diffusion. Fig. 1 depicts the significant inhibition zones observed against *B. subtilis* and *E. coli* bacterial strains when utilizing *Annona muricata* root extracts combined with various solvents, assessed on Muller Hinton Agar.

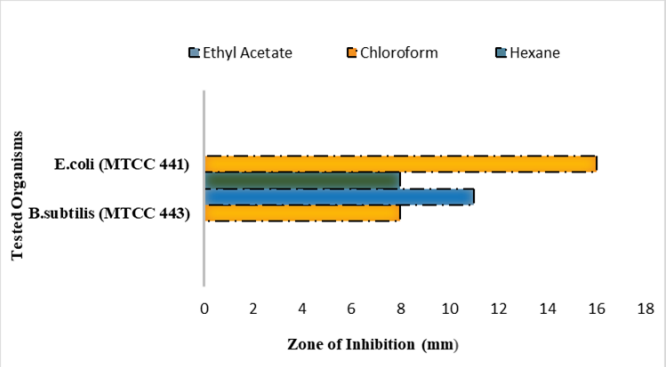

Fig. 1. Zone of inhibition of root extract against pathogenic organisms.

Antibacterial assay of root extract

Combining the root extract with five different solvents showed antibacterial action against the two types of bacteria. Specifically, the hexane root extract showed an 8mm inhibition zone against *E. coli*. The chloroform root extract displayed an 8mm inhibition zone against *B. subtilis* and a 16mm inhibition zone against *E. coli*. The ethyl acetate root extract also exhibited an 11 mm inhibition zone against *B. subtilis*. In contrast, the methanol and aqueous root extracts exhibited no antibacterial activity against these two bacterial strains.

Determination of MIC and MBC

The MIC of the chloroform root extract of *Annona muricata* against *E. coli* was determined as it exhibited the most significant zone of inhibition in the antibacterial analysis. The MIC was found to be 300µg/mL simultaneously; the MTT assay confirmed that this concentration also served as the Minimum Bactericidal Concentration (MBC) for *E. coli*. Table 1 represents the MIC and MBC of the chloroform extract against *E. coli*, as depicted in Fig. 2.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *E. coli*

MIC AND MBC		
Organism	MIC(µg/ml)	MBC(µg/ml)
E. coli	300	300

Determination of antioxidant activity

The DPPH free radical scavenging assay, a widely used technique for determining antioxidant capacity, was used to evaluate the extracts' antioxidant qualities. This assay assesses a compound's capacity to neutralize the extremely reactive and stable DPPH radical. The-scavenging activity was estimated spectrophotometrically at 517 nm, and the percentage of inhibition was calculated relative to a control sample. For comparison, ascorbic acid was used as a standard. The IC50 value, which denotes the sample concentration required to scavenge half of the DPPH radicals, was employed to assess the antioxidant potential of the extract. This assay offers insightful information about the beneficial properties of antioxidant extracts and their ability to reduce oxidative stress.

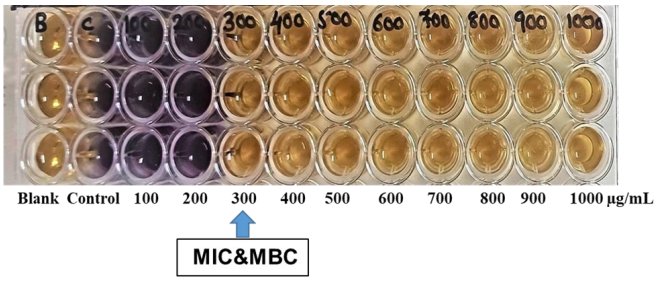

Fig. 2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of root extract against *E. coli.*

DPPH free radical scavenging assay

Analyzing the antioxidant activity of samples is commonly accomplished using the DPPH test. Absorbing UV-Vis light at 517 nm is DPPH, a stable free radical. A sample's absorbance decreases when an antioxidant is present because it lowers the DPPH radical. The extent of inhibition indicates the sample's antioxidant capacity, with the IC50 value representing the concentration required for 50% inhibition. The percentage inhibition of DPPH by methanolic extracts of *Annona muricata* root is shown in Table 2, along with the percentage inhibition of ascorbic acid, a common antioxidant, using the DPPH assay method. This information provides information about the extracts' scavenging activity and capacity to inhibit DPPH radicals. These findings highlight the proton-donating capacity of the methanolic root extracts and their substantial inhibition of DPPH radicals. The antioxidant capacity of the extracts is demonstrated clearly in Fig. 3, which compares their scavenging activity to that of normal ascorbic acid.

Table 2. Percentage inhibition and Half-maximal inhibitory concentration (IC50) value of ascorbic acid, a standard antioxidant using the DPPH assay method

Samples	Concentration					1C50 Value
	0.2	0.4	0.6	0.8	1	
Ascorbic Acid (Standard)	48.40	59.64	68.14	80.75	95.60	0.24µg/mL
Root Extract *(Annona muricata)*	31.44	44.44	55.46	66.89	80.99	0.56µg/mL

Quantification of total phenolic content (TPC)

The total phenolic content of *Annona muricata* root was evaluated and compared with that of the standard (gallic acid). The Fig. 4 indicates that the phenolic content in the root is 38%.

Quantification of total alkaloid content (TAC)

The TAC of the *Annona muricata* root sample was

quantified as 62% and is presented graphically in Fig. 4.

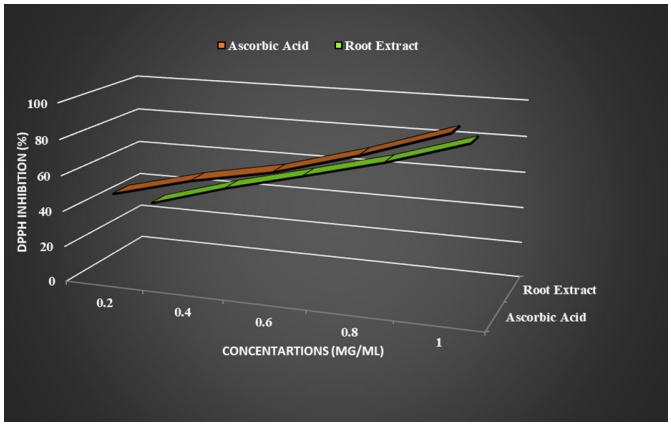

Fig. 3. DPPH radical scavenging activity (%) (Mean + Standard deviation) of *Annona muricata* root extract.

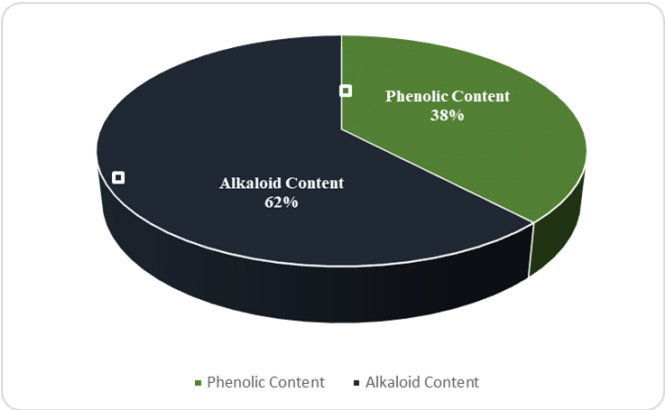

Fig. 4. Total phenolic and alkaloid contents of *Annona muricata* root extract.

Discussion

The increasing demand for plant-derived goods for medicinal and nutraceutical applications worldwide has sparked research endeavors to clarify the chemical components found in plants and their varied pharmacological characteristics. Concurrently, there is a growing imperative among researchers to explore safer antioxidants sourced from natural reservoirs as alternatives to synthetic counterparts like butylated Hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate, and tert-butylhydroquinone, known for their carcinogenic potential (45). Therefore, eating naturally occurring foods, such as fruits and vegetables with potent antioxidant properties, is crucial for preventing heart disease and many types of cancer (46). The antimicrobial analysis revealed the presence of potential novel compounds within the root, suggesting they could be isolated for therapeutic applications. Numerous scientific reports have demonstrated the significant therapeutic potential of various compounds found in the root. The pharmacological effects of plants are mostly linked to flavonoids, alkaloids, and phenolic compounds, as illustrated by the fact that the pharmacological activities of major and minor bioactive chemicals originating from plants are rarely reported (47). The antioxidant activity of *Annona muricata*, as indicated by its IC50 value of 0.56 mg/ml, was corroborated by a previous study, which demonstrated that Annona muricata root extract exhibits stronger antioxidant activity than other parts of the same plant (48).

Furthermore, as the root extract in this study is 65.5%, it can be deduced that alkaloid chemicals were responsible for suppressing the test organisms. The extract's MIC against *E. coli* MTCC 443 (0.3 mg/mL) indicated stronger antimicrobial activity against gram-negative bacteria. This finding aligns with research that demonstrated the capability of *Annona muricata* root extract to suppress the tested organism's growth (*E.coli*) (49). This suggests that the extract could be a potent therapeutic agent for infections such as dysentery, diarrhea, and food poisoning, where the test organism (*E. coli*) is commonly implicated (50).

Conclusion

The present study elucidated the antibacterial, antioxidant, and phytochemical profiles of *Annona muricata* root extracts. Evaluation of various solvents revealed distinctive activities, with chloroform extracts displaying notable antibacterial efficacy and methanolic extracts demonstrating pronounced antioxidant activity. Moreover, the analysis revealed a higher percentage of alkaloids than phenols in the root extracts. The study aimed to determine whether *Annona muricata* root extracts could be antibacterial agents to treat digestive disorders. Through this study revealed that the percentage alkaloid concentration was higher than that of phenols. The findings emphasize A's robust antibacterial and antioxidant properties. Further exploration into *Annona muricata*, particularly its prophylactic potential, may unveil additional therapeutic mechanisms and beneficial properties warranting continued scientific inquiry.

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Authors' Contributions

KRBR was responsible for conceptualization, writing, reviewing, and editing, while GMMG handled materials and methodology, field exploration, data curation and analysis, and original draft writing; both authors have verified and approved the final version of the manuscript.

Compliance with Ethical Standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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