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Original Article

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Studies on the antidiarrhoeal, antimicrobial and cytotoxic activities of ethanol-extracted leaves of yellow oleander (*Thevetia peruviana)*

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Abstract
This study screened the antidiarrhoeal, antimicrobial and cytotoxic effects of ethanol-extracted leaves of yellow oleander (*Thevetia peruviana)*. The extract was tested against castor oil-induced diarrhoea in a model of albino rats and showed significant antidiarrhoeal activity ($P<0.01$). Disc diffusion technique was used to test the *in vitro* antibacterial activities of the extract and exhibited poor antibacterial activities against both Gram positive and Gram negative bacteria (mainly *Bacillus* sp). Ethanol-extracted leaves of yellow oleander showed narrow zone of inhibition in the bacterial lawns of *Shigella flexineri, Salmonella typhi, Klebsiella sp, Staphylococcus aureus* and *Shigella sonnei*. Cytotoxicty was determined against brine shrimp nauplii and LC_{50} of the plant extract was determined as 627.21µg/ml. The wide range of LC_{50} value denotes the safety effect of the extract.
Keywords: Antidiarrhoeal, Antimicrobial, Cytotoxicity, Albino rats, Brine shrimp, Yellow oleander, LC_{50}

Introduction

In Bangladesh, medicinal plants are abundantly available at relatively low cost. The plant drugs prove relatively nontoxic, safe and even free from serious side effects (Momin, 1987). On the other hand, some toxic plants have life threatening properties. In Nigeria and Ghana, the bark is used as an antipyretic, however, it is both emetic and poisonous in excess (Oliver-Bever, 1986). Insecticidal, molluscidal and antibacterial properties of the leaves and seed oil have been reported (Obasi and Igboechi, 1991; Panigrahi and Raut, 1994).

The phytochemical screening is mainly applied to the quality control of traditional medicine. In recent years, secondary plant metabolites, previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents (Krishnaraju and Nigam, 1970).

Thus it is anticipated that phytochemicals with adequate antibacterial efficacy will be used for the treatment of the bacterial infections (Balandrin *et al*., 2006).

According to WHO, medicinal plants are the best sources to obtain a variety of new herbal drugs. About 80% of individuals from developing countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated for better understanding of their properties, safety and efficacy.

The frequency of animal life threatening infections caused by pathogenic micro-organisms has increased worldwide and is becoming an important cause of morbidity and mortality in immunocompromised animals in both developing and developed countries. In developing countries such as Bangladesh, Nepal and Nigeria, irrational use of antimicrobial agents is a major cause of antibiotics resistance.

In recent years, attempts have been made to investigate the indigenous drugs against infectious diseases. Research in the field of indigenous plants is a significant aspect of developing a safe antimicrobial principle through isolation, characterization, identification and biological studies. *Thevetia peruviana* (*T. peruviana*) is widely distributed throughout Bangladesh. *T. peruviana* belongs to the family Apocynaceae plant and has been referred with different names as Digoxin, Lucky nut, Nerium oleander and yellow oleander.

This plant is native of Central and South America, but now frequently grown throughout the tropical and sub-tropical regions. It is a small ornamental tree, which grows about 10 to 15 feet high. The leaves are spirally arranged, linear and about 13 to 15 cm in length.

The absorption of the equivalent of two *T. peruviana* leaves may be sufficient to kill a 7.5 kg dog bitch (Arnold and Ayuso, 1935). Many cytotoxic compounds have been investigated in the leaves of *T. peruviana*, such as Thevetin A and B, Thevetoxin, Peruvoside, Ruvoside and Nerifolin (Arnold and Ayuso, 1935).

The leaves of yellow oleander might possess antimicrobial, antidiarrhoeal and mainly cytotoxic activities. The purpose of the present study was to find out the antidiarrhoeal, antimicrobial screening and cytotoxic activities of ethanol-extracted leaves of yellow oleander.

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Materials and Methods

Preparation of extract:

The whole leaves of yellow oleander (*T. peruviana)* were collected from Nandail thana under the district of Mymensingh. The collected samples were cleaned, air dried and ground into powder. The dried powder (500 gm) was extracted with 3 L of ethanol. The extract was filtered and the filtrates were evaporated to dryness at 40ºC under vacuum and finally freeze-dried to get ethanol extract at 23.6 gm.

Antidiarrhoeal assay:

Male and female albino rats obtained from the animal house of Bangladesh Council of Scientific and Industrial Research (BCSIR) laboratory, Chittagong, weighing 180-200 gm, at the age of two months were used in this study. The rats were acclimatized to standard laboratory conditions (temperature 24 ± 1ºC, relative humidity 55 ± 5% and a 12 hours photoperiod) in suspended wire-meshed galvanized cages (4-6 rats/cage) for one week before the commencement of the experiment.

During the study, the rats were supplied with a semi-purified basal diet and water adlibitum. Rats were maintained according to the NIH guidelines of care and use of laboratory Animals published by Saha *et al*. (2001).

The experiment was conducted by castor oil model as described by Yegnanarayan and Shostri, (1982). Fifteen male and female rats were randomly divided into 3 groups: control group, treated group and positive control group. One ml of castor oil was given to induce diarrhea in each rat. Thirty minutes later, distilled water, plant extract and loperamide drugs were orally administered in the rats in the control group, treated group, and positive control group, respectively (Table 1).

Table 1. Doses of the drug in different groups of rats

Group	Dose (Concentration)
Control	2ml distilled water/rat
Treated	2ml plant extract/rat (200 mg/ml)
Positive control	2ml loperamide Sol^n/rat (0.2 mg/ml)

After that, the animals were caged individually and examined for the presence of diarrhoea every one hour for six hours. Diarrhoea was defined as the presence in the stool of fluid material that stained the absorbent paper placed beneath the cage. The number of stools passed during the six-hour period was noted for each rat.

Cytotoxicity assay:

The cytotoxicity assay was performed on brine shrimp nauplii as reported (Meyer *et al.*, 1982). Brine shrimp nauplii were obtained by hatching brine shrimp eggs (Carolina Biological Company, Burlington, NC, USA) in artificial sea-water (22.5 gm sodium chloride dissolved in 500 ml distilled water) for 48 hours.

Dissolution of extract was performed in artificial sea-water by using twine. Each 5 ml solution of different concentrations (62.5, 125, 250, 500, 1000, 2000 µg/ml) of the extract was taken in different beakers where brine shrimp nauplii were given and observed for mortality for 24 hours.

The resulting data were transformed to probit analysis for the determination of LC_{50} values of the extract. Artificial sea-water and artificial sea-water medium containing twine were used as controls.

Antimicrobial assay:

Organism: Ethanol-extracted leaves of yellow oleander were tested for their antibacterial activity against the following bacteria: *Staphylococcus aureus* BTCC 43, *Shigella sonnei* ICDDR'B, *Klebsiella sp* ICDDR'B, *Bacillus polymyxa* BTCC 16, *Bacillus cereus* BTCC 19, *Bacillus megaterium* BTCC 18, *Bacillus subtilis* BTCC 17, *Salmonella typhi* ICDDR'B, *Proteus sp* ICDDRB'B and *Shigella flexineri* ICDDR'B.

Disc diffusion method: The bioassay for bacterial strains was employed by disc diffusion method (Ergene *et al*., 2006).

Filter paper discs (Whatman No. 1) of 5 mm diameter were loaded with crude extracts. Discs were completely dried and sterilized. 100 µl of cultures were spread on sterilized nutrient agar media; impregnated discs were placed on it and incubated for 24 h at 37ºC.

Tetracycline (30 µg/disc) was used as a standard drug. The diameter of zone of inhibition in mm was recorded after incubation. The experiment was performed in triplicates and the average diameter of zone of inhibition was obtained.

Statistical Analysis:

All values of antidiarrhoeal test were expressed as Mean ± SEM (standard error of mean). Statistical difference between the mean of the various groups were analyzed by using student's "t" test. *P* value <0.01 or less were considered as significant. The LC_{50} values for cytotoxicity valued were calculated by "Probit Analysis". In this case "BioState Software - 2007" was used for probit analysis and Chi-sqare test.

Results and Discussions

Antidiarrhoeal Activity:

The ethanol extract of yellow oleander leaves significantly reduced castor oil-induced diarrhoea in albino rats (Table 2).

All control group responded to castor oil-induced diarrhoea in albino rats, while 66.7% of ethanol extract-treated group and 75% of loperamide (positive control) group responded to the relevant treatment (Table 2).

The mean latent period of ethanol extract-treated group (2.4 ± 1.66) and of the positive control (1.8 ± 1.11) decreased diarrhoea significantly (P<0.01) compared to the control group (Fig. 1 and Table 3). Zakaria and Mohd (1994) stated that response to ethanol extract of leaves of yellow oleander was 57.7% against diarrhoea, which is lower than present study.

Table 2. Effect of ethanol-extracted leaves of yellow oleander on castor oil-induced diarrhoea

Group	Control	Positive control	Treated
Treatment	Distilled water	Loperamide	Ethanol-extracted leaves of yellow oleander
Dose	2 ml/rat	2 ml/rat	2 ml/rat
No. of rats with diarrhoea	5/5	3/5	3/5
Protection (%)	0 %	40 %	40 %
Mean defecation within 6-h	7.2 ± 0.49	1.8 ± 1.11	2.4 ± 1.66
Inhibition of defecation (%)	0%	75 %	66.7%

Table 3. Comparison of effect of yellow oleander and loperamide on mean defecation of albino rats using Student's t-test

Mean defecation (6-h study period)

Control	Loperamide (2 ml/rat)	Yellow oleander leaves extract (2 ml/rat)
7.2 ± 0.49	1.8 ± 1.11 **	2.4 ± 1.66 **
Student's t test		
T calculated	4.44	3.79
T tabulated	3.35	3.35
degrees of freedom	8.0	8.0
P value	*<0.01*	*<0.01*

All values are expressed as mean ± SEM (n=5).
***P<0.01 significant compared to control Students "t" test.*

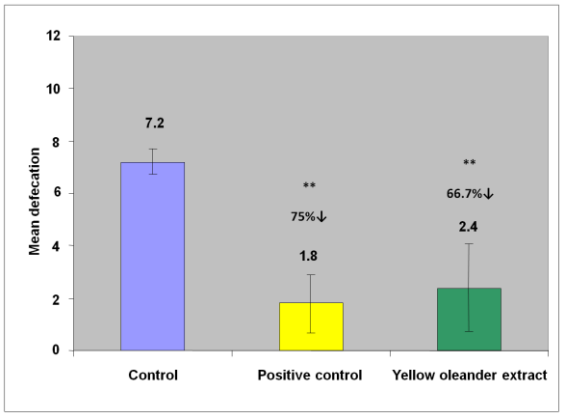

Fig. 1 Effect of ethanol-extracted leaves of yellow oleander (200 mg/ml) and commercially available antidiarrhoeal drug loperamide (0.2 mg/ml) on castor oil-induced diarrhoea. % activities are shown within parentheses. ** P<0.01(Table. 3) compared with control group (Student's't' test). ↓= decrease

Cytotoxicity Activity:

Cytotoxic activity of yellow oleander leaves extract was determined by brine shrimp lethality assay. Percentage mortality of brine shrimp at six different concentrations of yellow oleander leaves extract shown lethality in a dose dependent manner.

More specifically, 0%, 5%, 10%, 35%, 65% and 100% mortality was observed at 62.5, 125, 250, 500, 1000 and 2000 µg/ml, respectively (Table 4). This is might be due to increase in active compound concentration and thus exhibiting concentration dependent activity.

From the percentage of lethality of brine shrimp, the probits were calculated for each concentration. Probits were then plotted against corresponding leaves extract log concentration and from the plot LC_{50} (log concentration 50) the value of 2.79 µg/ml was obtained (Table 5 and Fig. 2). LC_{50} value of leaves extract of yellow oleander was found to be 672.21 µg/ml with 95% confidence limit, where the lower and upper limits were 489.79 and 813.71 µg/ml, respectively. Percentage of mortality of brine shrimp reported here (LC_{50} 3.12µg/ml) were higher than that reported by Rajapakse (2009) (3.75).

Table 4. Brine shrimp lethality bioassay of ethanol extract of yellow oleander leaves

Dose µg/ml	Log dose	Total	Alive	Death	Lethality %	Actual %	Probit
62.5	1.796	20	20	0	0	0.01	1.66
125	2.097	20	19	1	5	0.05	3.67
250	2.398	20	18	2	10	0.10	3.72
500	2.699	20	13	7	35	0.35	4.62
1000	3.000	20	7	13	65	0.65	5.37
2000	3.301	20	0	20	100	0.99	7.04

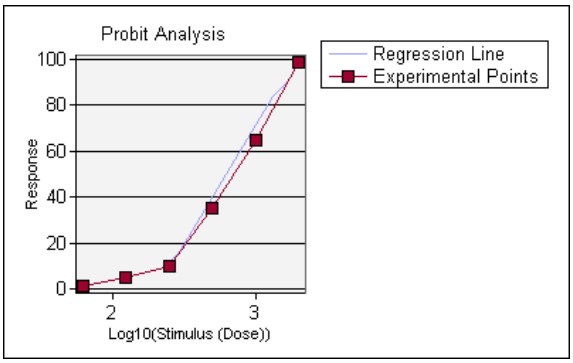

Fig. 2 Probit analysis. Regression line and experimental points.

Table 5. Calculation of LC_{50} value, regression equation and confidence limit by probit analysis

Log10 LC_{50} (µg/ml)	2.79
LC_{50} (µg/ml)	627.21
95% confidence limit (µg/ml)	489.79-813.71
Regression equation	Y = - 3.6121 + 3.1229 * X
Chi square	3.75

Antimicrobial Activity:

Antibacterial activity of extract of yellow oleander leaves was studied on five Gram positive and five Gram negative bacteria by disc diffusion method and compared with the standard antibiotic disc.

Antibacterial activity of yellow oleander leaves extract was measured at 100 µg/disc concentration. Poor activity was exhibited against Gram positive and Gram negative bacteria.

More specifically, yellow oleander leaves extract showed 2.7, 1.5, 2.5, 3.6 and 4.2 mm diameter of

zone of inhibition against *Staphylococcus aureus, Shigella sonnei, Klebsiella sp, Salmonella typhi* and *Shigella flexineri*, respectively and no activity against the four *Bacillus sp* and *Proteus sp* tested (Table 6). This result is in partial agreement with Ravikumar *et al.* (2007) description of antibacterial test against *Proteus vulgaris* culture. The methanolic extract of yellow oleander (leaves), however, showed significant inhibitory activities against the growths of *Salmonella*, *Shigella sp*; *Shigella flexneri*, *Shigella Virchow* and *Shigella dysenteriae* (Zakaria and Mohd, 1994). Standard antibiotic tetracycline (30 µg/disc) showed significant antibacterial activity against all Gram positive and Gram negative bacteria tested except *Proteus sp.* Manna *et al.* (2000) suggested that yellow oleander may have applications for various diseases, including arthritis, but all require further investigation.

Table 6. Antimicrobial activity against 10 bacterial isolates

Name of the bacteria	Ethanol extract 10 µg/filter paper disc. (Zone of inhibition) mm in diameter	Tetracycline 30 µg/disc (Zone of inhibition) mm in diameter
Staphylococcus aureus	2.7	16.5
Shigella sonnei	1.5	7.5
Klebsiella sp	2.5	28
Bacillus polymyxa	0	15.5
Bacillus cereus	0	10.5
Bacillus megaterium	0	12
Bacillus subtilis	0	10
Salmonella typhi	3.6	14.5
Proteus sp	0	0
Shigella flexineri	4.2	7

Conclusion

We reported here that yellow oleander leaves extract can be very effective in preventing castor oil-induced diarrhoea as well as to possess moderate cytotoxic and poor antimicrobial activities. However, further investigation warrant to isolation, identification and characterization of different active compounds from the extract and their mode of action responsible for these properties on different biological system is required.

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