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Assessment of phytochemical, antioxidant, and antibacterial properties of *Xylocarpus granatum* leaves

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Abstract
Xylocarpus granatum Koen is traditionally used to treat fever, cholera, colic, diarrhoea and other abdominal affections. The present study aims to phytochemicl screenings and assessment of potential antioxidant and antimicrobial activities of *X. granatum* leaves. The DPPH free radical scavenging assay was used to quantify antioxidant activity and total content of phenolic, flavonoids, tannin was also determined. Besides, disc diffusion assay was used to evaluate antibacterial effect. Phytochemical analysis of this extract showed the existence of glycosides, tannins, protein, saponin, steroids & flavonoids. Total phenolic, flavonoid and tannin content for extract were 195.9 mg gallic acid, 555.57 mg quercetin and 122.0 mg gallic acid equivalent per gram of dry extract, respectively. In the antibacterial activity test the extract showed mild activity against *Vibrio cholerae, E. coli, Staphylococcus aureus* and *Streptococcus pyogenes* at the dose of 250 µg/disc and 500 µg/disc in comparison with Kanamycin (30 µg/disc) but was resistant to *Shigella dysenteriae* or *Shigella flexneri.*

Keywords: Antioxidant, Total phenolic content, TLC, Antibacterial activity, *Xylocarpus granatum*

Introduction
The Sundarbans, the world's biggest deltaic swamp mangrove forest, were formed around 7000 years ago when sediments from the Himalayan foothills were deposited through the Ganges river system. It is located southwest of Bangladesh and south of West Bengal, India. Of the total 10,000 square km area, the recorded total area of Bangladeshi Sundarban mangrove forest covers about 6017 square km. A total of 70 species from 34 families have been identified in the Sundarbans. Sixty five (65) species from 37 families have been documented from the Bangladesh part of the Sundarbans. Strong winds, muddy anaerobic soil, high salinity, high and low water tides, high temperatures, high humidity, and an abundance of live insects and microbes are just a few of the extreme environments in which sundarban plants thrive. To survive in such harsh environments, Because of their highly evolved morphological and physiological adaptations, they are able to produce unique chemical compounds that protect them from these toxic substances [1]. A number of these compounds have unique bioactivities. Plant-derived compounds have long been recognized for their diverse pharmacological properties, including antioxidant and antimicrobial activities [2]. Among the vast array of plant species, *Xylocarpus granatum*, commonly known as the Crabapple Mangrove or Cannonball Mangrove, is a prominent member of the mangrove ecosystem, particularly found in regions like the Sundarbans of Mongla range, Khulna, Bangladesh. This plant has drawn attention due to its potential therapeutic benefits attributed to various phytochemical constituents [1]. Traditionally different parts of the plant are used for different diseases. Fever, cholera, colic, diarrhea, and other stomach ailments are all treated with the bark [3]. The fruits are also applied topically to reduce inflammation and prevent diarrhea. An Indian patent application explains how to make an antidiarrheal medication using X. granatum fruit seed coatings. The bitter and astringent oily fluid from the seeds is used as an illuminant, hair oil, and a remedy for diarrhea and dysentery. The seed kernels are used in tonics. Furthermore, different parts of the plant contain different phytocompounds. Tannin, xylomollin, a secoiridoid hemiacetal acetal, and the limonoids methyl angolensate and xyloccensis A–F are found in unripe fruits. In addition, limonoids, methyl angolensate, xylocarpin, xyloccensis A-F, 7-α-O-Ac-dihydronomilin, mexicanolide, and fatty oil are found

in seeds. The seeds have yielded a variety of compounds, including long chain fatty acids, alcohols, steroids, and xyloccensin K, a lignoid [4]. Gedunin, an antifungal chemical, is found in wood. The root bark contains alkaloids, including N-Me-flindersine compound, which has anti-micorbial and insect antifeedant properties [5]. Among the compounds found in bark are triacontanol, methyl-3β-acetoxy-1-oxomeleacate, β-sitosterol, and methyl-3β-isopropyl-1-oxomeliacate. From leaves, two unidentified tetranortriterpenoids and frieselin have been extracted [6].

The exploration of medicinal plants, particularly those from diverse ecosystems like mangrove forests, presents an invaluable avenue for discovering new therapeutic agents [7, 8]. *Xylocarpus granatum* Koen., a member of the mangrove ecosystem found in the Sunderbans, holds promise for pharmacological investigation due to its rich biodiversity and potential medicinal properties [9, 10]. Moreover, to the best of our knowledge, there are a few reports on anti-oxidants and anti-microbial activities from leaf extract of *X. granatum*. Therefore, the present study aims to elucidate the bioactive compounds through phytochemicl screenings and evaluation of potential antioxidant and antimicrobial activities of *X. granatum* leaves.

Materials and Methods

Plant Collection and Extract preparation

The leaves of *X. granatum* were collected from the Sundarbans of Mongla range, Khulna, Bangladesh. The fresh leaves were collected from the trees from the bank of river. Adulteration of any kind was outlawed during collection & plant was identified by renowned botanist at Mongla range. The plant part was subjected to shade drying. The leaves were dried, then pounded into a coarse powder and kept in an airtight receptacle. Next, a cold extraction method was employed, where 250 grams of the powdered leaves were soaked in 1000 mL of ethanol for fifteen days with regular shaking. After filtration to remove plant debris, the residue underwent a second round of soaking in 250 mL of ethanol for three days before another filtration step. The ethanol extract obtained was then concentrated using a rotary evaporator, followed by further evaporation under normal environment until a deep red paste-type concentrate, designated as crude ethanolic extract of leaves, was obtained. The yield of the dried extract of *X. granatum* was 21.1% (w/w). Then the extract was stored at -4 ^{0}C until further analysis.

Phytochemical Test

The kingdom of plants contains a vast reservoir of chemically structured physiologically active molecules as well as phytochemicals that are protective or disease-preventive. These phytochemicals include the alkaloids, steroids, flavonoids, terpenoids, tannins, and many more; they are frequently secondary metabolites found in reduced amounts in higher plants. The initial phytochemical investigations are represented by the testing of several chemical groups found in the extract. Unless specified differently in each test, a 5% (w/v) extract in ethanol solution was used in each assay. The tests for reducing sugar, tannins, flavonoids, saponins, gums and polysaccharides, steroids, alkaloids, glycosides, and proteins are known as chemical group tests [11].

In-vitro Qualitative Antioxidant Test

Thin Layer Chromatography (TLC) is a quick and simple method for separating organic compounds. It's often used to monitor reactions and check purity. A sample is spotted onto a plate coated with a solid stationary phase, then a liquid mobile phase moves across the plate via capillary action, separating compounds. TLC is used to detect polar, non-polar, and medium polar groups in plant extracts under UV light. The presence of antioxidants is indicated by the color change of 0.02% DPPH sprayed onto the chromatogram [12].

In-vitro Quantitative Antioxidant Test

The extract was found to have a quantitatively significant radical scavenging action when tested against the free radical 2, 2-diphenyl-1-picryl hydrazyl (DPPH) (24). Initially, a stock solution of the samples (1024 µg/mL) was made. Various concentrations of the material (512–1 µg/mL) were made from that solution. 3 mL of an alcoholic DPPH solution containing 0.1 mM was added to 1 mL of each concentration. After being incubated at room temperature for 30 minutes in the dark, the absorbance at 517 nm was measured. The standard was ascorbic acid. Percent radical scavenging activity = [(A0−A)/A0] x 100 was used to determine the percentage of DPPH free radical scavenging activity in each extract and standard.

Here, A is the absorbance of the DPPH solution including plant extract or standard, and A0 is the absorbance of the control solution with all reagents except plant extract. Finally, the plot of inhibition (%) against extract concentration allowed for the calculation of the sample concentration needed to scavenge 50% of the DPPH free radical (IC50) [13].

Determination of total phenolic content

Using a modified Folin-Ciocalteu technique, the extracts' total phenolic content was ascertained [14]. 5 mL of 10% (v/v) Folin-Ciocalteu reagent (FCR) was combined with 1 mg/mL of the extract. The mixture was then mixed with 4 mL of sodium carbonate (75 g/L). 30 minutes were spent with it at 40 °C. At 765 nm, the reaction mixture's absorbance was measured. The standard calibration curve, from which the total phenol content was calculated and represented as mg gallic acid equivalent per gram of dry extract was prepared using various doses (0.1–0.5 mg/mL) of gallic acid.

Total flavonoids content

The aluminum chloride colorimetric assay was used to quantify the total flavonoid content [15]. 0.3 mL of 5% w/v $NaNO_2$ and 4 mL of distilled water were added to 1 ml of the extract solution (1 mg/mL). After adding 0.3 mL of 10% w/v AlCl3 and 2 mL of 1M NaOH, the volume was increased to 10 mL after five minutes. After that, it was left at room temperature for 15 minutes, and the absorbance at 510 nm was determined. The technique employed quercetin (0.25-1 mg/mL) to generate a standard calibration curve, and the extract's total flavonoid concentration was quantified as milligrams of quercetin equivalent (QE) per gram of dried extract.

Total tannin content

The Folin Ciocalteu technique was used to determine the extracts' tannin content [16]. 1 mL of 35% (w/v) Na2CO3 solution was added, and it was diluted to 10 mL with distilled water, 0.5 mL of FCR, and 0.1 ml of the extract solution. After giving the mixture a good shake, it was left at room temperature for half an hour. To generate the standard calibration curve, a set of reference standard solutions containing gallic acid (20-100 µg/mL) was created. At 725 nm, the absorbance of the test and standard solutions was

measured in relation to a suitable blank. The tannin concentration was given as milligrams of GAE per gram of dry extract.

Antibacterial Activity

Using the disc diffusion method, the extract's antibacterial activity was evaluated [17]. This method prepared solutions with the necessary concentration (250 and 500µg/disc) by dissolving a determined amount of the test samples in specific volumes of solvent. Using a micropipette, a known quantity of test chemicals were deposited onto the sterile Matricel (BBL, Cocksville, USA) filter paper discs, which were then dried. After that, the test organisms were seeded via a sterile transfer loop onto an appropriate agar medium in petridishes containing the sample, positive control, and control disk. After that, the plates were maintained at 40 °C to allow for optimal diffusion, and they were then placed in an incubator for 12 to 18 hours at 37 °C to promote the growth of the bacteria. The development of microorganisms will be inhibited if the test substance has any antimicrobial activity, producing a distinct and well-defined zone known as the "zone of inhibition." By measuring the zone of inhibition's diameter in millimeters and comparing it to the reference antibiotic, the test agent's antibacterial activity is ascertained.

Results

Phytochemical Test

Some of the pharmacologically active phytochemicals were found in the extract during the phytochemical screening. Reducing sugar, gums, and alkaloids were absent from the phytochemical examination, but tannins, flavonoids, glycoside, steroids, saponins, and proteins were detected (Table 01).

Table 1: Preliminary phytochemical composition of ethanol leaf extract

Serial Number	Test groups	Results
1	Reducing sugar	-
2	Tannins	+
3	Flavonoids	+
4	Saponin	+
5	Gums	-
6	Steroids	+
7	Alkaloids	-
8	Glycoside	+
9	Proteins	+

Here, (+) indicates presence and (-) indicates absence

Qualitative Antioxidant Test

Upon examination under UV detectors at both short (254 nm) and long (360 nm) wavelengths, numerous coloured and fluorescent positive components were detected. Notably, shorter wavelengths revealed a plethora of coloured components, while longer wavelengths unveiled fluorescent ones. Subsequent application of DPPH on the TLC plate resulted in a yellow color against a purple background, indicative of the presence of antioxidant compounds within the *X. granatum* extract (**Figure 1, 2**). In the qualitative antioxidant assay conducted via TLC, the *X. granatum* extract demonstrated robust free radical scavenging properties, as evidenced by a prominent yellow spot against the purple background of the TLC plate. This encouraging outcome prompted further investigation into the quantitative antioxidant activity of the extract.

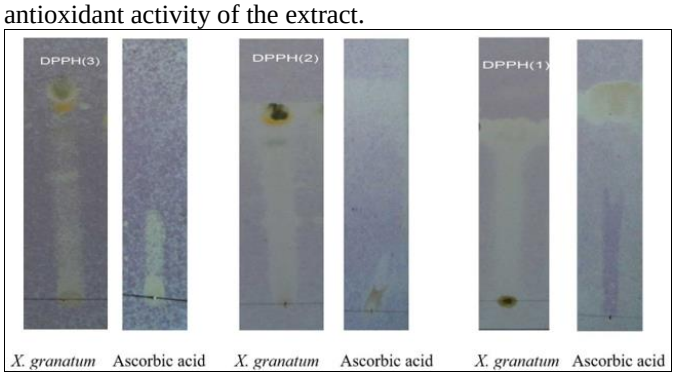

Fig 1: TLC plate for *X. granatum* leaves extract after applying DPPH

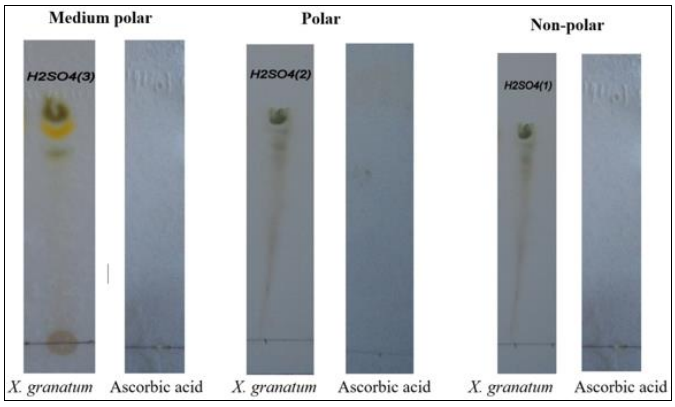

Fig 2: TLC plate for *X. granatum* leaves extract after applying 10% H_2SO_4.

Quantitative Antioxidant Test

Antioxidant activity increased progressively with increasing extract concentration in the DPPH radical scavenging experiment. In the quantitative assay, *X. granatum* leaves displayed a DPPH free radical scavenging activity (IC_{50} = 73.99 µg/mL) which is comparable to that of L-ascorbic acid (IC_{50} = 10.69 µg/mL), a well-known standard antioxidant.

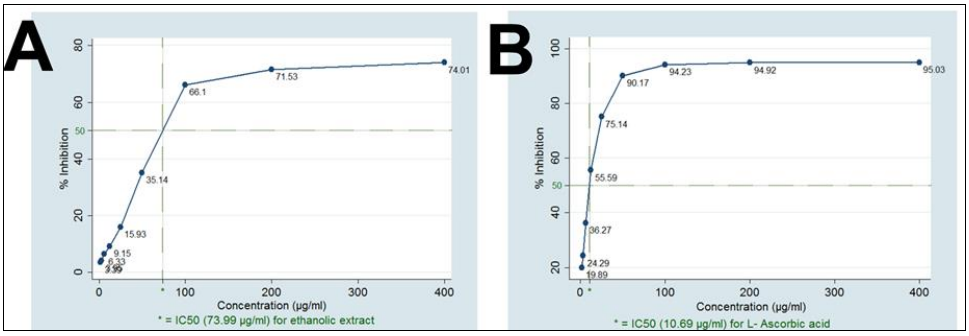

Fig 3: (A) *X. granatum* leaf DPPH scavenging experiment (% inhibition vs. conc.). (B) DPPH scavenging assay of L- Ascorbic acid (% inhibition vs Conc.).

Determination of total phenolic content

The total phenolic content of *X. granatum* leaves extract revealed to be 195.9 mg GAE/g of dried plant extract. Standard gallic acid calibration curve was used to determine phenol content in where y axis indicates absorbance and x is the concentration. The phenolic content was found in the ethanol leaf extract is 195.9 mg GAE/g dried extract.

Determination of total Tannin content

Total tannin content in *X. granatum* was estimated to be 122.0 mg gallic acid equivalents per gram of the dried plant extract. The standard curve was prepared using 100, 80, 60, 40, and 20 µg/mL solutions of gallic Acid in water, in where absorbance was plotted on the Y-axis and the concentration of gallic acid on the X-axis. Total tannin content was found in the ethanol leaf extract is 122.0 mg GAE/g *X. granatum* dried pant extract.

Determination of Total Flavonoid content

Total flavonoid content in *X. granatum* was estimated to be 555.57 mg QE/g extract. The standard curve was prepared using 300, 250, 200, 150, 100 and 50 µg/mL solutions of Quercetin in water, in where absorbance was plotted on the Y-axis and the concentration on the X-axis. Total flavonoid content was found to be 555.57 mg GAE/g experimental extract.

Determination of zone of inhibition

The antibacterial activity of the extract was tested against both Gram-negative bacteria (*Escherichia coli*, *Shigella dysenteriae*, *Vibrio cholerae*) and Gram-positive bacteria (*Staphylococcus aureus*, *Shigella flexneri*, *Streptococcus pyogenes*). The results of the disc diffusion assay, which measures the diameter of inhibition zones (mm), are summarized in the table below (Table 02).

Table 2: Antibacterial activity of crude extract expressed the result in zone of inhibition in mm

Bacterial Strains	Zone of Inhibition in mm			
	Blank	Kanamycin (30 µg/disc)	Experimental Extract	
			(250 µg/disc)	(500 µg/disc)
E. coli	0	28.80	11.50	12.50
Shigella dysenteriae	0	19.50	0	0
Vibrio cholerae	0	25.50	7.00	10.50
Staphylococcus aureus	0	31.00	6.50	7.50
Shigella flexneri	0	21.00	0	0
Streptococcus pyogenes	0	22.00	7.60	14.00

The results showed that the extract had varying degrees of antibacterial activity against the tested bacterial strains. The positive control, kanamycin (30 µg/disc), demonstrated strong antibacterial activity against all bacterial strains, forming large inhibition zones.

Among the Gram-negative bacteria, *E. coli* showed moderate susceptibility to the extract, with inhibition zones ranging from 11.50 mm to 12.50 mm at concentrations of 250 µg/disc and 500 µg/disc, respectively. *Vibrio cholerae* exhibited a similar pattern of susceptibility, with inhibition zones ranging from 7.00 mm to 10.50 mm. In contrast, the extract did not inhibit the growth of *Shigella dysenteriae* or *Shigella flexneri*, even at the highest concentration tested (500 µg/disc).

Among the Gram-positive bacteria, *Staphylococcus aureus* showed moderate susceptibility to the extract, with inhibition zones ranging from 6.50 mm to 7.50 mm. *Streptococcus pyogenes*, on the other hand, demonstrated a higher degree of susceptibility, with inhibition zones ranging from 7.60 mm to 14.00 mm.

Discussions

Since ancient times, native communities in Bangladesh have used a variety of native medicinal plants as their main source of healthcare. These plants contain a complex mixture of chemical compounds known to play important role in several biological activities. The preliminary phytochemical screening of *X. granatum* leaves revealed the presence of several secondary metabolites, including flavonoids, tannins, steroids, glycosides, saponins, and proteins [36]. These phytochemicals are known for their diverse biological activities, such as antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. Flavonoids, in particular, are potent antioxidants that scavenge free radicals and protect against oxidative stress-related diseases [18]. Tannins possess antimicrobial properties and contribute to the astringent taste of plants, while steroids exhibit anti-inflammatory and immunomodulatory effects [19]. Glycosides and saponins have demonstrated antimicrobial and cytotoxic activities, making them potential candidates for drug development [20]. The presence of these phytoconstituents underscores the medicinal significance of *X. granatum* as a valuable source of bioactive compounds.

The qualitative and quantitative antioxidant assays confirmed the significant antioxidant potential of *X. granatum* leaves. Thin-layer chromatography revealed the presence of antioxidant compounds, which were evidenced by the yellow spot against a purple background upon DPPH staining [21]. This observation aligns with previous studies demonstrating the antioxidant activity of plant extracts through TLC-based assays. Moreover, the quantitative DPPH scavenging assay demonstrated a notable IC_{50} value of 73.99 µg/mL, which is comparable to that of standard (IC_{50} = 10.69 µg/mL). The high DPPH scavenging activity of *X. granatum* extract can be attributed to its rich phenolic and flavonoid content. Phenolic compounds are known for their potent antioxidant properties. The determination of total phenolic, tannin, and flavonoid contents further supported the antioxidant potential of the extract, with substantial quantities detected [22]. These findings are also consistent with previous studies highlighting the correlation between phenolic content and radical scavenging activity of plant extracts.

The antibacterial activity of *X. granatum* extract was evaluated against both Gram-negative (*Escherichia coli*, *Shigella dysenteriae*, *Vibrio cholerae*) and Gram-positive (*Staphylococcus aureus*, *Shigella flexneri*, *Streptococcus pyogenes*) bacterial strains [23]. The extract exhibited mild antimicrobial activity against selected bacterial strains, with varying degrees of susceptibility observed. Gram-negative bacteria *E. coli* and *V. cholerae* showed moderate susceptibility to the extract, while Gram-positive bacteria *S. aureus* and *S. pyogenes* demonstrated a higher degree of susceptibility. These findings corroborate previous reports on

the antimicrobial activity of *X. granatum* extracts against pathogenic bacteria. The antimicrobial efficacy of plant extracts is attributed to their complex chemical composition, including phenolic compounds, flavonoids, and other bioactive constituents that disrupt microbial cell membranes and inhibit vital enzymatic processes [24].

The results of this study underscore the therapeutic potential of *X. granatum* leaves as antioxidants and antimicrobial agents [25]. The significant antioxidant activity of the extract suggests its potential application in combating oxidative stress-related diseases, including cardiovascular disorders, cancer, and neurodegenerative diseases [26, 27]. Additionally, the moderate antibacterial activity against pathogenic bacteria highlights the prospect of *X. granatum* as a natural alternative for the treatment of bacterial infections. It is necessary to conduct more study to clarify the underlying mechanisms of action and to explore the synergistic effects of phytochemical constituents present in *X. granatum* extract. Additionally, *in vivo* studies are necessary to validate the therapeutic efficacy and safety profile of the extract before clinical translation. Overall, this study support the medicinal value of *Xylocarpus granatum* and underscores the importance of harnessing natural resources for drug discovery and development.

Conclusion

The study explores the phytochemical composition and pharmacological activities of *Xylocarpus granatum* leaves, revealing its rich chemical diversity. The extract showed significant antioxidant activity and was found to have antibacterial properties against pathogenic bacterial strains. The extract showed mild antimicrobial activity against few bacteria, but no inhibition against *Shigella dysenteriae or Shigella flexneri*. These findings highlight the extract's potential as a natural alternative for combating bacterial infections. The study contributes to the growing literature on the pharmacological properties of mangrove plants, emphasizing the importance of exploring natural resources for drug discovery and development. The bioactive compounds identified warrant further investigation to elucidate their mechanisms of action and potential therapeutic applications in medicine and allied fields. The findings underscore the medicinal potential of *Xylocarpus granatum* leaves and the importance of preserving mangrove ecosystems for their biodiversity and pharmacological significance. Future research may involve isolation and characterization of bioactive compounds, elucidation of their pharmacological mechanisms, and clinical studies to evaluate their efficacy and safety profiles.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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