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Calamus leptospadix Griff. a high saponin yielding plant with antimicrobial property

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ABSTRACT

There are many scientifically unrevealed plant species in the Sub-Himalayan region, which have been used by the local tribes for their day-to-day use. *Calamus leptospadix* Griff. is a non-climbing palm predominantly found in North-East India. The objective of this study is to isolate and identify saponin extracted from tender shoots of *C. leptospadix* and the determination of its antimicrobial activity. In the present investigation, the total saponin content was found to be 9.5% when the tender shoots were extracted in methanol followed by *n*-butanol. Saponin isolated from *C. leptospadix* was further characterized by HPLC, FT-IR, ^{1}H NMR and ^{13}C NMR which confirm the presence of only one type of ursolic acid (3-beta-3-hydroxy-urs-12-ene-28-oic-acid) which is a triterpenoid saponin with olefinic carbon at C-12 and C-13. Antimicrobial properties of the isolated saponin were tested by disc diffusion method and by determining the minimum inhibitory concentration (MIC). Lowest MIC was found to be 60 μg/ml for *Escherichia coli* and *Candida albicans*. The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) for *E. coli* and *C. albicans* were recorded at 140 μg/ml and 120 μg/ml respectively. Highest MIC of 160 μg/ml was recorded for *Staphylococcus aureus*. This study showed that shoots of *C. leptospadix* contains a significant amount of saponin, which was confirmed as ursolic acid type of triterpenoid saponin with antimicrobial properties.

1. Introduction

Calamus leptospadix Griff. (Arecaceae) is commonly found in Sub-Himalayan region hold a significant importance for its varied application in medicinal as well as commercially. *C. leptospadix* seeds are used in indigestion and stomach disorders (Rongsensashi et al., 2013) and the young shoots are used as vegetable, preparing rope, baskets and other handicraft (Sarmah, 2010). Previous studies have confirmed its anthelmintic properties (Borah et al., 2013) and the antioxidant activity in the tender shoots with saponin as major phyto-constituents (Borah et al., 2014). Saponin are well known for their diverse biological properties like spermicidal, molluscicidal, anticancer, cholesterol lowering properties, antimicrobial, anti-inflammatory and significantly for foam stabilizing and cleaning capacity (Hostettmann and Marston, 1995; Zhang et al., 1993; Matsuura, 2001; Mroczek, 2015; Selim and Al Jaouni, 2015). *C. leptospadix* plants are of extensive ethnomedicinal use but no proper scientific investigation and biochemical profiling is a big bottle neck for industries to take up this plant for commercial use. To overcome this impediment, present study was undertaken to isolate and identify saponin from of *C. leptospadix* and their probable involvement in antimicrobial activity.

2. Materials and methods

2.1. Collection and preparation of the extract

The plant was collected from vicinity of CSIR-Northeast Institute of Science & Technology (NEIST) campus, upper Assam region of

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India, in 2013. The young shoots (2–3 months old) of *C. leptospadix* were collected. These shoots were shade dried at room temperature until crunchy enough to be fit for grinding. Powdered *C. leptospadix* shoot samples (20 g) were transferred to soxhlet apparatus with petroleum ether (250 ml) for removal of soluble fat. The material was air dried again and put in the soxhlet apparatus with 250 ml of methanol until the siphon tube of the soxhlet becomes colorless. Finally the extract was concentrated by evaporating the solvent using rotary evaporator at 50 °C (Kuljanabhagavad et al., 2008).

2.2. Determination of total saponin contents

The total saponin content was determined by gravitimetric method (Harbone, 1973). At first, the methanolic extract was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added, shaken vigorously and separated the aqueous layer. The aqueous layer was then mixed with 60 ml of *n*-butanol. The combined *n*-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. Evaporated the solvent using rotary evaporator to get the saponin content and was dried in an oven. The saponin content was calculated as follows

$$\text{Percentage of saponin} = \frac{FW}{IWS} \times 100$$

where IWS = initial weight of the sample; FW = finale weight of the sample.

2.3. Identification and characterization of saponin

2.3.1. Foam test

Froth forming capacity of isolated saponin was demonstrated by performing foam test (Chen et al., 2010). A small amount of dried extract containing saponin was shaken with distilled water in a test tube for 10 min. To monitor the positive result, diluted saponin solution was shaken and let it to be stable for 15 min for the formation of froth.

2.3.2. Liebermann–Burchard test

Crude extract (2 ml) of saponin was mixed with 1 ml of chloroform and few drops of acetic anhydride. To this solution, few drops of concentrated sulphuric acid was then added from the sides of test tube and observed for the formation of a brown ring at the junction of two layers. The appearance of red, brown color in the lower layer indicates a positive test for triterpenoid (Kenny, 1952).

2.3.3. Thin layer chromatography (TLC)

Thin layer chromatography was carried out to isolate the principal components that were present in most effective extracts of plant. Various solvents such as chloroform, ethyl acetate, hexane, methanol and water with different proportions were used for optimizing mobile phase and aluminium plates precoated with silica gel (Pore size 60 Å) was used as stationary phase. The best separation was found to be with mobile phase of hexane and ethyl acetate in 3:7 proportions respectively.

2.3.4. High performance liquid chromatography (HPLC)

The chromatographic separation was carried out on reverse-phase C-18 column. HPLC analysis was performed using Waters HPLC machine with injection valve of 20 ml loop and a UV variable-wavelength detector (at 210 nm) operated at room temperature (25 ± 1 °C). 1 mg of saponin extract was dissolved in 1 ml of methanol and filtered under vacuum through a 0.45 μm membrane. The mobile phase used for HPLC analysis was a mixture of acetonitrile: water (7:3 v/v). The flow was adjusted to 1.0 ml/min and the sample injection volume was 10 μl.

2.3.5. Fourier transform infrared spectrophotometer (FTIR) analysis

Dried powder 10 mg was encapsulated in 100 mg of KBr pellet, in order to prepare translucent discs (Kareru et al., 2008). The powdered sample was loaded in FT-IR system 2000 (PerkinElmer, USA, ANC 1). The spectra were recorded in the range of 400–4000 cm^{-1} range.

2.3.6. Nuclear magnetic resonance (NMR) analysis

Approximately 3 mg of the sample was dissolved in $CDCl_3$ solution. The sample was placed into 5 mm NMR tube. To confirm the chemical structure of saponin, ^1H NMR (600 MHz) and ^{13}C NMR (500 MHz) spectra were measured with Bruker Avance DPX NMR spectrophotometer. The chemical shifts (δ) were expressed in ppm.

2.4. Screening for antimicrobial activity

The test organisms used for screening antimicrobial activities of the *C. leptospadix* were two gram negative bacterial strains *Pseudomonas putida* ATCC 17642 and *Escherichia coli* MTCC 739, two gram positive bacterial strains *Staphylococcus aureus* MTCC 6908 and *Bacillus toyonensis* BCT 7112 and one fungal strain *Candida albicans* MTCC 3007 as test microorganisms. All microorganisms were obtained from Microbial Type Culture Collection (MTCC) and Gene Bank, Chandigarh, India. Antimicrobial activity of the extracted saponin was screened by disc diffusion method and by determining minimum inhibitory concentration against the microbes. Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were also performed.

Bacterial strains and fungal strain were grown in nutrient broth at 30 °C and in potato dextrose broth at 28 °C for 24 h respectively. The turbidity of resulting culture broth was adjusted to 0.5 McFarland standard of approximately 1×10^8 CFU/ml for bacterial strains and 1.5×10^6 CFU/ml for fungal strain using sterile distilled water. About 100 μl of the bacterial and fungal suspensions were spread respectively on nutrient agar and potato dextrose agar plates respectively by evenly spreading over the entire surface of the agar plates. Sterile filter paper discs (6 mm diameter) were impregnated with 20 μl of three different concentrations (50 μg/ml, 100 μg/ml and 250 μg/ml prepared in DMSO) of the tested saponin sample and allowed to dry. Dried discs were placed under UV light for 30 min and then placed onto the inoculated plates and incubated at 30 ± 2 °C for 24 h. Standard disc of ciprofloxacin 5 mcg and filter paper disc of nystatin (10 mg/ml) were used as positive control for bacterial and fungal strains respectively. Filter paper disc containing only the carrier i.e., DMSO was used as negative control. Diameter of zone of inhibitions was measured in millimeters (mm). All the experiment was performed in triplicate and expressed as mean ± SD.

2.4.1. Minimum inhibitory concentration (MIC) determination

MIC values were determined by following the micro broth dilution technique using 96-well micro-plate (Andrews, 2001). The values were recorded at their lowest concentration which is required for >90% inhibition of the tested microbial growth. Different concentrations of saponin viz. 10, 20, 40, 60, 80, 100, 120, 140 and 160 μg/ml were used to determine MIC values. Twenty micro-liters of both bacterial and fungal strain to at 1×10^8 and 1.5×10^6 CFU/ml (0.5 McFarland standard) respectively were added to 980 μl of their respective broth containing different concentrations of saponin. Saponin free solution was taken as negative control and the blanks were prepared for each concentration of saponin without any microbial culture. MIC values were determined by taking absorbance in micro plate reader (Biotek Epoch 2) at 600 nm after incubating for 24 h at 30 ± 2 °C.The lowest concentration of saponin in the broth, which inhibits >90% of the growth