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Research Article

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Phytochemical and Antimicrobial Activity of the Leaves of *Croton bonplandianum* Bail

Goutam Kumar Basak[1,2], Tanmay Chowdhury[3], Susama Chakraborty[1], Ankana Karmakar[1] and Amitava Mnadal[1*]

[1]*Molecular Complexity Laboratory, Department of Chemistry, Raiganj University, Raiganj, Uttar Dinajpur, West Bengal 733134, India*

[2]*Department of Microbiology, Raiganj University, Raiganj, Uttar Dinajpur, West Bengal 733134, India*

[3]*Department of Sericulture, Raiganj University, Raiganj, Uttar Dinajpur, West Bengal 733134, India*

ABSTRACT

Plants are always been the sources of medicine from prehistoric ages. Croton bonplandianum Bail is an exotic weed of Euphorbiaceae family. It has medicinal use in folk medicinal practices. Herein, we report the antibacterial activities of the leaf extracts of croton against some antibiotic resistance bacteria and have isolated stigmasterol as the active component. Structure of stigmasterol was established from detail spectral analyses (IR, NMR, etc.). The leaf extracts showed promising antibacterial activities and could be a suitable alternative against antibiotic resistance bacteria.

Keywords: Croton; Antibacterial activity; NMR; Stigmasterol

INTRODUCTION

Croton bonplandianum Bail is a monoecious exotic weed and belongs to the family Euphorbiaceae. It is native to Southern Bolivia, Paraguay, South Western Brazil, North Argentina etc. [1]. Other reports also claimed that it is native to India, Bangladesh, Pakistan and South America [2]. In India it is most often found in the Sub-Himalayan region of West Bengal and across the country in sandy alluvial soil in abandoned places, river basins, railway fields, etc. The plant is commonly called as Ban Tulsi traditionally in West Bengal because of resemblance of the flower cymese and laves to that of Tulsi. The plant is normally 40-50 cm in height with whorled ranches.

Traditionally, the whole plant is used for treatment of liver disorders and different skin disease that includes itching, ring worm infection, etc. and also to cure body swelling [3]. Leaves have antiseptic properties and are medicinally used to treat cuts and wounds, venereal sores and sometimes against cholera [4]. Seeds are useful for the treatment of acute constipation, jaundice, internal abscesses and abdominal dropsy [5]. Croton is considered as chologogue

and purgative [6]. It is reported to have potent anti-helmenthic and hepatoprotective properties [7]. Leaves are also used to control high blood pressure, [8] and leaves infusion is applied to cure severe fever caused by infection of glands [9]. Plant latex has wound healing properties [10] and the fresh leaf juice is used for headache [11]. Interestingly, the plants' latex is used in folk medicine practices by tribal people of Tamil Nadu to treat wasp sting [12]. Croton was reported to have repellent property against the mosquito, *A. aegypti* [13].

All these observations lead to undertake diverse research activities with croton [14]. Different researchers from different parts of the globe have reported the isolation of biologically active natural products from croton [12-14]. But no such study was yet reported from the croton plants from this part of India. This type of study is essential as the nature and content of naturally occurring compounds may vary from one site to another depending upon climate, soil type, average rain fall, type(s) of microorganisms present, different type(s) of environmental stresses at the plants' habitat, etc. Therefore the present research work was aimed to determine the antibacterial activity of various solvent extracts of the leaves of *C. Bonplandianum* Bail collected from North and South Dinajpur district, West Bengal, India against bacteria like *Bacillus cereus*, *Staphylococcus aureus, Lactobacillus plantarum, Enterobacter aerogenes, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Escherichia coli* which were isolated from wound infections of hospitalized patients and the phytochemical investigation of the active components. This type of work are now getting mileage as treatment of various human diseases is becoming more and more complicated since the pathogenic microorganisms are now showing multiple drug resistance. Hence, there is a dire need to identify plant based antimicrobial compounds which has very less possibility to get drug resistance.

MATERIALS AND METHODS

General

Solvents used for the study was reagent grade and were purified prior to their use. All solvents, silica gel (60-120 mesh) was purchased from Thomas Baker Co. Ltd. Rotary evaporator used for the study was Buchi Rotavapor R-3; Buchi Labortechnik AG, Flawil, Switzerland. All the bacterial strains were obtained from the Institute of Microbial Technology, Chandigarh, India. The nutrient agar was purchased from HiMedia Laboratories Limited, Mumbai, India. Water used throughout the study was distilled and de-ionised. Melting point was recorded in an open capillary method and was uncorrected. FT-IR was carried out in Perkin Elemer FT-IR Spectrometer (RX-1) in KBr discs. NMR spectra were recorded in Bruker-Avance 300 MHz FT-NMR spectrometer at 5 mm BBO probe in $CDCl_3$.

Collection of plant materials

The plants were collected from AASM (Medicinal and Aromatic plants) garden, Raiganj University, Raiganj, North Dinajpur, West Bengal, India and from different parts of North and South Dinajpur districts, West Bengal, India during December, 2017 to March 2018 and the herbarium was deposited in the AASM garden Herbarium, Raiganj University.

Preparation of extracts

Leaves of the collected plats were dried under shade at room temperature for several days. The dried plant materials (leaves) were crushed and make powdered by mechanical grinder. The powder leaves (50 g each) were soaked in different solvents (100 mL) like water and methanol and kept in shaker for 72 hours at room temperature. The extracts were then filtered using Whatmann filter paper No. 41. Solvent was recovered using a rotary evaporator

(Buchi Rotavapor R-3; Buchi Labortechnik AG, Flawil, Switzerland) at 40°C with a vacuum controller coupled to a cooling unit. Finally, the extracts were lyophilized and kept in a sealed labeled vial at 4°C in dark until tested and analyzed.

Test microorganisms

The antibacterial activity was evaluated against seven microorganisms including Gram-positive *Bacillus cereus* (MCC 2128), *Staphylococcus aureus* (MCC 2408) and *Streptococcus pneumonia* (MCC2425) and Gram-negative *Enterobacter aerogens* (MCC3092), *Pseudomonas aeruginosa* (2080), *Klebsiella pneumonia* (MCC 2730) and *Escherichia coli* (MCC 2413). All the bacterial strains were obtained from the Institute of Microbial Technology, Chandigarh, India. Bacterial strains were maintained on nutrient agar (HiMedia, Mumbai, India) slants and the cultures were stored at 4°C with a subculture period of 30 days.

Determination of antimicrobial activity by disc diffusion method

In vitro antibacterial activity of aqueous extracts (say) of Croton *Bonplandianus Bail* was studied against three Gram positive and four Gram negative bacterial strains by agar disc diffusion method by National Committee for Clinical Laboratory Standards, 1997. The nutrient agar (HiMedia Laboratories Limited, Mumbai, India) was autoclaved at 121°C and 1 atm for 15-20 minutes. The sterile nutrient media was kept away maintaining temperature at 45-50°C, after that 100 µL of bacterial suspension containing 108 Colony-Forming Units (CFU)/mL were mixed with sterile liquid nutrient agar and poured into the sterile petri dishes. Upon solidification of the media, filter discs (5 mm diameter) were individually soaked with different concentration (as for example, 10, 25, 50, 75 and 100 mg/mL) of the extract and placed on the solidified nutrient agar media plates. The plates were incubated for 24 hours at 37°C. The diameter of the zone of inhibition (including disc diameter of 5 mm) was measured with a scale. Each experiment was performed three times to minimize the error and the mean values were presented.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The Minimum Inhibitory Concentration (MIC) of was aqueous and methanol extracts of *C. bonplandianus* Baill. was determined by Broth dilution method (CLSI M07-A9). Each tube contained an inoculums density of 1 × 106 CFU/mL of each tested bacterial strain. All the tested bacteria were grown in Muller Hinton broth. Then the suspension of all the seven cultures was added into tubes containing diluted sample of the leaf extracts of 4-2048 µg/mL. Then the tube of diluted sample with bacteria was incubated overnight at 37°C on the shaker. The growth of the bacteria was determined by turbidity.

The Minimal Bactericidal Concentration (MBC) was determined by using standard method (CLSI M26-A). To determine the MBC of the extracts against the tested bacteria, the plates of the MIC that showed no growth of the bacteria were sub-cultured by striping using wire loop on sterile Muller Hinton agar plates. The plates were then incubated at 37°C for 24 h. The MBC was taken as the lowest concentration of the extract that exhibited not microbial growth on the agar plates.

Extraction and isolation

Extraction and isolation were carried out as described below. In brief, dried leaves (1.80 kg) of *Croton bonplandianum* were extracted by maceration at room temperature methanol (2 × 500 mL) and water (2 × 500 ml). This process involved soaking the plant materials (leaves) for 72 hours. Extracts were then obtained by removing

solvents from the filtrates through rotary evaporation at 40°C water bath temperature. Five grams of extracts from leaves was then dissolved in 100 mL distilled chloroform and pre-adsorbed in column silica gel (60-120 mesh). It was then purified by column chromatography and eluted with ethyl acetate:n-hexane solvent systems starting with ratios of 10:90, 30:70, 40:60, 50:50, 60:40, 80:20 and 100:0 to obtain 42 different fractions. Fractions 20-34 were combined and further purification using preparative TLC with 20:80 ethyl acetate:n-hexane solvent system was done to obtain a white solid compound 1.

RESULTS AND DISCUSSION

Antimicrobial assay

In-vitro screening of antibacterial activity of *Croton bonplandianum* leaves in both the aqueous and methanol extracts showed very promising results against all the tested bacterial strains used in the present investigation (Figures 1 and 2). The results also showed that both the aqueous and methanolic extracts were very sensitive against all the gram positive bacteria. Among them *B. cerus* is more susceptible in methanolic extracts (MIC 64 µg/mL, MBC 128 µg/mL) and *L. plantarum* is more susceptible in aqueous extracts (MIC 64 µg/mL, MBC 128 µg/mL). In case of gram negative bacteria *K. pneumoniae* showed significant antibacterial activity in both aqueous and methanolic extracts with MIC of 256 µg/mL and MBC of 512 µg/mL and MIC of 128 µg/mL and MBC 256 µg/mL respectively (Figures 1 and 2). MIC and MBC values are tabulated in Tables 1 and 2.

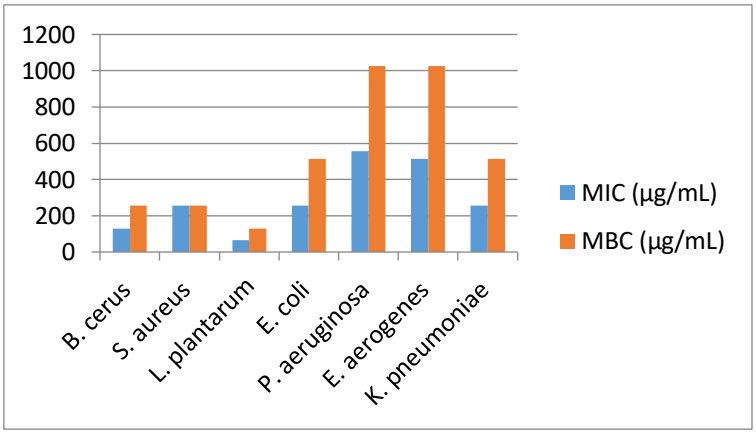


Figure 1. Antimicrobial activity of the aqueous extract of leaves of *C. bonplandianum*.

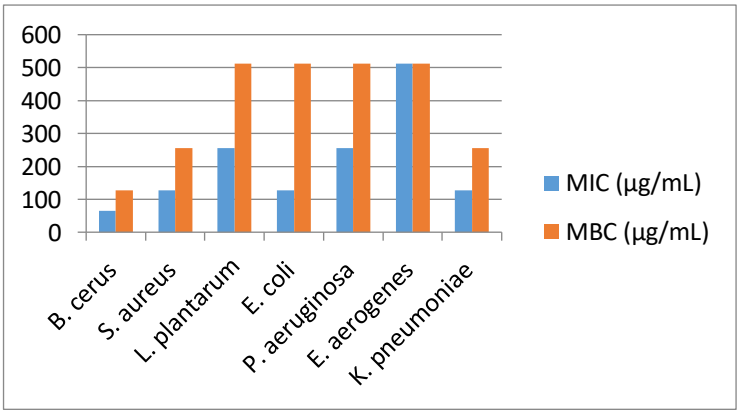


Figure 2. Antimicrobial activity of the methanolic extract of leaves of *C. bonplandianum*.

Table 1. MIC and MBC values of aqueous extract of *C. bonplandianum* against the tested organisms.

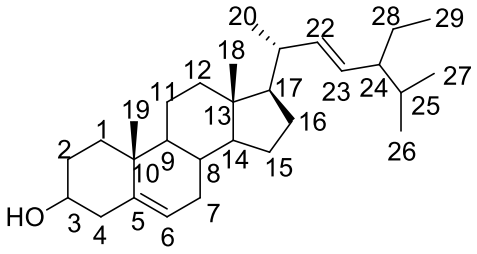
Bacteria	Aqueous extract	
	MIC (µg/mL)	MBC (µg/mL)
B. cerus	128	256
S. aureus	256	256
L. plantarum	64	128
E. coli	256	512
P. aeruginosa	556	1024
E. aerogenes	512	1024
K. pneumoniae	256	512

Table 2. MIC and MBC values of methanolic extract of *C. bonplandianum* against the tested organisms.

Bacteria	Methanolic extract	
	MIC (µg/mL)	MBC (µg/mL)
B. cerus	64	128
S. aureus	128	256
L. plantarum	256	512
E. coli	128	512
P. aeruginosa	256	512
E. aerogenes	512	512
K. pneumoniae	128	256

Characterization of compound 1

Compound 1 was isolated as white solid with melting point 162°C. In the FT-IR spectrum, taken in KBr disc, strong absorptions were found at 3422 (-OH), 2850 (C-H stretching), 1462, 1376, 1170, 1053 (olefinic double bond), 970, 838 cm^{-1} respectively. 1H NMR spectrum showed the presence of six methyl groups at δ 0.68 (s, 3H), 0.81 (d, 3H), 0.82 (d, 3H), 0.84 (t, 3H), 0.91 (d, 3H) and 1.03 (s, 3H) ppm. C-3 proton appeared at δ 3.51 (m, 1H). It showed the presence of three olefinic protons at δ 4.98 (oblate quartet, 1H), 5.14 (oblate quartet, 1H) and at δ 5.34 (s, 1H). The former represents a disubstituted olefinic bond and the latter a trisubstituted double bond characteristic to the presence of one C-5/C-6 double bond in steroid skeleton. Compound 1 also gave positive Libermann-Burchard test for steroid skeleton. ^{13}C NMR spectrum showed the presence of 29 carbon atoms, thus confirming the presence of steroid skeleton "stigmastane". C-3 appeared at δ 71.8 ppm. Four olefinic carbon atoms all appeared at δ 140.7 (C-S), 121.7 (C-6), 138.5 (C-20) and 129.6 (C-21) ppm. Six methyl carbons appeared at δ 21.0 (C-19), 11.9 (C-24), 19.8 (C-26), 19.4 (C-27), 18.7 (C-28) and 12.2 (C-29). All these confirmed that the isolated compound 1 was stigmasterol that could be the molecule for promising antibacterial activities of the leaf extracts (Figure 3).



Stigmasterol

Figure 3. Structure of the isolated compound 1, stigmasterol with numbering of carbon atoms.

CONCLUSION

In a nutshell, we have described the antibacterial activities of aqueous and methanolic extracts of leaves of *Croton bonplandianum* Bail against few gram positive and gram negative antibiotic resistance bacteria. Both the extracts showed good results. Both MIC and MBC values are reported in the manuscript. Phytochemical analysis of the active part (extract) leads to the isolation of steroid. The structure of which was characterized by different spectral techniques, viz. IR, NMR etc. Since most of the antibiotics in the present day market are getting resistance by bacteria, natural products could be a suitable alternative. And in this regard this kind of studies worth huge significance.

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