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Antibacterial, antifungal and cytotoxic activities of 3,5-diacetyltambulin isolated from *Amorphophallus campanulatus* Blume ex. Decne

[1]Khan A., [2]Rahman M., [2]Islam M.S.

[1]*Department of Pharmacy,* [2]*Department of Animal Husbandry and Veterinary Science, University of Rajshahi, Rajshahi 6205, Bangladesh*

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ABSTRACT

Background and purpose of the study: Amorphophallus campanulatus is widely distributed in Bangladesh, India, and Africa and the tuberous roots of the plant has many traditional uses and is an important source of biologically active compounds. In the present study *in vitro* antibacterial, antifungal and cytotoxic activities of 3,5-diacetyltambulin which is a flavonoid isolated from *Amorphophallus campanulatus* was studied.

Materials and Methods: *In vitro* antibacterial and antifungal activities was evaluated by disc diffusion and MICs technique was determined by serial dilution technique. Cytotoxicity was determined against brine shrimp nauplii.

Results and Major conclusion: The compound showed significant antibacterial activities against four Gram-positive bacteria (*Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, *Streptococcus β-haemolyticus*) and six Gram-negative bacteria (*Escheichia coli*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella flexneri*, *Pseudomonus aeruginosa*, *Salmonella typhi*). The MIC values against these bacteria ranged from 8 to 64 µg/ml but had weak antifungal activity against a number of fungi. In cytotoxicity determination, LC_{50} of the compound against brine shrimp nauplii was 10.02 µg/ml.

Keywords: Flavonoid, Gram-positive, Gram-negative, MIC, Cytotoxicity.

INTRODUCTION

The Frequency of life threatening infections caused by pathogenic microorganism is increased worldwide and is becoming an important cause of morbidity and mortality in immunocompromised patients in the developing countries (1). Although huge numbers of antimicrobial agents have been discovered, the pathogenic microorganisms are developing resistance against these agents day by day (2,3). In third world countries irrational use of antimicrobial agent is a major cause of such resistance (3). In recent years, attempts have been made to investigate the indigenous drugs against infectious diseases (4). Research in the field of indigenous plant is a significant aspect to develop a safer antimicrobial principle through isolation, characterization, identification and biological studies (4).

Amorphophallus campanulatus Blume ex. Decne (Fam. Araceae), locally known as Ol Kachu, is a perennial herb with rounded tuberous root stock (corm) that is widely distributed in Bangladesh, India, and Africa (5-7). The tuberous roots of the plant have been used traditionally for the treatment of piles, abdominal pain, tumors, enlargement of spleen, asthma and rheumatism (5-7). The tuberous roots of the plant also have tonic, stomachic and appetizer properties (6,7). It is evident from its traditional uses that the root of the *Amorphophallus campanulatus* is an important source of biologically active compounds. The analgesic activity (8), tyrosinase and laccase activities (9) and inhibitory activities against amylase, trypsin and chymotrypsin (10) for *Amorphophallus campanulatus* have been reported. Its traditional uses in the treatment of tumors, enlargement of spleen indicate that the tuberous roots of the plant might contain antimicrobial, antifungal or cytotoxic constituents. The present study was designed to determine antibacterial, antifungal and cytotoxicity of the isolated flavonoid 3,5-diacetyltambulin.

MATERIALS AND METHODS

Plant materials

The tuberous roots of *Amorphophallus campanulatus* Blume ex. Decne was collected during January 2004 from Katakhali area of Rajshahi district of Bangladesh and identified by

Correspondence: alamkhan792002@yahoo.co.in

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Prof. A. T. M. Naderuzzaman, Department of Botany, University of Rajshahi, Bangladesh where a voucher specimen (No. AC9642) was deposited. The tuberous roots were cut, air-dried and ground into powder.

Plant materials extraction and fractionation
Powdered dried roots (600 g) of the plant were extracted with ethanol (4 L) in flat bottom glass containers, through occasional shaking and stirring for 10 days (11). The whole extract was filtered and the solvent were evaporated to dryness *in vacuo* by a rotary evaporator at 40 - 50° C to afford a blackish green mass (34 g) which was further extracted with petroleum ether (3 x 50 ml), chloroform (3 x 50 ml) and methanol (3 x 50 ml) to afford petroleum ether (17 g), chloroform (8 g) and methanol (7 g) fractions, respectively (12). The preliminary phytochemical screening of different fractions was carried out by chemical tests and thin layer chromatographic method (13).

Isolation of compound
All fractions (petroleum ether, chloroform and methanol) were subjected to antibacterial screening and of which chloroform fraction showed better antibacterial activity. The chloroform soluble fraction (3 g) was subjected to column chromatography using n-hexane, chloroform and methanol of increasing polarity. Column chromatography fractions eluting with 100% chloroform to 90% chloroform in methanol which showed good antibacterial activity were subjected to preparative TLC (Silica gel PF_{254}) using solvent system: chloroform: n-hexane (20: 1) to afford compound **X** (27.6 mg). Its structure was confirmed on the basis of various spectroscopic methods (IR, liquid chromatography/electrospray-mass spectroscopy (LC/ES-MS), ^{1}H and ^{13}C NMR including JMOD, COSY, NOESY, HMBC, HSQC). The liquid chromatography/electrospray-mass spectroscopy (LC/ES-MS) in the positive ion mode of **X** showed molecular $[M+H]^+$ peak at m/z 429.3 corresponding to a molecular formula of $C_{22}H_{20}O_9$. The ^{1}H-NMR of compound **X** showed three methoxyl groups protons [δ 3.95 (6H, s), δ 3.89 (3H, s)], four aromatic protons [δ 7.03 (2H, d, J = 9 Hz, H-3´, H-5´), δ 7.88 (2H, d, J = 9 Hz, H-2´, H-6´)], one aromatic proton [δ 6.67 (1H, s)], and two acetoxyl groups protons [δ 2.34 (3H, s), δ 2.44 (3H, s)]. ^{1}H and ^{13}C NMR data (Table 5 and 6) of compound **X** were in good agreement with ^{1}H and ^{13}C NMR data of 3,5-diacetyltambulin reported in literature (14). In solubility test compound **X** was sparingly soluble in water and freely soluble in chloroform, methanol, ethanol and acetone.

Column chromatography of fractions which eluted with 70-90% chloroform in n-hexane also showed good antibacterial activity and subjected to preparative TLC (Silica gel PF_{254}) with solvent system: chloroform, n-hexane (2:3) to afford compound **M** (19.4 mg). The spectral data of compound M was indicated that it was an impure and hence failed to define by structure.

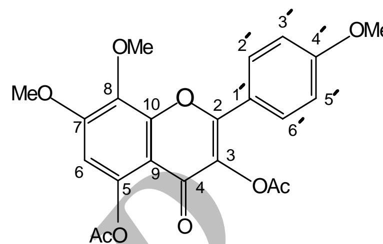

Figure 1. Structure of 3,5-diacetyltambulin (**X**).

Organisms
Antibacterial activity and MIC were determined against four Gram-positive bacteria (*Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, *Streptococcus β-haemolyticus*) and six Gram-negative bacteria (*Escheichia coli*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella flexneri*, *Pseudomonus aeruginosa*, *Salmonella typhi*). These organisms were available in the Microbiology Research Laboratory of Pharmacy Department, Rajshahi University, Bangladesh. The pure cultures of these bacteria were collected from the Microbiological Laboratory of the Institute of Nutrition and Food Science (INFS) and Department of Microbiology, University of Dhaka, Bangladesh. Antifungal screening was carried out against four fungi (*Aspergillus flavus*, *Aspergillus niger*, *Candida albicans* and *Rhizopus aurizae*). These organisms were available in the Microbiology Research Laboratory of Pharmacy Department, Rajshahi University, Bangladesh. The pure cultures of these fungi were collected from the Department of Botany, University of Rajshahi, Bangladesh. Cytotoxicity was determined against brine shrimp nauplii. Brine shrimp nauplii were obtained by hatching brine shrimp eggs (Carolina Biological Supply Company, Burlington, NC, USA) in artificial sea-water (3.8% NaCl solution) for 48 hrs.

Media
Nutrient agar medium (Difco laboratories) of pH 7.2, nutrient broth medium (Difco laboratories) of pH 6.8, Sabouraud dextrose agar medium (Biolife Vole Monza) of pH 5.6 and artificial sea-water(3.8% NaCl solution) of pH 8.4 were used for antibacterial screening, MIC determination, antifungal screening and cytotoxicity determination, respectively. .

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Antibacterial screening

In vitro antibacterial screening was carried out by disc diffusion method (15-16), which is a qualitative to semi quantitative test. Briefly, 20 ml quantities of nutrient agar were plated in petri dish with 0.1 ml of a 10^{-2} dilution of each bacterial culture (18 h old). Filter paper discs (6 mm in diameter) impregnated with various concentration of 3,5-diacetyltambulin were placed on test organism seeded plates. Methanol was used to dissolve the compound and was completely evaporated before application on test organism seeded plates. Blank disc impregnated with methanol followed by drying off was used as negative control. The activity was determined after 18 hrs of incubation at 37 °C. The diameters of zone of inhibition by 3,5-diacetyltambulin were then compared with chloroform fraction (160 μg/disc) and kanamycin as standard antibiotic (30 μg/disc). Each sample was used in triplicates for the determination of antibacterial activity.

Minimum inhibitory concentration (MIC) determination

Serial tube dilution technique (2, 3, 17, 18) was used to determine MIC of compounds. 3,5-Diacetyltambulin (1.024 mg) was dissolved in 2 ml distilled water (3 drops Tween 80 was added to facilitate dissolution) to obtain stock solution having concentration of 512 μg/ml. In serial dilution technique, 1 ml of the prepared stock solution was transferred to test tube containing 1 ml nutrient broth medium to give concentration of 256 μg/ml from which 1 ml was transferred to another test tube containing 1 ml of nutrient broth medium to give concentration of 128 μg/ml and so on up to concentration 2 μg/ml. After preparation of suspensions of test organisms (10^7 organism per ml), 1 drop of suspension (0.02 ml) was added to each broth dilution. After 18 hrs incubation at 37^0 C, the tubes were then examined for the growth. The MIC of 3,5-diacetyltambulin was taken as the lowest concentration that showed no growth. Growth was observed in those tubes where concentration of the 3,5-diacetyltambulin was below the inhibitory level and the broth medium was observed turbid (cloudy). Concurrently, like 3,5-diacetyltambulin serial tube dilution technique was carried out for kanamycin as standard to take as a positive control. Distilled water with 3 drops of Tween 80 was used as negative control.

Antifungal screening

In vitro antifungal screening was carried out by disc diffusion method (15, 16). In this method, 20 ml quantities of Sabourand dextrose were plated in petridish with 0.2 ml of a 10^{-2} dilution of each fungal culture (10 h old). Filter paper discs (6 mm in diameter) impregnated with various concentration of 3,5-diacetyltambulin were placed on test organism seeded plates. Methanol was used to dissolve the compound and was completely evaporated before application on test organism seeded plates. Blank disc impregnated with solvent methanol followed by drying off was used as negative control. The activity was determined after 72 h of incubation at 30^0 C. The diameters of zone of inhibition produced by the 3,5-diacetyltambulin were then compared with chloroform fraction (160 μg/disc) and kanamycin standard antibiotic (30 μg/disc). Each sample was used in triplicate for the determination of antifungal activity.

Cytotoxicity assay

The cytotoxicity assay was performed on brine shrimp nauplii using Mayer method (19-20). Brine shrimp nauplii were obtained by hatching brine shrimp eggs (Carolina Biological Supply Company, Burlington, NC, USA) in artificial sea-water (3.8% NaCl solution) for 48 hrs. Dissolution of 1 mg of compound was performed in 2 ml of artificial sea water containing 20% DMSO to give concentration of 0.5 μg/μl. From this solution 5, 10, 20, 50, 100, 200 and 400 μl were transferred to each 5 ml vial and using artificial sea water volume was adjusted to 5 ml by artificial sea water to give concentrations of compound of 0.5, 1, 2, 5, 10, 20 and 40 μg/ml, respectively. Brine shrimp nauplii were grown in these solutions and observed for mortality for 24 h. The resulting data were transformed to probit analysis (21) for determination of LC_{50} values of the compound. Cytotoxicity of chloroform fraction was also determined using this method. Artificial sea-water medium containing DMSO that was used for the analysis employed as control. Gallic acid and vincristine sulfate were used as standards in this assay.

RESULTS

The results of antibacterial activity of 3,5-diacetyltambulin against the test bacteria are presented in Table 1. In comparison to kanamycine as reference standard (30 μg/disc) and chloroform fraction (160 μg/disc), 3,5-diacetyltambulin exhibited significant antibacterial activity at 160 μg/disc which was higher against *Streptococcus-β-haemolyticus* and lower against *Salmonella typhi*. The MIC values of 3,5-diacetyltambulin against these Gram-positive bacteria ranged from 8 to 16 μg/ml and against Gram-negative bacteria ranged from 16 to

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Table 1. *In vitro* antibacterial activities of 3,5-diacetyltambulin isolated from *Amorphophallus campanulatus*. Data are presented as Mean ± SEM

Test organism	Strain No.	Diameter of zone of inhibition (mm)				
		Chloroform Fraction 160 µg/disc	**X** 30 µg/disc	**X** 80 µg/disc	**X** 160 µg/disc	Kanamycin 30 µg/disc
Gram-positive						
Bacillus subtilis	QL 40	16 ± 1.3	9 ± 0.8	20 ± 1.6	21 ± 1.9	30 ± 2.1
Bacillus megaterium	QL 38	14 ± 1.5	11 ± 1.2	18 ± 1.6	23 ± 1.8	28 ± 0.9
Staphylococcus aureus	ATCC 259233	17 ± 1.8	12 ± 0.9	18 ± 1.5	24 ± 1.4	31 ± 1.4
Streptococcus-β-haemolyticus	CRL	16 ± 1.6	13 ± 0.8	22 ± 1.7	25 ± 2.0	25 ± 1.7
Gram-negative						
Escheichia coli	FPFC 1407	13 ± 1.5	9 ± 1.2	16 ± 0.9	19 ± 1.4	24 ± 1.4
Shigella dysenteriae	AL 35587	15 ± 1.2	10 ± 1.2	18 ± 1.2	20 ± 1.3	30 ± 2.4
Shigella sonnei	AJ 8992	18 ± 0.7	10 ± 1.0	16 ± 1.3	17 ± 0.9	32 ± 1.7
Shigella flexneri	AL 30372	16 ± 1.7	11 ± 0.8	18 ± 1.2	21 ± 1.5	28 ± 1.2
Pseudomonus aeruginosa	CRL	17 ± 1.4	9 ± 1.1	15 ± 0.9	16 ± 1.2	31 ± 1.7
Salmonella typhi	B 56	11 ± 0.9	7 ± 1.2	11 ± 0.8	14 ± 1.4	28 ± 2.1

The control disc used for solvent (dried off before application) had no zone of inhibition, so their data was omitted from the above data. Data are represented in the form of mean of three tests ± SEM of the standard kanamycin group. **X** = 3,5-diacetyltambulin. QL, ATCC, CRL, FPFC, AL, AJ and B are culture collection number given by different culture collection organizations e.g. ATCC number is given by American type culture collection organization, America; CRL number is given by Centro de Referencia Para Lactobacilos, Germany; AJ number is given by Central Research Laboratories, Ajinomoto Co, Inc., Kawasaki, Japan".

Table 2. Minimum inhibitory concentration of 3,5-diacetyltambulin isolated from *Amorphophallus campanulatus*.

Bacteria	MIC values of Chloroform Fraction (µg/ml)	MIC values of 3,5-diacetyltambulin (µg/ml)	MIC values of Kanamycin (µg/ml)
Bacillus subtilis	64	16	2
Bacillus megaterium	32	8	4
Staphylococcus aureus	32	16	8
Streptococcus β-haemolyticus	64	8	8
Escheichia coli	32	16	8
Shigella dysenteriae	32	16	2
Shigella sonnei	64	32	4
Shigella flexneri	32	16	16
Pseudomonus aeruginosa	64	64	16
Salmonella typhi	128	64	4

The negative control used for solvent had no MIC values, so their data was omitted from the above data.

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Table 3. *In vitro* antifungal activity of 3,5-diacetyltambulin isolated of *Amorphophallus campanulatus* Data are presented as Mean ± SEM

.Test organism	Strain No.	Diameter of zone of inhibition (mm)				
		Chloroform Fraction 160 µg/disc	**X** 30 µg/disc	**X** 80 µg/disc	**X** 160µg/disc	Nystatin disc 30 µg/disc
Aspergillus flavus	MC 21	10 ± 1.2	6 ± 0.7	8 ± 0.6	9 ± 1.2	17 ±1.3
Aspergillus niger	ATCC 9142	9 ± 0.9	0	7 ± 0.7	8 ± 0.8	16 ± 1.4
Candida albicans	SC 5314	7 ± 1.0	0	8 ± 0.7	8 ± 1.1	18 ± 0.9
Rhizopus aurizae	IFO 4707	8 ± 1.3	7 ± 0.8	7 ± 1.0	10 ± 1.2	15 ± 1.1

The control disc used for solvent (dried off before application) had no zone of inhibition, so their data was omitted from the above data. Data are represented in the form of mean of three tests ± SEM of the standard nystatin group. **X** = 3,5-diacetyltambulin. MC, ATCC, SC, and IFO are culture collection number given by different culture collection organizations e.g. MC and ATCC are given by American type culture collection organization, America; SC is given Squibb institute for medical research, New Jersey, USA and IFO number is given by Institute for fermentation Osaka, Japan.

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Table 4. Cytotoxicity of 3,5-diacetyltambulin isolated from *Amorphophallus campanulatus* expressed by LC_{50} value (calculated using probit analysis).

Sample	LC_{50} (µg/ml)	95% confidence limits (µg/ml)	Regression equation
3,5-diacetyltambulin	10.02	6.20 - 16.18	Y = 2.43 + 2.56 X
Chloroform fraction	14.31	11.84 - 17.30	Y = 1.14 + 3.33 X
Gallic acid	4.53	3.33 - 6.15	Y = 3.93 + 1.62 X
Vincristine sulfate	0.76	0.57 - 0.82	Y = 3.16 + 2.98 X

Table 5. ^1H-NMR Data ($CDCl_3$, 400 MHz) of compound **X**.

Position of Protons	Δ Values in ppm (*J* in Hz)
H-6	6.67 (1H, s)
H-3´, H-5´	7.03 (2H, d, *J* = 9)
H-2´, H-6´	7.88 (2H, d, *J* = 9)
7-OCH_3, 8-OCH_3	3.95 (6H, s)
4´-OCH_3	3.89 (3H s)
3-$OCOCH_3$	2.34 (3H. s)
5-$OCOCH_3$	2.44 (3H, s)

Table 6. ^{13}C-NMR Data ($CDCl_3$, 100 MHz) of compound **X**.

Position of Carbon	Δ Values in ppm
C-2	148.7
C-3	132.2
C-4	170.5
C-5	144.2
C-6	110.5
C-7	134.8
C-8	155.6
C-9	155.1
C-10	112.1
C-1´	122.2
C-2´	130.2
C-3´	114.3
C-4´	162.2
C-5´	114.3
C-6´	130.2
7-OCH_3	55.2
8-OCH_3	61.4
4´-OCH_3	54.9
3-$OCOCH_3$	20.7
5-$OCOCH_3$	21.6
3-$OCOCH_3$	171.4
5-$OCOCH_3$	173.5

64 µg/ml where MIC values of chloroform fraction against Gram-positive bacteria ranged from 32 to 64 µg/ml and against Gram-negative bacteria were 32 to 128µg/ml (Table 2).

3,5-diacetyltambulin showed weak antifungal activity against a number of test fungi (Table 3). The antifungal activity of chloroform fraction was also insignificant (Table 3). In cytotoxicity assay with brine shrimp nauplii, the LC_{50} value of 3,5-diacetyltambulin was 10.02 µg/ml. The cytotoxicity of 3,5-diacetyltambulin was comparable to those of chloroform fraction, standard gallic acid and vincristine sulfate whose LC_{50} values were 14.31, 4.53 and 0.76 µg/ml, respectively (Table 4). No mortality was found in the control group. An approximate linear correlation was observed when logarithm of concentrations versus percentages of mortality was plotted on graph paper.

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DISCUSSION

Isolation of 3,5-diacetyltambulin from the genus *Amorphophallus* as well as from the family Araceae is reported for the first time. Isolation of this flavinoid from fruit *Zanthoxylum integrifoliolum* Merr. (Rutaceae) and its antiplatelet aggregation and vasorelaxing effects (14) and from *Zanthoxylum Armatum* DC (Rutaceae) (22) have previously been described.

This compound showed significant antibacterial activity against both Gram-positive and Gram-negative bacteria. The result of antifungal screening indicate that its antifungal application is clinically insignificant. Moderate cytotoxicity of 3,5-diacetyltambulin indicate that it can be selected for further cell line assay, since many scientists have shown a correlation between cytotoxicity and activity against the brine shrimp nauplii using extracts or isolated compounds from terrestrial plants (23-26). Like chloroform fraction, 3,5-diacetyltambulin showed antibacterial and cytotoxic activities but potency of 3,5-diacetyltambulin was observed higher than chloroform fraction.

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