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Characterization and Biological Screening of a Triterpenoid from *Nymphoides cristatum*

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Abstract: A triterpenoid, Bet-20(29)-en-3-ol-28-oic acid (1) was isolated from the ethyl acetate extract of *Nymphoides cristatum* (Roxb.) O.kuntze and its structure was elucidated on the basis of spectral evidences. This is the first report of isolation of this compound from this plant. The compound was screened for antimicrobial activity against a number of pathogenic bacteria and fungi and the cytotoxic activity by brine shrimp lethality bioassay. The test agent exhibited significant antimicrobial activity against most of the bacteria and fungi. The minimum inhibitory concentration (MIC) of the test agent determined against *Bacillus cereus, Bacillus subtilis, Escherichia coli and Shigella sonnei* was found to be 64, 128, 64 and 32 μg/ml respectively. The compound also showed prominent cytotoxic activity. The LC_{50} (median lethal concentration) was 15.39 μg/ml.

Key words: *Nymphoides cristatum*, triterpenoid, antimicrobial activity ,cytotoxicity

Introduction

Nymphoides cristatum (Roxb.) O.kuntze (Menyanthaceae), an aquatic herb, belonging to the genus *Nymphoides* Hill (14 species in fresh water) is widely distributed through the tropical and temperal zones (Hooker, 1985). This plant is found in India, Srilanka and China (Kirtikar and Basu, 1994). In Bangladesh, it is found to grow in ponds, tanks, beels, jheels and haors (Khan and Halim, 1987). The plant is commonly used as a substitute for chiretta and in the treatment of fever and jaundice (Chopra and Nayer, 1956). Milk is increased when it is fed on the milked cow (Biswas, 1973). The plant juice is also used in the treatment of epilepsy, convulsion of infants, palpitation and to relief the tiredness of the body (Bhattacharjee, 1991). Recently a report has shown that the ethyl acetate extract of *Nymphoides cristatum* (Roxb.) O.kuntze possesses the significant antimicrobial activity and cytotoxicity (Rahman *et al.*, 2001). Therefore we have studied further for the isolation of bioactive principles from this plant. The characterization of the structure, antimicrobial activity and brine shrimp lethality directed cytotoxicity of a triterpenoid isolated from the ethyl acetate extract of *Nymphoides cristatum* (Roxb.) O.kuntze.

Materials and Methods

Plant materials: The plant, *Nymphoides cristatum* (Roxb.) O.kuntze was collected from different beels and jheels of Paksey in the district of Pabna during October-November 1999. The plant was identified by both Bangladesh National Herbarium, Dhaka and Department of Botany, University of Rajshahi, Bangladesh.

Extraction and isolation: The powdered plant material (960 gm) was successively extracted with petroleum ether (40-60^{0}C) and ethyl acetate in a soxhlet apparatus. The extracts were concentrated by a vacuum rotary evaporator under reduced pressure and the ethyl acetate extract (14 gm) was subjected to column chromatography over silica gel (70-230 mesh). The column was successively eluted with petroleum ether, increasing amounts of ethyl acetate in petroleum ether and finally with methanol which provided fractions from 1 to 25. The eluents were subsequently tested by analytical thin layer chromatography under UV light, in iodine chamber, by dragendorff's reagent and vanillin-sulfuric acid spray. Multiplate preparative TLC using petroleum ether : ethyl acetate (9:4) of fraction 10 afforded compound (1) (10 mg).

Compound (1), white crystalline solid, m.p.194-195^{0}C. IR ν_{max}: 3624, 3074, 1705, 1641 and 884 cm^{-1}. EIMS m/z (ret. int.): 456 [M$^+$], 408, 218, 208, 203 and 189. ^{1}H NMR: δ : 3.12 (1H, m, $W_{1/2}$ = 6.8 H_Z, H-3), 2.947 (1H, d, J = 10.5, 4.2 H_Z, H-19), 4.543 and 4.675 (1H each, d, J = 2.0 H_Z, C = CH_2 -29), 0.963 (3H, s, Me-23), 0.823 (3H, s, Me-24), 0.862 (3H, s, Me-25), 0.987 (3H, s, Me-26), 0.916 (3H, s, Me-27), 1.634 (3H, s, Me-30) ; ^{13}C NMR: δc 39.599 (C-1), 34.131(C-2), 218.124(C-3), 47.307(C-4), 544.913(C-5), 19.667(C-6), 33.555(C-7), 40.764(C-8), 49.778(C-9), 36.865(C-10), 21.467(C-11), 25.170(C-12), 38.167(C-13), 42.877(C-14), 27.409(C-15), 35.507(C-16), 42.792(C-17), 47.946(C-18), 48.230(C-19), 150.833(C-20), 29.817(C-21), 39.961(C-22), 26.634(C-23), 21.018(C-24), 15.771(C-25), 15.846(C-26), 14.462(C-27), 17.996(C-28), 109.378(C-29), 19.667(C-30) (Rahman *et al.*, 2000).

Antibacterial screening: Twelve pathogenic bacteria were selected for the test. The isolated compound (1) was dissolved methanol to get a concentration of 200 μg per 10 μl. Then *in vitro* antibacterial activity of the test agent was carried out by the standard disc diffusion method (Barry, 1980; Berghe and Vlietnck, 1991; Buer *et al.*, 1966) against selected test organisms. The diameters of zone of inhibition produced by the agent were compared with those produced by the standard antibiotic (Kanamycin, 30 μg/disc).

Minimum inhibitory concentration (MIC): The MIC value of the compound (1) was determined against two gram positive (*Bacillus cereus, Bacillus subtilis*) and two gram negative (*Escherichia coli* and *Shigella sonnei*) bacteria. The test was carried out by serial dilution technique (Reiner, 1982). Nutrient agar and nutrient broth were used as bacteriological media.

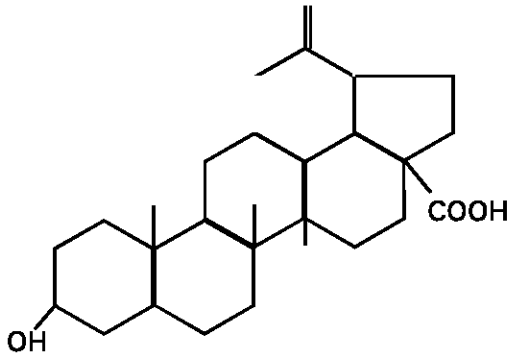
Antifungal screening: Five pathogenic fungi were selected for the test. PDA was used as fungicidal media. The compound (1) was dissolved in sufficient volume of methanol to get a concentration of 400 μg/disc. Then *in vitro* antifungal activities of the compound was performed by disc diffusion method (Beur, 1966). Clotrimazole was used as a standard one.

Brine shrimp lethality bioassay: The cytotoxic effect of compound (1) was evaluated by LC_{50} of brine shrimp Lethality test (Persone, 1980; Meyer *et al.*, 1982). The compound was dissolved in DMSO and five graded doses 5, 10, 20, 40 and 80 μg/ml respectively were used for 5 ml sea water containing 10 brine shrimp nauplii in each group. The number of survivors was counted after 24 hours and LC_{50} was determined by probit analysis. The experiment was carried out in quadruplicate and the mean LC_{50} value was measured.

Results and Discussion

The ethyl acetate extract of *Nymphoides cristatum* (Roxb.) O.kuntze after chromatography over silica gel yielded a pure compound (1) which was obtained as a white crystalline solid having melting point 193-195^{0}C. It produced a single spot on the TLC plate after spraying with vanillin-sulfuric acid and heating. The IR spectrum of the compound showed strong band at 3624 and 1705 cm^{-1} which could be assigned for hydroxyl function. The spectrum also revealed stretching at 1641 and 884 cm^{-1} (C=CH_2). The mass spectrum displayed a highest ion peak (M^+) at m/z 456 corresponding to $C_{30}H_{48}O_3$. Other peaks included at m/z 408, 218, 208, 203 and 189.

The ^{1}H NMR spectrum of compound (1) showed six singlets at δ 0.823 (3H, Me-24), 0.862 (3H, Me-25), 0.916 (3H, Me-27), 0.963 (3H, Me-23), 0.987 (3H, Me-26) and 1.634 (3H, Me-30), each integrating for three protons which was assigned for six methyl groups in the compound. A carbinylic proton at C-3 was assigned by the presence of characteristic signal at δ 3.12 (1H, m, $W_{1/2}$ = 6.8 H_Z, H-3). This carbinylic proton is highly de-shielded due to the OH substituent at C-3. Two doublets at δ 4.543 and 4.675 (1H each, J = 2.0 H_Z) represents two protons at C-29 (C=CH_2) A single proton at C-19 was assigned by the double triplet (dt) at δ 2.947 (J = 10.5 and 4.2 H_Z). All the proton peaks in ^{1}H NMR data of the compound are in good agreement with those of betulinic acid (Herz *et al.*, 1972).



Bet-20 (29)-en-3-ol-28-oic acid (1)

On the basis of ^{1}H NMR spectral data coupled with physical and chemical evidence the structure of the compound was thus determined as Bet-20(29)-en-3-ol-28-oic acid (1). This is the first report of isolation of this compound from this plant.

The result of antibacterial and antifungal activity of the compound (1) are presented in Table, 1 and respectively. The compound showed significant antibacterial activity against almost all the tested bacteria and produced zone of inhibition in between 15-23 mm. It showed highest inhibitory activity against *Bacillus cereus, Bacillus subtilis, Escherichia coli, Shigella sonnei* and the MIC values against these organisms were 64, 128, 64 and 32 μg/ml respectively are presented in Table 3. The compound also showed activity against the tested pathogenic fungi.

Table 1: Antibacterial activity of compound (1) isolated form *Nymphoides cristatum* (Roxb.)O.kuntze

	Diameter of zone of inhibition in mm	
Test organism	Compound (1)	SK
Gram positive		
Bacillus cereus	23	24
Bacillus subtilis	20	26
Bacillus megaterium	19	28
Staphylococcus aureus	15	27
Streptococcous β-haemolyticus	17	24
Gram negative		
Escherichia coli	22	27
Shigella dysenteriae	18	26
Shigella shiga	17	22
Shigalla flexneriae	16	27
Shigella sonnei	21	23
Shigella boydii	16	25
Klebsiella species	14	19

Compound (1) = Bet-20(29)-en-3-ol-28-oic acid (200 μg/disc)
SK = Standard Kanamycin (30 μg/disc)

Table 2: Antifungal activity of the compound (1) isolated form *Nymphoides cristatum* (Roxb.) O.kuntze

	Diameter of zone of inhibition in mm	
Test organism	Compound (1)	SC
Aspergillu fumigatus	11	22
Hensinela califomica	14	23
Phizopus arizae	10	25
Schizosporum species	12	24
Rhizopus arizae	13	21

Compound (1) = Bet-20(29)-en-3-ol-28-oic acid (400 μg/disc)
SC = Standard Clotrimazole (30 μg/disc)

Table 3: The minimum inhibitory concentration (MIC) values of the compound (1) against test organisms

Organisms	*Bacillus cereus*	*Bacillus subtilis*	*Escherichia coli*	*Shigella sonnei*
μg/ml	64	128	64	32

Compound (1) = Bet-20(29)-en-3-ol-28-oic acid

In brine shrimp lethality bioassay the cytotoxicity of the compound (1) is presented in Table 4. The compound showed positive result, indicating the biological activity. The 50% mortality (LC_{50}) of the compound was found to be 15.39 μg/ml and 95% confidence limits is 8.87-26.68. A regression equation, Y = 3.06 + 1.63X and χ^2 value 0.215 are observed from probit analyses which were compared with galic acid (Saker *et al.*, 1998) as a standard one. Although there was no mortality in the control group, the test sample showed different mortality rate at different concentrations and found to be increased with increasing concentration of the sample. It is evident that the compound was moderately lethal to brine shrimp nauplii. An evaluation of cytotoxicity is also an important study for possible clinical use i.e., indicative of wide range of pharmaceutical activities of the drugs. In that sense, the mortality rate of the compound (1) with highest concentration suggest that the drug can be used at higher

Table 4: Cytotoxicity of the compound (1) by brine shrimp lethality bioassay

Test sample	Concentration μg/ml	% Mortality	LC_{50} (ppm)	95% Confidence limits	Regression equation	χ^2 Value
	5	20				
	10	30				
Compound (1)	20	50	15.39	8.87-26.68	Y = 3.06 + 1.63X	0.215
	40	70				
	80	90				
Galic acid	-	-	4.53	3.33-6.15	Y = 3.93 + 1.62X	1.25

Compound (1) = Bet-20(29)-en-3-ol-28-oic acid

doses and also suitable for further clinical trial.

In conclusion, the present study reports for the first time the antibacterial, antifungal activity and cytotoxicity of the compound (1) isolated form the *Nymphoides cristatum* (Roxb.) O.kuntze. However, further and specific studies are needed to better evaluate the potential effectiveness of the isolated the compound from the *Nymphoides cristatum* (Roxb.) O.kuntze as an antimicrobial agent.

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