



AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: <http://www.ajptr.com/>

Observation of Cytotoxic Activity and Antimicrobial Effect of Crude Extracts of *Diospyros peregrina gurke*

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ABSTRACT

This dissertation describes the biological investigations of *Diospyros peregrina gurke*; a plant belonging to the family Ebenaceae. Present study was designed to test the cytotoxic activity and antimicrobial effect of crude extract. The study protocol consists cold extraction of powdered unripe fruit and seed of *Diospyros peregrina gurke* for cytotoxicity effect using Brine shrimp bioassay lethality and tested against four Gram negative and three Gram positive bacteria for antimicrobial activity. From the results of the brine shrimp lethality bioassay it can be well predicated that crude extracts of *Diospyros peregrina gurke* possess cytotoxic principles (with LD₅₀ 9.12011 μg/ml and 10.8893 μg/ml). For antimicrobial activity, the extracts were tested against standard microbial strains of gram positive and gram-negative, by means of Agar-Disc Diffusion Method and minimum inhibitory concentration (MIC) was noted. Although, both extracts were found to be effective in inhibiting pathogens to varying degrees to the tested organisms, the unripe fruit pulp extract of *Diospyros peregrina gurke* was found to be more effective than seed extract of *Diospyros peregrina gurke*. When both extracts were used in combination, they have shown strong synergistic effect against all the pathogens tested in the present study. Plant extracts can be used to source antitumor and antibiotic substances for possible treatment.

Keywords: Cytotoxicity, synergism effect, antimicrobial, *Diospyros peregrina gurke*, deshi ghab, Gaub persimmon .

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Received 09 January 2014, Accepted 24 January 2014

Please cite this article in press as: Yousuf T *et al.*, Observation of Cytotoxic Activity and Antimicrobial Effect of Crude Extracts of *Diospyros peregrina gurke*. American Journal of PharmTech Research 2014.

INTRODUCTION

The Ebenaceae is a family of flowering plants, which includes ebony and persimmon. The family has approximately 500 species of trees and shrubs in two genera, *Diospyros* and *Euclea*. The species are mostly evergreen and native to the tropics and subtropics, with a few deciduous species native to temperate regions. *Diospyros* contains 450-500 species and a pantropical distribution, with the greatest diversity of species in Indomalaya. *Euclea* contains 20 species, native to Africa, the Comoro Islands, and Arabia. A persistent calyx on the fruits is characteristic of the family.¹

Distribution throughout India; Bangladesh, Malaysia and other South-East Asian countries, also in Australia.² Ebenaceae are recognized by their fruits, which appear like little persimmons, often brownish, and seated on a persistent calyx of significant hardness. The wood of Ebenaceae is dense, very hard and blackens upon exposure to light. Approximately 20 species of Ebenaceae are used for medicinal purposes in Asia-pacific, especially to expel intestinal worms and to treat viral infections.

Botanical names : *Diospyros peregrine*(Gaertn.) gurke

Synonymous Name : *Diospyros malabarica*(Desv.)Kostel, *Diospyros embiopteris* pers.

Vernacular names

Unani name :Tendu.

Hindi :Gabh,Tedu.

Bangla :Desi gabh

Ayurvedik names :Tinduka, Nilasaara (charaka); Tinduki (sushruta), Sphuurjak, Kaalaskandh, Asitkaarak.

Tamil : *Paniccai, Panicika, Tumbika*

Malayalam : *Paniccha, Panichi, Panacci, Panicci, Panitsjika, Vananci, Vananchi*

Kannada : *Bandadamara, Holetupare, Kusarta*

English :Gaub persimmon, Indian persimmon, Wild mangosteen, river ebony⁴

Taxonomy

Kingdom *Plantae*

Phyllum *Magnoliophyta*

Class *Magnoliatae*

Order	<i>Ebenales</i>
Family	<i>Ebenaceae</i>
Genus	<i>Diospyros</i>
Species	<i>Diospyros peregrina gurke</i>

Deshi ghab is a deciduous and evergreen tree. Trees up to 12 m tall. Bark greyish black, flaky; blaze dull red. Young branchlets terete to slightly angular, glabrous. Leaves simple, alternate, distichous; petiole up to 1.5 cm long, canaliculate, glabrous; lamina 19 x 6.5 cm, oblong-lanceolate, apex acute to obtuse, base acute to attenuate, margin entire, coriaceous, glabrous, strongly reticulate on both surfaces; midrib canaliculate; secondary nerves ca. 8 pairs. Flowers unisexual, dioecious; male inflorescence 3-7 flowered axillary cymes; female flowers solitary, axillary. Berry, globose, 4 cm across, reddish velvety, fruiting calyx large, reflexed; seeds 4-8, smooth, pulp viscid, turning black when exposed.



Figure-1: *Diospyros peregrina gurke* seed and unripe fruit pulp

Medicinal use

Different parts of ghab have great medicinal value. The plant is useful both, internally as well as externally. The barks are useful as Astringent, Dysentery and in Intermitten fever.^{5,6} The Fruit can be used as astringent and useful in Aphthae, Sore throat in form of infusion where fruit juice can be applied for wound healing and ulcers. Seed oil of the leaves is suggested for dysentery and diarrhoea.³

Reported phytoconstituents

The Ghab fruit pulp and seed yielded Betulin, Lupeol, Gallic acid, Betulinic acid, Hexacosane, Hexacosanol, Sitosterol, A terpene keton. Leaves contain Triterpenes, anthrocyanin.^{7,8} Beta-sitosterol, Betulin and Oleanolic acid. A dihydroflavonol glycoside 5, 7, 3, 5 – Tetra hydroxyl – 3– methoxy flavones can be extracted from its root.⁹ Bark contains myricyle alcohol, saponin, triterpenes²

MATERIALS AND METHOD

Brine Shrimp Lethality Bioassay

Meyer BN, Ferrigni NR, Putnam JE, Jacobsen JB, Nicholsand DE, Mclaughlin JL. Brine shrimp;

a convenient general bioassay for active plant constituents. Plant Med. 1982;45:31–34. [PubMed]

Brine shrimp eggs were hatched in simulated sea water to get nauplii. Sample solutions were prepared by dissolving the test materials in pre-calculated amount of Dimethyl sulphoxide (DMSO). Ten nauplii were taken in vials containing 5ml of simulated sea water. The samples of different concentrations were added to the pre-marked vials with a micropipette. The assay was performed using three replicates. Survivors were counted after 24 hours. These data were processed in a simple program for analysis to estimate LD₅₀ values with 95% confidence intervals for statistically significant comparisons of potencies.

Materials

Artemia salina leach (Brine shrimp eggs), Sea salt (NaCl), Small tank with perforated dividing dam to hatch the shrimp, Lamp to attract shrimps, Pipettes (5.25ml) and micropipette (5µl-40µl), Glass vials, Magnifying glass, Test samples of experimental plants (Table-1)

Table-1: Test samples of experimental plants

Plant	Test samples	Measured Amount (mg)
<i>Diospyros</i>	Crude extract of seed	4.00
<i>peregrina gurke</i>	Crude extract of fruit pulp	4.00

Preparation of sea water

38 gm sea salt (pure salt) was weighed, dissolved in one liter of distilled water and filtered to get clear solution.

Hatching of brine shrimps

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Sea water was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. Two days were allowed¹⁰ to hatch the shrimps and to be matured as nauplii. Constant oxygen was provided throughout the hatching line. The hatched shrimps were attracted to the lamp through the perforated dam and with the help of a Pasteur pipette 10 living shrimps were added to each of the vials containing 5 ml of sea water.

Preparation of test samples of experimental plants

Clean test tubes were taken. These test tubes were used for five different concentration (one test tube for each concentration) of test samples and test tubes were taken for standard drug Vincristine for five concentration of it and another one test tubes for control test. Then 100µl of solution was taken in test tube each containing 5ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400µg/ml. Then a

series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In each case 100 μ l sample was added to test tube and fresh 100 μ l DMSO was added to vial. Thus the concentration of the obtained solution in each test tube were as-

Table-2 : Concentration of test sample in each test tube

Test tube number	Concentration (μ g/ml)
1	400
2	200
3	100
4	50
5	25



Figure-2 : Brine shrimps eggs are hatched in simulated sea water(Top view)

Preparation of control group

Control groups were used in cytotoxicity study to validate the test method and ensure that the results are obtained only due to the activity of the test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used

Positive control, Negative control

Preparation of positive control group

Positive control in a cytotoxicity study is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In the present study Vincristine sulphate is used as the positive control. Measured amount of the vincristine sulphate is dissolved in DMSO to get an initial concentration of 20 μ g/ml from which serial dilutions are made using DMSO to get 10 μ g/ml, 5 μ g/ml, 2.5 μ g/ml, 1.25 μ g/ml, and 0.6225 μ g/ml. Then the positive control solutions are added to the pre-marked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups.

Preparation of the negative control group

100 μ l of DMSO was added to each of two pre-marked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show

a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reasons other than the cytotoxicity of the compounds

Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

TEST FOR ANTIMICROBIAL ASSAY

Plant materials and preparation of extract

Diospyros peregrina gurke (unripe fruit) of fresh condition was collected from Noakhali district of Bangladesh in August 2011. The collected plant parts (unripe fruit) were washed with water, fruit pulp was separated from seed of plant. Unripe fruit pulps and seeds were sun-dried for 15 days after cutting into small pieces. The plant parts were ground into a fine powder with the help of blender. Fine powder was stored in air-tight container with marking for identification and kept in cool, dark and dry place until analysis commenced. The powdered 88.47gram powders of fruit pulp were soaked in 1.66 liters(600ml) methanol in october 2011 in a 800 ml beaker through occasional shaking and stirring for 7 days. The extract was then filtered through filter-cloth. The filtrate was kept to dry in fresh and clean air to afford a mass of antimicrobial investigation.19.88gm powders of seed was soaked in 200ml methanol in September 20 in a 500ml beaker through occasional shaking and stirring for 7 days. The extract was then filtered through filter-cloth. The filtrate was kept to dry in fresh and clean air to afford a mass of antimicrobial investigation.

Preparation of sample

In the study of antimicrobial activity, extracts were dissolved in Dimethyl sulphoxide (DMSO) with the concentration of 200µg/ml . The corresponding concentration was expressed in term of µg of extract per ml of solvent (µg/ml).

Test microorganisms

The test microorganisms used in this study (three gram-positive bacteria; *Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus cereus* and four gram-negative bacteria; *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholera*, and *Salmonella typhi*) were obtained from the Department of Pharmacy, Noakhali Science and Technology University, Noakhali, Bangladesh. The bacterial strains were first subcultured in a nutrient broth (Oxoid) and incubated at 37°C for 18 hours.

Preliminary screening for antimicrobial activity

The test was performed by disc diffusion assay.¹¹ The nutrient agar plates containing an inoculum size of 10⁶ cfu / ml for bacteria was used¹². Dried and sterilization filter paper disc (6mm diameter) were then impregnated with previously prepared extracts using micropipette and residual solvents were completely evaporated. Antibiotic discs of ciprofloxacin(200µg/ml) was used as standard antibacterial antibiotics for bacteria. Discs containing the test materials were placed on to Muller Hinton agar medium uniformly seeded with the test microorganisms. These plates were then Kept at low temperature (4°C) for 18- 24 hrs.¹³ to allow maximum diffusion of the test material. Then these plates were incubated in inverted position at 37°C for 24 hrs to allow maximum growth of the organism. Sensitivity was recorded by measuring the clean zone of growth inhibition on agar surface around the disc.

Determination of minimum inhibitory concentration (MIC):

A minimum inhibitory concentration (MIC) is the lowest attentiveness of an antimicrobial culture that stops the growth of a microorganism after 18 to 24hr.^{14,15,16} MIC was determined by a broth dilution method.¹⁷ The MIC was defined as the minimum level of the extract that produced a 90% reduction in growth of the bacteria.

The synergism effect of the two extracts:

1ml from the MIC of each extract was taken, then mixed by vortex to determine the synergism effect in both methods, except bacteria that did not show any sensitivity by extracts, 1ml was taken from first concentration.

RESULTS AND DISCUSSION

(a)Cytotoxic activity

The brine shrimp test (BST) represents a rapid, inexpensive and simple bioassay for testing plant extract lethality which in most cases correlates reasonably well with cytotoxic and anti-tumor properties. Following the procedure¹¹ the lethality of crude extract and the summary of the result is expressed in table 3.

Table3:Results of the test samples of *Diospyros peregrina gurke*

Sample	LD₅₀ (µg/ml)	Regression equation	R²
Vincristine sulphate (positive control)	0.283204	y = 23.24x + 62.74	0.942
Seed	9.12011	y = 36.45x + 14.91	0.643
Unripe fruit pulp	10.8893	y = 23.21x + 15.56	0.925

The LD₅₀ values of crude extract (seed and Unripe fruit pulp) found to be 9.12011µg/ml and 10.8893µg/ml respectively. The positive control vincristine sulphate showed LD₅₀ at a concentration of 0.283204µg/ml.

From the result of the brine shrimp lethality bioassay it can be well predicted that seed extracts possess moderate cytotoxic principles. Unripe Fruit pulp extracts possess moderate cytotoxic activity. Comparing with positive control vincristine sulphate signifies that cytotoxicity exhibited by the seed extraction and unripe fruit pulp extraction has moderate anti-tumor activity. However this can be confirmed with further higher and specific tests.

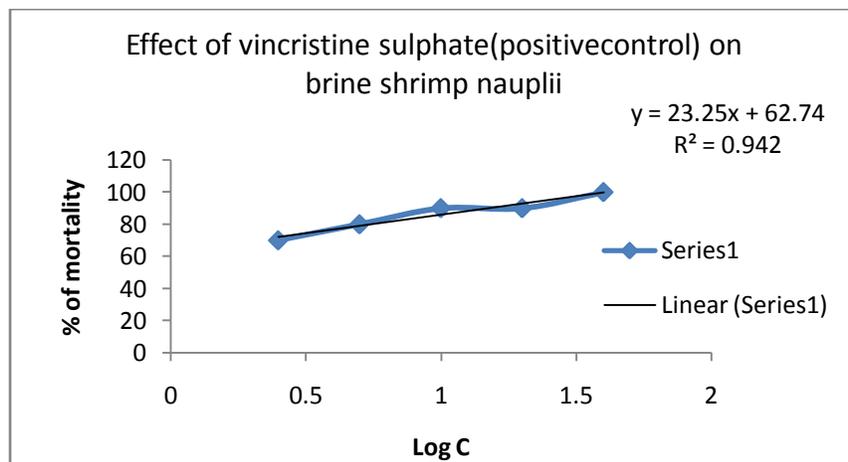


Figure-5: Effect of vincristine sulphate on brine shrimp nauplii

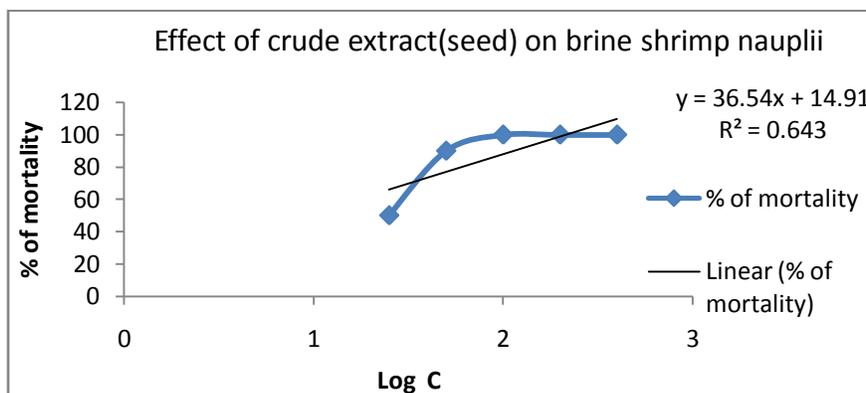


Figure-6: Effect of crude extract (seed) on brine shrimp nauplii

Table-4: Effect of crude extract (seed and unripe fruit pulp) on brine shrimp nauplii

Conc (C) (µg/ml)	Log C	%Mortality		LC ₅₀ (µg/ml)		Vincristine Sulphate				
		seed	Unripe fruit pulp	Seed	Unripe fruit pulp	Conc (C) (µg/ml)	Log C	% Mortality	LC ₅₀ (µg/ml)	
400	2.602059991	2.60206	100	100						
200	2.30102996	2.30103	100	90						
100	2	2	100	90	9.12011	10.8893	10	1	90	0.283204
50	1.698970004	1.69897	90	70			5	0.69897	80	
25	1.397940009	1.39794	50	60			2.5	0.39794	70	

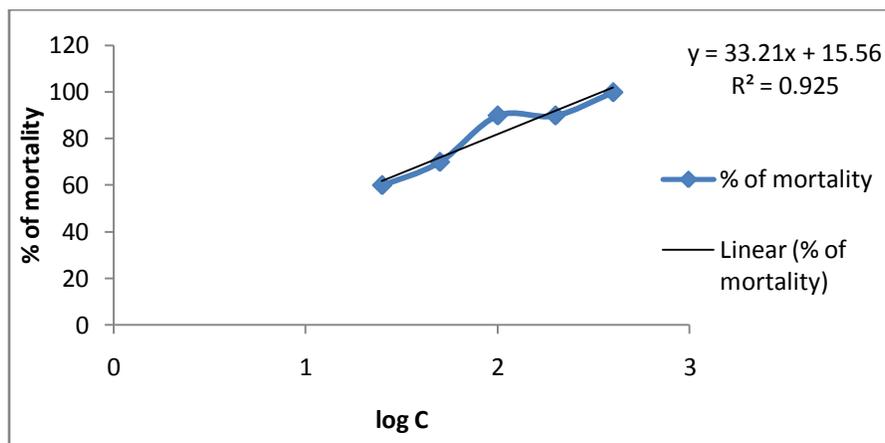


Figure-7: Effect of crude extract (unripe fruit pulp) on brine shrimp nauplii

(b)Antimicrobial activity

All two extracts of the plant tested showed varying degree of antibacterial activities against the test bacterial species (Table 5 and 6). The antibacterial activities of the methanol extracts compared favorably with that of standard antibiotics (ciprofloxacin) and has appeared to be broad spectrum as its activities were independent on gram reaction.

The *in vitro* antimicrobial and synergism activity of *Diospyros peregrina gurke* extract (unripe fruit pulp and seed) shown in table 5.

Table 5: Antimicrobial activity and synergism effect of *Diospyros peregrina gurke* unripe fruit pulp and seed:

Bacterial Strains	Zone of inhibition diameters in mm			
	Unripe fruit pulp (200 µg/ml)	Seed (200 µg/ml)	Unripe fruit pulp-seed (200 µg/ml)	Ciprofloxacin (200 µg/ml)
Gram Positive				
<i>Staphylococcus aureus</i>	10.50	9.70	11.00	14.14
<i>Bacillus subtilis</i>	8.00	-	8.50	13.58
<i>Bacillus cereus</i>	8.60	8.00	9.00	14.00
Gram Negative				
<i>Escherichia coli</i>	12.60	11.50	12.80	13.50
<i>Pseudomonas aeruginosa</i>	8.70	8.00	9.00	16.08
<i>Vibrio cholera</i>	10.00	10.30	10.00	14.62
<i>Salmonella typhi</i>	11.50	10.50	12.00	18.05

(-) negative sign has indicated that seed extract has no zone of inhibition on *Bacillus subtilis*.

There was antibacterial activity of plant extract against *E. coli* was observed with *Diospyros peregrina gurke* (unripe fruit pulp) with inhibition zone =12.60 mm, where was observed for the extracts of *Diospyros peregrina gurke* (seed) with inhibition zone=11.50, which were most near antibiotic ciprofloxacin with inhibition zone =13.50mm. But against *S. aureus*, antibacterial

potentials were observed for the extracts of *Diospyros peregrina gurke* (unripe fruit pulp) with inhibition zone =10.50mm, *Diospyros peregrina gurke* (seed) inhibition zone=9.70mm which were most similar to antibiotic ciprofloxacin with inhibition zone=14.14mm. Synergistic effect of antibiotics and plant extracts showed synergistic antibacterial activity against antibiotic-resistant bacteria. The results obtained with *E. coli* were particularly interesting, since it was inhibited by *Diospyros peregrina gurke* (unripe fruit pulp and seed) extracts. In case of *S. aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Vibrio cholera* *Salmonella typhi*, those showed synergistic activity of all antibiotics and plant extracts used. This inhibition was observed when they were used with low effective or ineffective antibiotics. The highest synergistic effect was observed by most plant extracts with ciprofloxacin.

In case of Minimum inhibitory concentration the results showed that extract of *Diospyros peregrina gurke* (unripe fruit pulp) is highly sensitive against *Escherichia coli* strains (MIC 25 µg/ml), moderately sensitive (MIC 100 µg/ml) to *Staphylococcus aureus* and *Vibrio cholerae* strains, less sensitive (MIC 200 µg/ml) to *Pseudomonas aeruginosa* and *Salmonella typhi* whilst resistant (MIC >200 µg/ml) to *Bacillus subtilis* and *Bacillus cereus*.

Table 6: Minimum inhibitory concentration of *Diospyros peregrina gurke* (unripe fruit pulp):

Bacterial Strains	Unripe fruit pulp
Gram positive	
<i>Staphylococcus aureus</i>	100
<i>Bacillus subtilis</i>	>200
<i>Bacillus cereus</i>	>200
Gram negative	
<i>Escherichia coli</i>	25
<i>Pseudomonas aeruginosa</i>	200
<i>Vibrio cholera</i>	100
<i>Salmonella typhi</i>	200

In the present bioactivity study, all the crude extracts showed positive results indicating that the test samples are biologically active. The overall results can be stated as:

1. Methanol extracts of two parts (seed and unripe fruit pulp) of *Diospyros peregrina gurke* of plant possess moderate cytotoxic activity against brine shrimps. Thus; it can be used in the treatment of anti-tumor activity.
2. Plant extracts especially unripe fruit pulp of *Diospyros peregrina gurke* has potential as antimicrobial compounds against microorganisms when compared with a broad spectrum antibiotic ciprofloxacin. Thus, it can be used in the treatment of infectious diseases caused by resistant microbes.

3. The synergistic effect from the association of antibiotic with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment.

CONCLUSION

Extracts of *Diospyros peregrine gurke* (unripe fruit pulp and seed) in this study demonstrated a moderate cytotoxic activity against brine shrimp and a broad-spectrum of activity against both gram-positive and gram-negative bacteria. Now our research will be directed to develop a further literature findings in future and a broad spectrum antimicrobial herbal formulation with this unripe fruit. Present investigation offers the potential for reaching on better understanding of *Diospyros peregrine gurke*'s therapeutic efficacy.

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