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BIOLOGICAL ACTIVITIES OF ISOLATED COMPOUNDS FROM *VITEX NEGUNDO* LEAF

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Abstract

Context: *Viyex negundo* Linn. (Verbenaceae) is a beautiful tree which is an erect, large aromatic shrub with quadrangular branchlets possess pesticidal, antibacterial and antifungal properties.

Objective: To determine the biological activities (antibacterial, antifungal, brine shrimp lethality bioassay) of the two isolated compounds from methanolic leaf extract.

Materials and Methods: Powdered leaves of nishinda were extracted with methanol using Soxhlet's apparatus and subsequent analyses isolated two compounds. Five gram-positive, eight Gram-negative bacterial strains were used for the antibacterial activity using the disc diffusion assay method. The antifungal activities of the isolated compounds were also performed on four pathogenic fungi. Each pure compound was dissolved in 200 µl of methanol to get a concentration 300 µg/10 µl. Minimum Inhibitory Concentrations were determined by serial dilution technique. For brineshrimp bioassay each compound and standard amphicilin trihydrate were dissolved in dimethylsulfoxide to get a five concentrations. Each concentration contained three vials consisting of 10 nauplii in 5 ml of treated sea water. The number of survived nauplii were counted after 24 h and the LD_{50} values were calculated.

Results: The zone of inhibition was prominent for the control (kanamycin) at concentration of 30 µg/disc. At 100 µg/disc Compound 1 exhibited bigger and more prominent clear zone of growth inhibition in all test microorganisms except *Shigella shiga*. On the contrary, Compound 2 at 100 µg/disc, showed clear zone of inhibition in all bacteria, but inhibition of zones were larger in Compound 1 than Compound 2. Antimicrobial effect of Compound 2 tested on different pathogenic bacteria (MIC 128 µg/ml) and fungi showed that it possesses growth inhibitory effect at various concentrations. MIC of Compound 1 for *B. subtilis*, *S. aureus* and *S. B-haemolyticus* was 64 µg/ml, whereas for *P. aeruginosa* it was 128 µg/ml (Table 2). MIC of Compound 2 was 128 µg/ml for *B. subtilis*, *S.-β-haemolyticus* and *P. aeruginosa* whereas it was 64 µg/ml for *S. aureus*. No fungal activity was observed for Compound 1. Clear inhibition zone was observed for Compound 2 at both concentrations for all of the pathogenic fungi tested. At 100 µg/disc Compound 2 exhibited bigger and prominent clear zone than 50 µg/disc.. Brine shrimp bioassay showed the toxic effect of the both the compounds.

Conclusion: The findings indicate promising antibacterial and antifungal activities of *V. negundo* against life treating pathogens which appears to be an effective material for development of antimicrobial drugs and ecofriendly biopesticides.

Keywords: *Vitex negundo,* Antimicrobial, MIC, bacteria, phytochemical, medicinal plant activity, brineshrimp

Introduction

The importance of natural products in modern medicine has been well recognized. Scrutiny of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87% of all categorized human diseases (infectious and non-infectious) (Chin *et al*. 2006). According to a report of World Health Organization, more than 80% of world's populations depend on traditional medicine for their primary health care needs (Duraipandiyan *et al*. 2006). The increased interest in plant derived drugs is mainly because of the wide spread belief that 'herbal medicine' is safer than costly synthetic drugs which possesses side effects. Hence, there is need to screen medicinal plants for promising biological activity. Further, there is a continuous development of resistant strains which pose the need for search and development of new drug to cure diseases (Silver 1993).

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Vitex negundo (verberaceae) is an important source of such natural drugs. It is a reputed medicinal herb and its parts have been employed as a traditional cure in Asian systems of medicine (Indian, Bangladesh, Pakistan, Chinese, Malaysian) for a variety of disease conditions. A number of pharmacological activities have been attributed to *V. negundo*, such as: analgesic and anti-inflammatory activity (Dharmasiri *et al*. 2003), enzymes inhibition (Azhar-Ul-Haq *et al*. 2006), nitric oxide scavenging activity (Jagetia and Baliga 2004), snake venom neutralization activity (Alam and Gomes 2003), antifeeding activity (Chandramu *et al*. 2003), antiradical and antilipoperoxidative (Munasinghe *et al*. 2001), CNS activity (Gupta *et al*. 1999), hepatoprotective activity (Avadhoot and Rana 1991), antibacterial activity (Perumal Samy *et al*. 1998), anti-fungal (Damayanti *et al*. 1996), larvicidal activity (Pushpalatha and Muthukrishnan 1995), antiandrogenic effects (Bhargava 1989), mosquito repellent activity (Hebbalkar *et al*. 1992).

Phytochemical studies on *V. negundo* have afforded several types of compounds, such as volatile oils (Dayal and Singh 2000), lignans (Azhar-Ul-Haq *et al*. 2006), Flavonoids (Banerji *et al.* 1988), iridoids (Chandramu *et al.* 2003), terpenes (triterpenes, diterpenes, sesquiterpenes) (Chawla *et al.* 1991), and steroids (Maurya *et al*. 2007). The most flavonoid glycoside isolated from leaves of *V. negundo* of ethanolic extract is 5-hydroxy-3,6,7-trimethoxy-2-(3,4-dimtoxyphenyl)-4H-chrome-4-on and 5 ,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4H-chromen-4-one (Gautam 2008). Hence, in the present experiment an attempt has been made to evaluate the biological activity of mthanolic leaf extract of *V. negundo* (two pure compounds first time reported in this plant) against five prominent Gram-positive and eight Gram-negative human pathogenic bacteria and the biological activities of the extracts in terms of MIC, brine shrimp bioassay and antifungal activities were also determined.

Materials and Methods

Plant: The leaves of *V. negundo* were collected from the Rajshahi University and authenticated by the authority of the Department of Botany, University of Rajshahi where a voucher specimen (# 528) has been deposited.

Preparation of Extracts and isolation of two compound: Powdered leave (500 g) was extracted with methanol (MeOH) (BDH, England) using a Soxhlet's apparatus. The crude extract were stored in a refrigerator at -20°C. The subsequent analyses led to the isolation, identification and structural elucidations of two compounds 22, 23-dihydro α-spinasterol-β-D-glucoside (Compound-1) and 2-hydroxy benzoic acid (salicylic acid) (Compound 2) (Fig. 1). Both compounds were first time isolated from this plant.

Antibacterial activity: Five gram-positive (*Bacillus subtilis*, *B. megaterium*, *Sarcina lutea*, *Staphylococcus aureus*, *St. β-haemolyticus*) and eight gram-negative (*Shigella dysenteriae*, *Sh. shiga*, *Sh. boydii*, *Sh. sonnei*, *Escherichia coli*, *Pseudomonus aeruginosa*, *Klebsiella* sp., *Salmonella typhi*) strains were used for the anti-bacterial activity using the disc diffusion assay method (Bauer *et al*. 1966). The bacteria were collected from the cultures maintained in the microbiology laboratory of the Department of Pharmacy, University of Rajshahi.

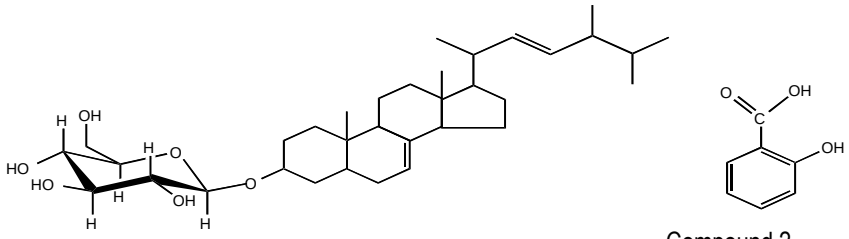
The test organism was transferred from the subculture to the test tube containing 20 ml sterile media. The bacterial suspensions were aseptically transferred to the sterile petri dish giving a uniform depth of media (4 mm). Kanamycin 30 μg/disc was used as control. Standard disc was prepared by pouring 10 μl of kanamycin stock solution (3 μg/μl). The sample disc, standard antibiotic disc and control disc were placed gently on the solidified agar plates. The plates were then inverted and kept in a refrigerator for about 24 h at 4°C to obtain maximum diffusion. Finally, the plates were incubated at 37.5°C for 18-24 h. The antibacterial activities of the test sample were determined by measuring the diameter of inhibitory zones in mm.

Brine shrimp bioassay: Brine shrimp bioassay (Mayer *et al*. 1982) of both the compounds was done. One milligram of each sample compound and standard antibiotic amphicilin trihydrate were dissolved in 200 μ1 of

dimethylsulfoxide (DMSO) to get a concentration of 5 µg/µl. The experiment was conducted into five groups. Each group contained three vials consisting of 10 nauplii in 5 ml of sea water. The concentrations of the sample were 5, 10, 20, 40 and 80 µg/ml, respectively. For control, three vials containing 10 brine shrimp nauplii in 5 ml seawater were taken and 20 µ1 DMSO was added in each vial. After 24 h, the vials were observed and the number of survived nauplii in each vial was counted. The LD_{50} was calculated by Probit analysis and regression lines were drawn according to Goldstein *et al*. (1974).

Determination of minimum inhibitory concentration: The minimum inhibitory concentration (MIC) of pure compounds was determined by agar dilution method (Vander-Berghe and Vlietinck 1991). The test organisms were *Bacillus subtilis, Staphylococcus aureus, St.-β-haemolyticus* and *Pseudomonus aerugonosa*. The compounds (Compound 1 and Compound 2) in various concentrations (2-512 µg/ml) and 10µl of bacterial culture (10^7cells/ml) were added in culture tubes containing 1 ml sterile nutrient broth medium. The cultures were mixed well and incubated at 37.5°C for 24 h and observed for growth of the bacteria.

Antifungal activity: The same procedure was followed as did for that of antibacterial activity. Nystatin was used as standard at and 50 µg/disc from the stock solution (5 µg/µl). The period of incubation was 48 h. The fungi (*Aspergillus niger, A. flavus, Candida albicans* and *Trichoderma* sp.) were collected from the Department of Botany, University of Rajshahi.



Compound 1 Compound 2

Fig. 1. Structure of isolated compounds (Compound 1. 22, 23-dihydro α –spinasterol- β -D-glucoside; Compound 2. 2-hydroxy benzoic acid

Results

Antibacterial activity: The zone of inhibition was prominent for the control (kanamycin) at concentration of 30 µg/disc (Table 1). At 100 µg/disc Compound 1 exhibited bigger and more prominent clear zone of growth inhibition in all test microorganisms except *Sh. shiga*. On the contrary, Compound 2 at 100 µg/disc, showed clear zone of inhibition in all bacteria, but inhibition of zones were larger in Compound 1 than Compound 2. Compound 2 did not develop any inhibition zone at 30 µg/disc against *Staphylococcus aureus* and *St. β-haemolyticus* and *Sh. shiga* and *Sh. sonnei*.

Minimum inhibitory concentration (MIC): The tested bacterial species did not show any growth when culture medium was supplemented with 128, 256 and 512 µg/ml of Compound 1and Compound 2. MIC of Compound 1 for *B. subtilis*, *St. aureus* and *St.-β-haemolyticus* was 64 µg/ml, whereas for *P. aeruginosa* it was 128 µg/ml (Table 2). MIC of Compound 2 was 128 µg/ml for *B. subtilis*, *St.-β-haemolyticus* and *P. aeruginosa* whereas it was 64 µg/ml for *St. aureus*. No inhibition zone was observed in test tubes containing Compound 1 and Compound 2 at a concentration less than 64 µg/ml. Three control tests were performed using nutrient C_M (medium), C_S (medium + sample) and C_1 (medium + inoculum) where bacterial growth was observed in C_1 only but the other two culture tubes were clear (Table 2). It is evident from the results that both the compounds have property to inhibit bacterial growth even at low concentration (64 µg/ml).

Table 1. Antibacterial activity of Ch-1 and Ch-2 and standard Kanamycin on the growth of Gram-positive and Gram-negative bacteria

Test organisms	Diameter of zone of inhibition (mm)				
	Compound 1 (µg/disc)		Compound 2 (µg/disc)		Kanamycin (µg/disc)
	30	100	30	100	30
Gram positive Bacteria					
Bacilus subtilis	14	16	11	16	25
B. megaterium	10	17	10	20	22
Sarcina lutea	15	20	10	15	28
Staphylococcus aureus	7	13	0	12	20
St.-β-haemolyticus	10	13	0	14	24
Gram Negative Bacteria					
Shigella dysenteriae	12	17	16	21	23
Sh. shiga	0	15	0	7	20
Sh. boydii	30	38	30	38	30
Sh. sonnei	10	15	0	14	24
Escherichia coli	25	30	23	30	26
Pseudomonus aeruginosa	8	17	7	12	20
Klebsiella sp.	10	15	7	12	25
Salmonella typhi	35	40	34	40	20

Table 2. Minimum inhibitory concentrations of Compound 1 and Compound 2 of *V. negundo* against five pathogenic bacteria.

Test tube No.	Diluted solution (µg/ml)	Bacterial growth observation against							
		Compound -1				Compound -2			
		Bacillus subtilis	*Staphylococcus aureus*	*St. -β-haemolyticus*	*Pseudomonus aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*	*St. -β-haemolyticus*	*Pseudomonus aeruginosa*
1	512	-	-	-	-	-	-	-	-
2	256	-	-	-	-	-	-	-	-
3	128	-	-	-	-	-	-	-	-
4	64	-	-	-	+	+	-	+	+
5	32	+	+	+	+	+	+	+	+
6	16	+	+	+	+	+	+	+	+
7	8	+	+	+	+	+	+	+	+
8	4	+	+	+	+	+	+	+	+
9	2	+	+	+	+	+	+	+	+
C_s	512	-	-	-	-	-	-	-	-
C_1	00	+	+	+	+	+	+	+	+
C_M	00	-	-	-	-	-	-	-	-

Table 3. Antifungal activity of Compound 2 and the standard drug Nystatin *in vitro*

Test Organisms	Diameter of zone of inhibition (in mm)		
	Ch-2 (µg/disc)		Nystatin
	50µg/disc	100µg/disc	50 (µg/disc)
Aspergillus niger	10	15	22
Aspergillus flavus	12	16	21
Candida albicans	12	15	20
Trichoderma sp.	9	14	24

Table 4. Effects of ampicillin trihydrate, Ch-1 and Ch-2 lethality bioassay on brine shrimp nauplii.

Group	Conc. (µg/ml)	Percent mortality	Regression equation	LD_{50} (µg/ml)
Control	20 µg DMSO	0	0	0
Ampicillin trihydrate	5	46.66	Y=4.388456 + 0.7314934X	6.85
	10	53.33		
	20	66.66		
	40	76.66		
	80	80.00		
Ch-1	5	10.00	Y=1.877592 + 2.229338X	25.15
	10	20.00		
	20	33.33		
	40	56.66		
	80	100.0		
Ch-2	5	6.666	Y=3.042883 + 1.044776X	74.68
	10	20.00		
	20	33.33		
	40	40.00		
	80	46.66		

Antifungal activity: No fungal activity was observed for Compound 1. Clear inhibition zone was observed for Compound 2 at both concentrations for all of the pathogenic fungi tested. At 100 µg/disc Compound 2 exhibited bigger and prominent clear zone than 50 µg/disc. However, Nystatin (control) showed prominent zone of inhibition at 50 µg/disc (Table 3).

Brine shrimp lethality bioassay: Bioactive compounds are always toxic in higher doses. The mortality rates of brine shrimps nauplii were found to be dose dependent. Regression lines indicated linear correlation between doses and mortality. The LD_{50} values of Compound 1, Compound 2 and standard Ampicilin trihydrate were found to be 25.153, 74.686 and 6.855 µg/ml, respectively (Table 4).

Discussion

The earlier work revealed that the methanol extract of nishinda leaves (*V. negundo*) possess pesticidal, antibacterial and antifungal properties (Chowdhury *et al.* 2009). The essential oil from fresh leaves of *V. negundo* was found to have significant antifungal activity against *Trichoderma viridae*, *Fusarium* sp., *Collectotrichum* and *Helminthosporium* (Uppalapani and Rao 1979); and antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* bacterial strains (Khokra *et al.* 2008). The extracts of *V. negundo* were found to be effective as antibacterial and antifungal against *Micrococcus pyogens* var. *aureus*, *Klebsiella aerogens*, *P. vulgaris*, *P. aerogens* (Patel *et al*. 2009) and *E. coli* (Patel *et al.* 2009).

Antimicrobial effect of Compound 2 tested on different pathogenic bacteria (MIC 128 µg/ml) and fungi showed that it possesses growth inhibitory effect at various concentrations. Salicylic acid is an important regulator of induced plant resistance to pathogens. This ingredient has multifunctional properties reducing the skin problems including acne, pimples and blemishes (Bradley 1992). It is an excellent exfoliant, anti-irritant quality as well as anti-inflammatory (Leung and Foster 1996). Moreover, salicylic acid helps with the treatment of breakouts because of its anti-microbial properties (Campbell 1990).

The results of the present investigation methanol extracts of leaves possesses compounds which might shows antibacterial activity. Although previously some reports concerning the antibacterial activity of *V. negundo* are present but our finding supports the efficacy. Kumar *et al.* (2006) studied the antibacterial activity of dichloromethane: methanol (1:1 v/v) extracts of *V. negundo* against different bacterial strains. Their finding conclude that none of the micro-organisms including the bacterial strains like *B. subtilis, S. aureus, S. epidermidis, E. coli* and *P. aeruginosa* were inhibited by dichloromethane: methanol extract.

Ahmad *et al*. (1998) studied the antibacterial activity of *V. negundo* whole plant of hexane, alcoholic and aqueous extracts against *B. subtilis, E. coli, Proteus vulgaris, Sa. typhimurium, P. aeruginosa* and *S. aureus* had no activity. Valasraj *et al.* (1997) studied antibacterial activity of ethanol extracts of *V. negundo* leaf using agar dilution method against four bacteria *B. subtilis, S. epidermidis, E. coli* and *P. aeruginosa.* They conclude that antibacterial activities against Gram-positive bacteria were more pronounced then against Gram-negative. Their finding showed that at concentration 6.25 mg/ml inhibition was found against *B. subtilis* where as other organism's viz. *S. epidermidis, E. coli* and *P. aeruginosa* were inhibited at a concentration of 25.0 mg/ml. Panda *et al.* (2009) obtained with ethanol and methanol extracts of leaves; petroleum ether and chloroform extract of bark exhibited significant antibacterial activity against three Gram-positive bacteria viz. *S. epidermidis, B. subtilis, S. aureus* and five Gram-negative bacteria viz. *E. coli, Sa. typhimurium, P. aeruginosa, V. cholerae* and *V. alginolyteus.*

So far the antibacterial activity on *V. negundo* tested by Kumar *et al.* (2006) and Ahmad *et al.* (1998) resulted in negative results. On the other hand, Valasraj *et al*. (1997) reported positive response with four strains only and Panda *et al.* (2009) reported eight strains.However, our results obtained have better inhibitory effect as

compared to Valasraj *et al*. (1997). Comparison of the data obtained in this study with previously published result is problematic. First, the composition of the plant extracts is known to vary according to local climatic and environmental conditions (Janssen *et al*. 1987, Sivropoulou *et al* 1995). Secondly, the method used to assess antibacterial activity and the choice of the test organisms also varies (Janssen *et al.* 1987). Most frequently used methods to antibacterial activity are agar diffusion techniques and broth dilution methods. The results obtained by each of these methods may differ as many factors vary between assays (Janssen *et al.* 1987, Hili *et al.* 1997). In vivo studies may be required to confirm the values of the some of the results obtained

Conclusion

The present results indicate that Compound 1 is highly active against Gram-positive bacteria (*Sarcina lutea*) and Gram-negative bacteria (*S. boydii* and *Klebsiella* sp.). The MIC results indicate that both the compounds are effective to inhibit bacterial growth at low concentration (64 μg mL^{-1}). Compound 2 showed strong fungicidal activities at 50 μg/disc. Compound 1 was more cytotoxic in brineshrimp bioassay (LD_{50} 25.153) than Compound 2. These findings lead to further *in vivo* studies, using animal model, to explore the potential application of this protocol for bacterial pathogen treatment in immune compromised patients and also in preservation of food, pharmaceutical and cosmetic formulations to protect product from microbial activity.

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