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Investigation of the Effect of *Croton bonplandianum* Baill on Multidrug Resistant Microorganisms

Tayya Raga Sudha

Department of Microbiology, Government City College, Nayapul, Hyderabad, India

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ABSTRACT

Bacteria protect themselves from commonly used antibiotics by acquiring resistance through horizontal gene transfer with the resistant bacteria present in the environment. This defensive mechanism of bacteria and excessive use of antibiotics resulted in the emergence of multidrug resistance in many disease-causing bacteria. *Staphylococcus aureus* and *Pseudomonas aerogenosa* are known to be the main causative organisms for skin and hospital acquired infections throughout the globe. In the current study multi drug resistant microorganisms isolated from the patient samples of King George Hospital, Visakhapatnam, Andhra Pradesh, India were tested against the crude methanol, chloroform, ethyl acetate and hexane extracts of *Croton bonplandianum*. These extracts were found to be significantly effective against the tested multi-drug resistant organisms.

Key words: *Croton bonplandianum, Staphylococcus aureus, Pseudumonas aerogenosa*, methanol extract, chloroform extract, multidrug-resistant bacteria

INTRODUCTION

The use of medicinal plants as source of remedies for the treatment of many diseases is dated back to prehistory and people of all continents have this old tradition (Newmann *et. al,* 2000). In developing countries where medicines are quite expensive, it is obvious that these medicinal plants will find their way in the arsenal of anti-microbial drugs (Cowan, 1999). The medicinal value of these plants lies in some chemical substances known as phytochemicals that produce a definite physiological action on the human body (Hill, 1952). A knowledge of the chemical constituents of the plants is desirable, not only for the discovery of the therapeutic agents, but also because such information may be of value in disclosing new sources of economical materials as tannins, oils, gums, precursors for the synthesis of complex chemical substances, etc. (Fransworth, 1966).

The enormous use of antibiotics in the treatment of various diseases and the rapid development of multi-drug resistant microorganisms is demanding an urgency to develop new antibiotics. Penicillin resistant *Staphylococcus aureus* confronted London civilian hospitals very soon after the introduction of penicillin in the year 1940. Then on the so called "super drug" penicillin had undergone a number of conformational changes to bypass the penicillin-resistant microorganisms like *Staphylococcus aureus* and *Neisseria gonorrhea*.

The genus Croton belonging to the family Euphorbiaceae, comprises of 750 species habituated in tropical and sub-tropical regions. Amongst them *Croton bonplandianum* Baill (*Croton sparsiflorus* Morong) a shrub found to be grown as a weed abundantly in waste lands of India is used in the present study. The vernacular names of the plant are Kala bhangra (hindi) or galivana mokka (telugu). Traditionally this plant is known to be used to cure acute arthritis, wounds, cuts and fungal diseases. The plant was found to have antimicrobial (Ganga Rao and Raga Sudha, 2009) and anti-carcinogenic (Asolkar *et.al,*1992) activities. Various potential bioactive compounds like crotsparine, dihyroproaporphines-crotsparinine, Rutin (Bakuni and Dharm, 1968); N-methylcrotsparine, N-O-dimethylcrotsparine, N-methylcrotsparinine, Beta-sitosterol (Acharya *et al.,* 1965), Phorbol derivative (I), Sparsiflorine, Crotoflorine (Chatterjee *et. al.,* 1976), Isicrotsparinine, its N-Me derivatives, (+) – tetra-hydroglazievine and phorbol esters (Rastogi & Mehrotra, 1993) were isolated from the leaves and aerial parts of *C. bonplandianum.*

The current study is an attempt to determine the antimicrobial activity of the crude methanol, chloroform, ethylacetate and hexane extracts of *Croton bonplandianum* on multi-drug resistant microorganisms isolated from the patient samples of King George Hospital, Visakhapatnam, Andhra Pradesh.

MATERIALS AND METHODS

Plant material

The plant material used for the study was collected from the waste lands of Andhra University campus, Visakhapatnam, Andhra Pradesh and authenticated by the taxonomist Prof. M. Venkaiah, Dept. of Botany, Andhra University. A voucher specimen (no. BGR/CBA-1) was deposited in the herbarium of Andhra University for further reference.

Extraction

The shade dried plant materials were pulverized into coarse powder and extracted in Soxhlet apparatus using methanol (96%) as solvent. The collected methanol extracts were concentrated under vacuum (50ºC) dried and weighed. The dried methanol extract was then subjected for qualitative phytochemical analysis. Then dried methanol extract was suspended in water and fractionated with chloroform, ethyl acetate and hexane. These fractions were then subjected for the qualitative phytochemical analysis. All the chemicals are of analytical grade purchased from Desai chemicals Pvt. Ltd., Visakhapatnam.

Phytochemical Analysis:

1. Test for alkaloids: A 3ml of each extract was evaporated to dryness and the residue was heated on a boiling water bath with 2N hydrogen chloride (5ml). After cooling the mixture was divided into two equal portions. One portion was treated with a few drops of Mayer's reagent and the other with equal amounts of Wagner's reagent (Rizk, 1982). The samples were then observed for the presence of turbidity or precipitate.
2. Test for flavonoids: A 5ml of each extract was treated with a few drops of concentrated hydrogen chloride and magnesium turning (0.5g). The presence of flavonoids was indicative if pink/magenta-red color developed within three minutes (Somolenski *et. al.,* 1972).
3. Test for tannins: 10ml of each extract was evaporated and the residue was extracted by 10ml of hot 0.9% sodium chloride solution, filtered and divided into three equal portions. One portion of the extract was added with sodium chloride solution, 1% gelatin solution to a second portion and gelatin-salt reagent to the third portion. Precipitation with the latter reagent or with both the second and third reagents is an indicative for the presence of tannins.

   Positive tests are confirmed by the addition of ferric chloride ($FeCl_3$) solution to the extract and should result in a characteristic blue, blue-black, green or blue-green color or precipitate (Segelman and Fransworth, 1969).

4. Test for saponins: About 2.5g of the dried powdered sample was extracted with boiling water. After cooling, the extract was shaken vigorously to froth and was then allowed to stand for 15-20 minutes and classified for saponin contents as follows: no froth=negative; froth less than 1 cm= weakly positive; froth=1.2cm high= positive; and froth> 2cm high= strongly positive (Kapoor *et. al.,* 1969 and Somolenski *et. al.,* 1974).
5. Test for cardiac glycosides: 5ml of each extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout this layer (Trease and Evans, 1989).
6. Test for steroids: Dried powdered sample was extracted with chloroform. 2ml of acetic anhydride was added to 0.5ml chloroform extract. Then 1ml of concentrated sulphuric acid was added from the sides of the test tube. A reddish brown ring at the junction of two layers indicates positive test for steroids (Trease and Evans, 1989).
7. Test for triterpinoids: Dried powdered sample was extracted with chloroform. 2ml of acetic anhydride was added to 0.5ml chloroform extract. Then 1ml of concentrated sulphuric acid was added from the sides of the test tube. A red ring at the junction of two layers indicates positive test for triterpinoids (Trease and Evans, 1989).

Determination of Antimicrobial Activity:

Preparation of Samples

All the extracts and the standard antibiotic were suspended in dimethyl sulfoxide to yield the concentrations of 25, 50, 100 and 200 µg/ml.

Microorganisms Tested

The bacterial cultures namely *Staphylococcus aureus* and *Pseudomonas aerogenosa* were purchased from IMTECH, Chandighar for the experimental. The test organisms also include two Multidrug resistant organisms namely Methicillin resistant *Staphylococcus aureus* and Multidrug resistant *Pseudomonas aerogenosa* isolated from patient samples at King George Hospital, Visakhapatnam.

Methicillin resistant *Staphylococcus aureus* was found to be resistant to penicillin, amoxicillin, carbenicillin, azithromycin and methicillin and sensitive to clindamycin, lincomycin, roxithromycin, linizolid, rifampicin, vancomycin and tiecoplanin. Multidrug resistant *Pseudomonas aerogenosa* was found to be resistant to all the tested twelve antibiotics namely ceftriaxone, ciprofloxacin, netilmicin, ceftazidime, amikacin, cefoperazone, gentamicin, sparfloxacin, cefadroxil, lomefloxacin, cefotaxime, and chloramphinicol.

Culture Media

The media used for the growth of bacteria nutrient broth and nutrient agar of Hi-media Pvt. Ltd., Mumbai, India.

Inoculum Preparation

The test bacteria were inoculated into liquid media (nutrient broth) and incubated at 37 ^{0}C for 8-10 hr for bacteria. The suspensions were checked to provide approximately 10^5- 10^7 CFU/ml.

Antimicrobial Testing

The plant extracts were tested for antibacterial activity by cup-plate method (Chung, *et. al.,* 1990) using four bacterial strains.

Bacterial cultures each of 20µl was poured over the basal plates containing 25ml of nutrient in sterile 9 cm petri plates and spread using L-shaped glass rod. A 50 µl of each of the extract was poured into the wells (4mm in size) bored with a sterile metal borer. Each extract was tested in triplicate. Nutrient agar plates were incubated at 37^{0}C for 24 hr. simultaneously, the positive (chloramphenicol) and the negative (dimethyl sulfoxide) controls were also tested for the activity and the zones of inhibition were recorded by measuring the diameter of zone of inhibition by the following formula:

Zone of Inhibition (mm) = D-d
Where,
D = diameter of zone of inhibition
D = diameter of the well (4mm)

RESULTS AND CONCLUSION:

The minimum inhibitory concentration (MIC) of the hexane and ethyl acetate extracts of aerial and root parts was greater than 200 µg/ml against all the tested

organisms while the MIC of chloroform and methanol extracts of the aerial parts and root of *C. bonplandianum* was 50 and 25 µg/ml respectively against the test organisms. The MIC of the standard antibiotic chloramphinicol is 10 µg/ml for *Staphylococcus aureus* (Sa) and *Pseudomonas aerogenosa* (Pa) and no inhibition by the isolates Methicillin resistant *Staphylococcus aureus* and Multidrug resistant *Pseudomonas aerogenosa* . On comparison with the positive control the antimicrobial activity of the methanol and chloroform extracts of *C. bonplandianum* (aerial parts and roots) have exhibited a significant zone of inhibition against all the four test organisms including the multi-drug resistant organisms. The hexane and ethylacetate extracts of aerial and root parts had exhibited no activity.

The effectiveness of the extracts was not due to one bioactive compound but due to a combination of various compounds present in it (Bai, 1990). The bioactive components like steroids, terpenoids, alkaloids, tannins, flavonoids and glycosides are classified as compounds with antimicrobial properties (Rojas *et. al.,*1992). The preliminary phytochemical screening has shown the presence of steroids, terpenes, alkaloids, flavonoids and glycosides in the aerial part extracts and steroids, alkaloids, flavonoids, tanins and glycosides in the root extracts of *C. bonplandianum*. The effectiveness of the extracts on the pathogenic microorganisms really shows the presence of significant bioactive compounds.

Table 1.Preliminary Phytochemical screening of aerial parts and root extracts of *C. bonplandianum*

Croton bonplandianum		**Aerial parts extracts**				**Root extracts**			
SNo.	**TESTS**	**H**	**E**	**C**	**M**	**H**	**E**	**C**	**M**
1	Steroids	+	-	+	+	+	+	+	+
2	Terpenes	-	+	+	+	-	-	-	-
3	Saponin	-	-	-	-	-	-	-	-
4	Steroidal Saponins	-	-	-	-	-	-	-	-
5	TerpenoidalSaponins	-	-	-	-	-	-	-	-
6	Alkaloids	-	-	+	+	-	-	+	+
7	Flavonoids	-	+	+	+	-	-	-	+
8	Tanins	-	+	-	-	-	-	-	+
9	Glycosides	-	-	+	+	-	+	-	+
10	Carbohydrates	-	-	-	-	-	-	-	-

Table 2: Minimum inhibitory concentration of the extracts of *C. bonplandianum* against microorganisms.

Extract	**Minimum inhibitory concentration (MIC) µg/ml**			
	Sa	**MR-Sa**	**Pa**	**MR-Pa**
Aerial parts				
Hexane	>200	>200	>200	>200
Ethylacetate	>200	>200	>200	>200
Chloroform	50	50	50	50
Methanol	25	25	25	25
Root				
Hexane	>200	>200	>200	>200
Ethylacetate	>200	>200	>200	>200
Chloroform	50	50	50	50
Methanol	25	25	25	25
Chloramphinicol	10	-	10	-
DMSO (control)	-	-	-	-

Table 3. Antimicrobial activity of the aerial parts and root extracts of *C. bonplandianum*

Extract	Concentration (µg/ml)	Diameter of Zone of Inhibition# (mm)			
		Sa	MR-Sa	Pa	MR-Pa
Aerial parts					
Chloroform	25	-	-	-	-
	50	16	11	15	12
	100	34	22	21	23
Methanol	25	14	9	7	9
	50	26	20	15	19
	100	53	41	30	38
Root					
Chloroform	25	-	-	-	-
	50	8	12	12	12
	100	18	24	25	26
Methanol	25	18	16	10	19
	50	36	35	21	39
	100	46	45	40	48
CA	25	20	-	21	-
DMSO	-	-	-	-	-

Note: Sa: *Staphylococcus aureus*, MR-Sa: multi-drug resistant *Staphylococcus aureus*, Pa:*Pseudomonasaerogenosa*, MR-Pa: multi-drug resistant *Pseudomonas aerogenosa*, CA: chloramphenicol (standard antibiotic), DMSO: dimethyl sulphoxide (control). "-" no zone of inhibition.

*The hexane and ethylacetate extracts of aerial and root parts had exhibited no activity.

Fig. 1.*Croton bonplandianum* plant.

Fig. 2.A) Fruit and B) Inflorescence of *Croton bonplandianum*

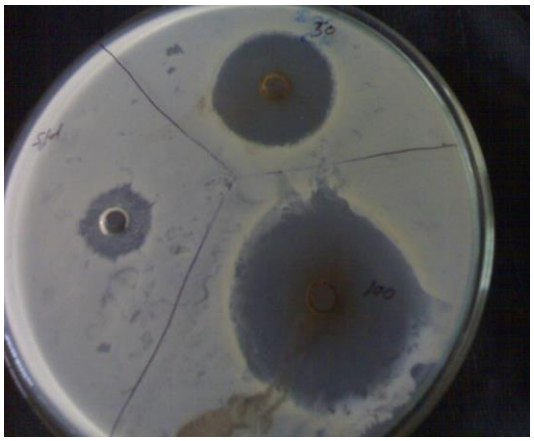

Fig. 3. Effect of *Croton bonplandianum* chloroform extract on multi-drug resistant *Staphylococcus aureus* (Methicillin resistant)

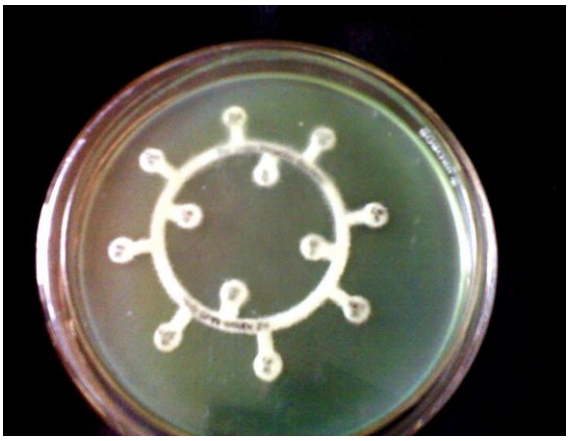

Fig 4.Nutrient agar plate with multi-drug resistant *Pseudomonas aerogenosa*.
Psuedomonasaerogenosa is resistant to twelve antibiotics namely: ceftriaxone(ctx), ciprofloxacin(cip), netilmicin(net), ceftazidime(cfz), amikacin(am), cefoperazone(cfp), gentamicin(g), sparfloxacin(sf), cefadroxil(cd), lomefloxacin(lm), cefotaxime(cf), chloramphinicol (cl).

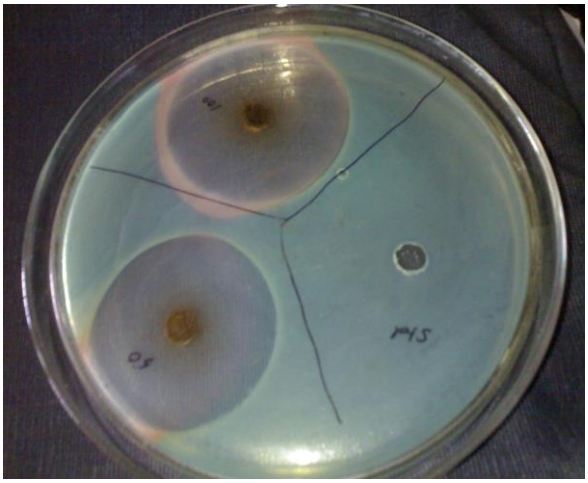

Fig. 5. Nutrient agar plate with the zone of inhibition produced by the methanol root extract of C. bonplandianum (50 and 100µg/ml) and the standard Chloramphenicol (25 µg/ml)against multi-drug resistant *Pseudomonas aerogenosa*.

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