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PHYTOCHEMICAL SCREENING, PLANT GROWTH INHIBITION, AND ANTIMICROBIAL ACTIVITY STUDIES OF *XYLOCARPUS GRANATUM*

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Phytochemical analysis of the methanolic extract of Xylocarpus granatum Koen indicated the presence of carbohydrates, saponins, tannins and flavonoid types of compounds. Alkaloids and glycosides were found to be absent from the extract. The primary methanolic extract exhibited a potent growth inhibitory effect. Inhibition of both the rootlet and shoot showed a dose-dependent response. The residual methanolic extract also has a growth inhibitory effect. Both methanolic extracts have a greater inhibitory effect on rootlet growth than shoot growth. The residual methanolic extract has a lesser inhibitory effect than the primary methanolic extract. Removal of the non-polar compounds (by n-hexane and chloroform) from the primary methanolic extract reduced the inhibitory activity on both the rootlet and shoot growth, which suggests that the non-polar fractions may contain growth inhibitory principles. The primary extract demonstrated antibacterial activity against the gram positive bacteria Staphylococcus aureus and Bacillus subtilis and the gram negative bacteria Proteus vulgaris. The primary methanolic extract was found to be inactive against Escherichia coli and Pseudomonas aeruginosa. The primary methanolic extract was more active against gram-positive bacteria than gram-negative bacteria. The residual methanolic extract was also found to be inactive against all the tested microorganisms.

Keywords: *Xylocarpus granatum, Antimicrobial activities, Methanolic extract*

INTRODUCTION

Disease is as old as life itself, and man has always been in search of agents to cure diseases. Medicinal plants and herbs have been used for the eradication of disease and human suffering since antiquity. Plants that possess therapeutic properties or exert beneficial pharmacological effects on an organism are generally known as "medicinal plants". Many indigenous medicinal plants are being discovered everyday. Medicinal plants used in traditional medicine should be collected at the right time, the right season, and the right stage of their growth so that the constituents can be optimally harvested.

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Xylocarpus granatum Koen (Family: Meliaceae) usually grows in the coastal forests of Bengal, Andaman, Burma, the Malay Peninsula, and Africa. In Bangladesh, this plant is found in low lying, swampy locality in the Sundarbans mangrove forests (Bandyopadhyay 1986). Mangroves are salt-tolerant forest ecosystems of tropical and subtropical intertidal regions of the world (Hamilton and Snedarker 1984). The Sundarbans cover 6017 sq. km in the southwest corner of Bangladesh (Brown 1997). It is the largest continuous mangrove forest in the world (Chaudhuri and Naithani 1985; Chaffey and Sandom 1985; Anon 1989).

A large number of research works have been previously done on *Xylocarpus granatum*. The seed of the plant contains xylocarpin (Okorie and Taylor 1970). An alkaloid named N-methylflindersine was identified as a component of root bark; several other alkaloids were also identified (Chou *et al.* 1977). Literature on *Xylocarpus granatum* also showed the presence of inorganic compounds Na^{+}, K^{+}, Ca^{++}, Cl^{-}, and Mg^{++} in the leaves (Shinoda, Ogisu and Tajima 1984). Four novel tetranortriterpenoids, xylogranatins A-D (1-4) with an unusual 9, 10-seco skeleton, were isolated from the seeds of a Chinese marine mangrove *Xylocarpus granatum.* Their structures were determined by spectroscopic and chemical means (Yin *et al.* 2006). Phytochemical screening of the plant helps to reveal the existence of carbohydrates, alkaloids, saponins, tannins, glycosides, and other components in the plant.

The plant belongs to the Family Meliaceae. Meliaceae contains various types of compounds, especially pentacyclic triterpenoid and limonoids (Shinoda, Ogisu and Iwata 1985). A considerable number of very different functions have been ascribed to plant terpenoids. Their growth regulating properties are very well documented. Two of the major classes of growth regulators are the sesquiterpenoid and diterpenoid base gibbrerellins. Based on these activities of the plant, the wheat rootlet and shoot growth inhibition bioassay can be performed to observe the effect of the methanolic extract on mitotic cell division of the root and shoot of wheat grain.

In addition to this, a large number of human, animal, and plant diseases are caused by pathogenic microbes. Infection by microbes has been a major cause of death in higher organisms. The discovery of the antibiotic penicillin is therefore considered to be one of the most important discoveries in the world. Historically, many of the new antibiotics were isolated from natural sources. Many more were later synthesised and introduced in clinical practices. The discovery and

development of new antimicrobial agents is an ongoing process. The remarkable diversity of chemicals present in biological samples has tremendous potential in the search for new antimicrobial agents (Rahman, Choudhary and Thomson 2001).

METHODS

Preparation of the Extract

Plant materials:
Xylocarpus granatum was collected from the Sunderbans of Bangladesh. The plant was identified by the Bangladesh National Herbarium, Dhaka, and a voucher specimen was deposited at the Department of Pharmacy. The stem barks were collected from the fresh tree. It was then dried in an oven tray for 2 days. The temperature was strictly maintained between 40°C to 45°C to ensure that the active constituents would not decompose. After drying, the plant part was ground into fine powder, which was almost red in colour.

Preparation of the extract:
The plant was extracted by the cold extraction method. 400 g of powder was soaked in 1300 mL of 80% methanolic in a glass container for 6 days. The extract was concentrated by evaporation and dried to a solid in an oven. Finally, reddish colour type of primary methanolic extract (PME) was found. Next, 90 g of primary methanolic extract was dissolved in 250 mL of 80% methanolic and was successively partitioned with n-hexane (3 x 100 mL, three times each with 100 mL) and chloroform (3 x 100 mL, three times each with 100 mL). The n-hexane and chloroform extract was obtained by evaporation and drying. The remaining methanolic extract was also concentrated by evaporation and finally freeze-dried.

Phytochemical Tests

Tests for carbohydrates:
Benedict's test (test for reducing sugar) and Fehling's test (standard test for reducing sugar) were performed to confirm the presence of carbohydrates (Harbon 1973; Ghani 1998).

Tests for alkaloids:
Mayer's reagent and Dragendorff's reagent were applied to 0.5 g of the extract (Harbon 1973; Ghani 1998).

Tests for saponins:
Frothing test was performed (Harbon 1973; Ghani 1998).

Tests for glycosides:
A small amount of alcoholic extract was dissolved in 1 mL of water and a few drops of aqueous sodium hydroxide solution were added. A yellow colour was taken to signify the presence of glycosides (Harbon 1973; Ghani 1998).

Test for tannins:
About 0.5 g of extract was dissolved in 5 to 10 mL of distilled water and filtered. A few drops of a 5% ferric chloride solution were added to the filtrate. A blue, blue-black, green, or blue-green colour or a precipitate was taken as an indication of the presence of tannins (Harbon 1973; Ghani 1998).

Tests for flavonoids:
A few drops of concentrated hydrochloric acid were added to a small amount of an alcoholic extract of the plant material. Immediate development of a red colour was taken as an indication of the presence of flavonoids (Harbon 1973; Ghani 1998).

Wheat Rootlet and Shoot Growth Inhibition Bioassay

Five wheat grains *(Triticum awstivum)* were selected at random and placed on filter paper in a Petri dish containing 5 mL of the primary methanolic extract and residual methanolic extract solutions of different concentrations (250, 500, 1000 and 1500 µg/mL). The Petri dishes were incubated for 5 days under normal conditions in a dark place. The experiments were performed in duplicate. The average rootlet and shoot length of the ten seeds for each concentration was used in the percentage of growth inhibition calculation. A log concentration vs. percent inhibition graph was drawn, from which the IC_{50} (Table 1) value was determined. The IC_{50} values were calculated from the straight line drawn from different log concentration vs. percent inhibition graphs (Mongelli *et al.* 1995).

Control group:
The longest rootlet length and shoot length of each seed was measured and the inhibition was calculated as a percentage relative to the length of the rootlets and shoots from the controls with tap water.

Table 1: Calculation of the IC_{50} Value.

General formula of a straight line: Y = mX + C So, X = (Y-c)/m in these equations Y = % inhibition, X = log conc, thus concentration = antilog X, C = intercept	
Y = 9.802X + 70.23	Concentration = antilog X = 0.009 µg/mL
Y = 13.14X + 59.18	Concentration = antilog X = 0.20 µg/mL
Y = 81.16X - 181.3	Concentration = antilog X = 707.83 µg/mL
Y = 39.50X - 74.43	Concentration = antilog X = 1412.94 µg/mL

Antimicrobial Activity Test

The antimicrobial activity test was performed at the Gono Shasthaya Vaccine Research and Diagnostic Laboratory using the standard agar dilution method. The assay medium was a Mueller-Hinton agar medium. Sterile medium was poured into a sterile Petri dish under aseptic conditions at room temperature. Before being used, the medium was incubated at 37°C for 48 h to check whether there was any infection in the medium. A culture of the test organism was uniformly streaked over the medium using an inoculating loop under aseptic conditions (Barry 1980; Berghe and Vlietnck 1997). The concentrations of the extracts used in the test were 1 mg/mL, 2 mg/mL, and 3 mg/mL. They were incubated at 37°C for 24 h for the culture sensitivity test (Colee 1967).

RESULTS AND DISCUSSION

Phytochemical Tests

From the phytochemical screening, we observed that the residual methanolic extract gave a positive result with Benedict's reagent and Fehling's solution, which indicated the presence of a reducing sugar in the residual methanolic extract. The Meyer's reagent and Dragendorff's reagent failed to show the presence of alkaloids in the extract. The Frothing test would indicate the presence of saponins in the extract and

glycosides were found to be absent from the extract using this test. Based on the ferric chloride test and general test for flavonoids, both tannins and flavonoids were found (Table 2) to be present in the extract, respectively.

Table 2: Results of phytochemical screening of residual methanolic extract.

Sample	Observation	Inference
Tests for Carbohydrates		
Benedict's test	A red-coloured precipitate was found	Present
Fehling's test	Brick red-coloured precipitate was found	Present
Tests for Alkaloids		
Mayer's test	White or creamy white precipitate was not found	Absent
Dragendroff's test	Orange or orange red precipitate was not found	Absent
Test for saponins		
Frothing test	Persistent frothing was found	Present
Test for glycosides		
General test	A yellow colour was not found	Absent
Test for tannins		
Ferric chloride test	A blue-green-coloured precipitate was found	Present
Test for flavonoids		
General test	A red colour was found immediately.	Present

Effect of the Primary Methanolic Extract and Residual Extract on Wheat Rootlet and Shoot Growth Inhibition Bioassay

The primary methanolic extract has a potent growth inhibitory effect on both wheat roots and shoots. It inhibited the growth of wheat rootlets by about 91.9% and shoots by 89.4%, even at the lowest concentration level (250 µg/mL) when compared to the control. The extract inhibited the growth of wheat rootlets by about 99.1% at 500 µg/mL and 100% at 1000 µg/mL. The shoot growth was inhibited by 96.3% at 500 µg/mL and 98.8% at 1000 µg/mL. The primary methanolic extract caused 100% inhibition of both rootlet and shoot growth at the highest dose level (1500 µg/mL) (Tables 3 and 4).

Table 3: Effect of primary methanolic extract from *Xylocarpus granatum* Koen. on the wheat rootlet growth inhibition bioassay.

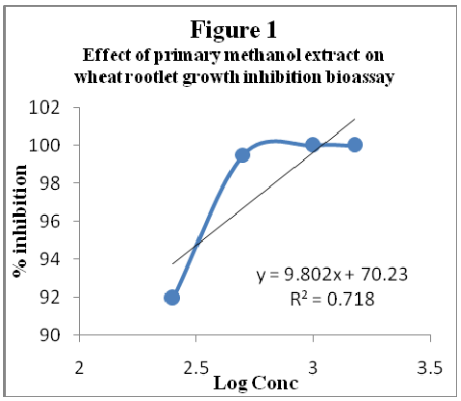
Concentration (µg/mL	Log concentration	% inhibition	Rootlet length (mm) Mean ± SEM	Control length (mm) Mean ± SEM
250	2.40	91.3	3.3 ± 2.93	40.9 ± 12.93
500	2.70	99.5	0.2 ± 0.19	
1000	3.00	100.0	0.0 ± 0	
1500	3.17	100.0	0.0 ± 0	

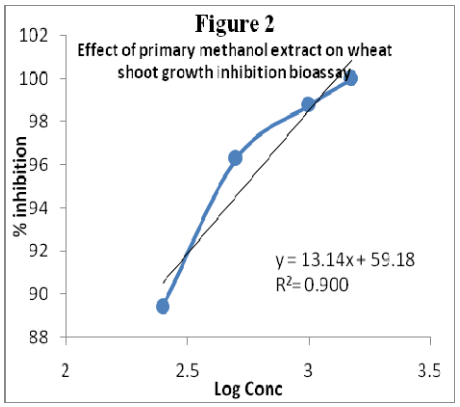
Table 4: Effect of primary methanolic extract from *Xylocarpus granatum* Koen on the wheat shoot growth inhibition bioassay

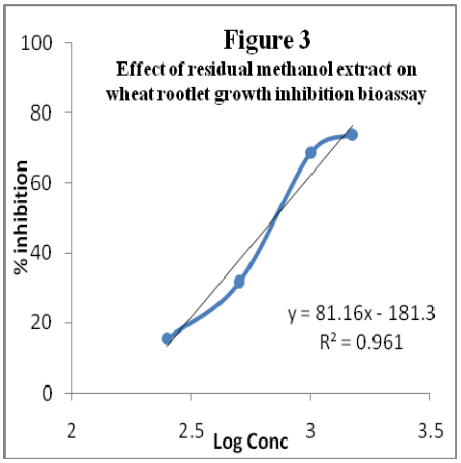
Concentration (µg/mL)	Log concentration	% inhibition	Rootlet length (mm) Mean ± SEM	Control length (mm) Mean ± SEM
250	2.40	89.4	6.0 ± 3.5	56.6 ± 10.15
500	2.70	96.3	2.1 ± 1.99	
1000	3.00	98.8	0.7 ± 0.66	
1500	3.17	100.0	0.0 ± 0	

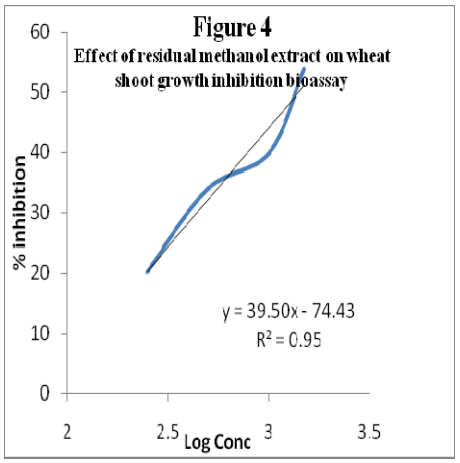
Shoot branching is a major determinant of plant architecture and is highly regulated by endogenous and environmental causes. Two classes of hormones, auxin and cytokinin, have long been known to have an important involvement in controlling shoot branching (Umehara *et al.* 2008). Furthermore, the application of strigolactones, a group of terpenoid lactones, inhibits shoot branching (Umehara *et al.* 2008). Thus strigolactones, or their biosynthetic precursors, may act as a new hormone class involved in regulating above-ground plant architecture. In addition, a literature survey also showed that methanolic extracts of *Cryptomeria japonica* wood yielded several terpenoids: ferruginol, sugiol, sandaracopimarinol, sandaracopimarinal, phyllocladanol, and β-sitosterol. The inhibitory activity of each terpenoid on the mycelial growth of the bark of *Lentinula edodes* was examined. Ferruginol, sugiol, and sandaracopimarinol were found to inhibit the mycelial growth. Mycelial growth inhibition is probably due to a synergistic effect of ferruginol and sandaracopimarinol, which are the major terpenoids in the wood (Takanao *et al.* 2001). Moreover, four novel tetranortriterpenoids, xylogranatins A-D, were isolated from the seeds of a Chinese marine mangrove *Xylocarpus granatum* (Wu *et al.* 2003; Yin *et al.* 2006). Again, the Meliaceae family contains various types of pentacyclic triterpenoid, and

these terpenoids have a growth inhibitory effect (Shinoda, Ogisu and Iwata 1985). The methanolic extract inhibited the growth of wheat rootlets and shoots. Since terpenoids display such activities, we can definitely conclude that some unknown terpenoid(s) must be present in the extract, which cause the wheat rootlet and shoot growth inhibitory effect by interfering with mitotic cell division.









Again, the residual extract inhibited growth of the wheat rootlet about by 15.7% and the shoot by 20.2% (Tables 6 and 7), even at the lowest concentration (250 µg/mL) when compared to the control. In contrast to the primary methanolic extract, 100% inhibition of both rootlet and shoot growth was not found in this case, even at highest dose level (1000 µg/mL). As the residual methanolic extract was prepared by partitioning with n-hexane and chloroform from the primary methanolic

extract, a significant percentage of terpenoids was removed. The remaining portion contained a small amount of terpenoids, and thus the growth inhibition ability of the residual methanolic extract was significantly reduced. Therefore, the IC_{50} values of the primary methanolic extract were found to be 0.009 µg/mL for rootlet and 0.20 µg/mL for shoot (Table 5), whereas these values were very large in the case of the residual methanolic extract, 707.83 µg/mL and 1412.94 µg/mL (Table 8) for the rootlet and shoot, respectively. In both cases, inhibition of both root and shoot, growth was found to be dose dependent. The activity increased with the increasing dose (Fig. 1, 2, 3, and 4).

Table 5: Effect of primary methanolic extract on wheat rootlet and shoot growth.

Experiment	IC_{50} (µg/ml)	Regression equation	R^2
Rootlet	0.009	Y = 9.802X + 70.23	0.72
Shoot	0.20	Y = 13.14X + 59.18	0.90

Table 6: Effect of residual methanolic extract from *Xylocarpus granatum* Koen. on the wheat rootlet growth inhibition bioassay.

Concentration (µg/mL)	Log concentration	% inhibition	Rootlet length (mm) Mean ± SEM	Control length (mm) Mean ± SEM
250	2.40	15.7	34.5 ± 11.7	40.9 ± 12.93
500	2.70	31.5	28.0 ± 6.49	
1000	3.00	68.7	12.8 ± 2.49	
1500	3.17	73.8	10.7 ± 4.89	

Table 7: Effect of residual methanolic extract from *Xylocarpus granatum* Koen. on the wheat shoot growth inhibition bioassay.

Concentration (µg/mL)	Log concentration	% inhibition	Rootlet length (mm) Mean ± SEM	Control length (mm) Mean ± SEM
250	2.40	20.2	44.6 ± 13.14	56.6 ± 10.15
500	2.70	33.9	37.4 ± 10.02	
1000	3.00	39.8	34.1 ± 6.88	
1500	3.17	53.7	26.2 ± 8.94	

Table 8: Effect of residual methanolic extract on wheat rootlet and shoot growth.

Experiment	IC_{50} (µg/mL)	Regression equation	R^2
Rootlet	707.83	Y = 81.16X - 181.3	0.96
Shoot	1412.94	Y = 39.50X - 74.43	0.95

Antimicrobial Activity of Primary and Residual Methanolic Extracts

The antimicrobial study (Table 9) of the primary extract showed that the extract demonstrated antibacterial activity only at the highest (3 mg/mL) concentration level against the gram positive bacteria *S. aureus* and *B. subtilis* and gram negative bacteria *P. vulgaris.* The extract was found to be inactive against *E. coli* and *P. aeruginosa*. The primary extract was inactive against all the tested microorganisms at the 1 mg/mL and 2 mg/mL concentration levels. The minimum inhibitory concentration (MIC) was found to be 3 mg/mL. The family Meliaceae is distinguished by the occurrence of characteristic substances called limonoids (Shinoda, Ogisu and Tajima 1984; Ambrozin *et al.* 2006). These substances have a wide spectrum of biological activities, particularly insecticidal action (Ambrozin *et al.* 2006). Some of the phytochemical compounds, including glycosides, saponins, tannins, flavonoids, terpenoids, and alkaloids have been reported to have antimicrobial activity (Ebi and Ofoefule 1997; Okeke *et al*. 2001). *Xylocarpus granatum* also possesses alkaloidal substances, which also have biological activities (Chou *et al.* 1977). Additionally, the ethanolic and partially extracted products of *Xylocarpus granatum* were examined and found to exhibit good antibacterial activity (Alam *et al.* 2006). The phytochemical screening of the extract in the present study revealed the presence of saponins, tannins, and flavonoids. From these findings, we can assume that saponins, tannins, and flavonoids in the extract may show these antibacterial activities. The residual extract was also found to be inactive against all tested microorganisms at all concentration levels. Fractionation of the primary methanolic extract (by n-hexane and chloroform) removes the antibacterial activity. This suggests that the active antibacterial principles might be relatively non polar compound(s).

Table 9: Antimicrobial activity of primary methanolic extract and residual methanolic extract from *Xylocarpus granatum* Koen.

Microorganisms	Primary methanolic extract			Residual methanolic extract		
	1 mg/mL	2 mg/mL	3 mg/mL	1 g/mL	2 g/mL	3 mg/mL
Gram positive bacteria						
Bacillus subtilis	-	-	+	-	-	-
Staphylococcus aureus	-	-	+	-	-	-
Gram negative bacteria						
Escherichia coli	-	-	-	-	-	-
Proteus vulgaris	-	-	+	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-

+ active; - inactive

CONCLUSION

The growth inhibition activity of methanolic extracts provides the opportunity to explore new compounds from the plant. Since different terpenoids had been identified earlier, comprehensive research on *Xylocarpus granatum* should be done. Although the extract itself has little antibacterial activity, different components with better antibacterial activity can be isolated from it.

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