SHORT COMMUNICATION

Antibacterial activity of *Croton roxburghii* Balak. against the enteric pathogens

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ABSTRACT

In this study, the antibacterial activity of crude (aqueous and alcoholic) extracts of the bark and leaf of *Croton roxburghii* Balak. (*Euphorbiaceae*) was tested against enteric pathogens causing urinary tract infection (UTI) using the agar cup method, minimum inhibitory concentration (MIC), time kill kinetics and synergy study. The ethanol extract exhibited a significant and broad spectrum of inhibition as compared to the aqueous extract of both the bark and leaf. The highest antibacterial activity was observed against *Staphylococcus aureus* followed by enteropathogenic and enterotoxigenic *Escherichia coli*. The diameter of inhibition zones varied from 10 to 18 mm for both aqueous and alcoholic extracts. The MIC value ranged from 356 to 625 μg/ml. This could justify the traditional use of this plant in dysentery and other infections.

Key words: *Croton roxburghii*, MIC, Similipal Biosphere Reserve, synergy, UTI

INTRODUCTION

In recent years, multiple resistance in human pathogenic microorganisms have developed due to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This situation, the undesirable side effects of certain antibiotic, and the emergence of previously uncommon infections have forced scientists into looking for new antimicrobial substances from various sources like medicinal plants.[1] The screening of plant extracts and plant products for an antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents.[2] Infectious diseases account for approximately one-half of all deaths in tropical countries. In industrialized nations, despite the progress made in the understanding of microorganisms and their control, the incidence of epidemics due to drug-resistant microorganisms and the emergence of unknown disease-causing microorganisms pose enormous public health concerns.[3]

Although more than 1000 species of *Croton* are reported, in India only five species are used in ethnomedicine for the treatment of various diseases, disorders, and ailments like antifertility, boils, bowel complaints, chicken pox, cholera, cold and cough, constipation, cuts and wounds, diarrhea, dysentery, eye diseases, epilepsy, fever, gastric disorders, insanity, jaundice, liver complaints, malaria, rheumatism, ringworms, scurvy, spasmolytic agent, snake bite, sprains, etc.[4] Tribal people in India used various parts of *C. roxburghii* against snake poisoning and to treat infertility, fever, and wounds.[5] The tribes of the Similipal Biosphere Reserve use the cold decoction of the root in sore throat. Two to three teaspoon decoction of leaves is given in dysentery. The paste of root-bark is heated and applied to boils for either subsiding or hastening suppuration. To the best of our knowledge, there is no report available on the antibacterial activity of *C. roxburghii* except our previous study on the preliminary screening of medicinal plants.[6] Therefore, a microbiological study was conducted to determine the antibacterial property of *C. roxburghii* bark and leaf extracts against enteric pathogens.

MATERIALS AND METHODS

The aqueous and the alcoholic extracts of the bark and leaf of *C. roxburghii* were prepared following the method of Panda *et al*.[7] The extraction was done by using cold percolation (steeping) by soaking the plant material in alcohol and distilled water for 3 days. Enteric pathogens like

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enteropathogenic and enterotoxigenic *Escherichia coli* (EPEC and ETEC, respectively), *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella flexneri*, *S. sonnei*, *Staphylococcus aureus* and *Vibrio cholerae* were tested in the present study. The agar cup method and MIC were used to study the antibacterial activity.[7] Broth microdilution technique adopted using 96-well microtiter plates and tetrazolium salt, 2,3,5-triphenyltetrazolium chloride (TTC), was carried out to determine the MIC following the methods as described by Eloff *et al*.[8] Selected extracts were serially diluted in the 96-well plate with an overnight culture of microorganisms (0.5 McFarland) grown at 37°C to obtain the final concentration of extracts ranging from 78 to 5000 μg/ml. The microplate was sealed and incubated at 37°C at 130 rpm and observed for the growth of the microorganism.

The antibiotic in combination with the crude drug was serially diluted along with antibiotic and crude drugs. According to the NCCLS guidelines for broth microdilution, the MIC was defined as the lowest concentration of an antibiotic that completely inhibits the growth of the organism as detected with the naked eye. Synergy is more likely to be expressed when the ratio of the concentration of each antibiotic to the MIC of that antibiotic was same for all components of the mixture. The ∑FICs were calculated as follows: ∑FIC=FIC A+FIC B, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic when the ∑FIC is ≤0.5 and antagonistic when the ∑FIC is ≥2.[9]

RESULTS AND DISCUSSION

The broad-spectrum activity of aqueous and alcoholic extracts of the leaf and bark of *C. roxburghii* was observed against a battery of enteric pathogens as shown in Table 1. The alcoholic extract of *C. roxburghii* (bark and leaf) exhibited an antibacterial activity against all the reference bacterial strains while the aqueous extract of both bark and leaf did not show any activity against *S. typhimurium*. The highest antibacterial activity was observed against *S. aureus* followed by enteropathogenic and enterotoxigenic *E. coli.* Similar observations were also noticed during the determination of the MIC with leaf extracts [Table 1]. However, the alcoholic extract of bark did not show any remarakable difference for MIC. The growth inhibition of the test bacteria ranged from 156 to 625 μg/ml (w/v). The lowest MIC value was recorded against *S. aureus* at 156 μg/ml (w/v). It is interesting to note that the solvent extract, i.e. ethanol, has a highly pronounced antibacterial activity compared to the aqueous extract. This indicates the presence of more than one active principle in *C. roxburghii*.

Even though the MIC for eight bacterial samples was determined, only five of them were considered for the synergism experiment [Table 2]. This was due to the loss of the resistance for few antibiotics observed in EPEC, ETEC, *S. typhimurium*, *S. aureus* and *V. cholerae* [Table 3], probably because of the loss of plasmids, where the resistance genes are usually located. Since the majority of bacteria were resistant to many antibiotics, only Amikacin was used in the synergism assays. This was because the resistance to at least one of these drugs was common in the entire test bacteria. In addition to this, only the aqueous extract of leaf was selected because this extract showed relatively less activity in comparison to the ethanol extract. The MIC of Amikacin was found to be ≥5000 μg/ml in all strains. The MIC of Amikacin in combination with the aqueous extract of leaf ranged from 48 to 192 μg/ml whereas the MIC for the aqueous extract alone ranged from 2500 to ≥5000 μg/ml. The FIC value was calculated in between 0.05–0.2. The time kill kinetics experimented for EPEC showed that the combination of Amikacin and *C. roxburghii* leaf (aqueous) remained in the lag phase for 1 h. After lag phase for the period of 1–4 h, it started multiplying at much slower rate as compared to the control strain as well as growth in the presence of Amikacin. During 4–8 h of incubation, this

Table 1: Inhibition of the growth of enteric bacteria by alcoholic and aqueous extracts of *Croton roxburghii*

Organism	Zone of inhibition (mm)						Minimum inhibitory concentration (μg/ml)					
	Alcoholic extracts		Aqueous extracts		Antibiotics		Alcoholic extracts		Aqueous extracts		Antibiotics	
	Bark	Leaf	Bark	Leaf	A	C	Bark	Leaf	Bark	Leaf	A	C
EPEC	16	14	13	14	13	15	312	625	625	625	20	10
ETEC	18	15	12	14	16	20	312	625	625	625	20	10
Pa	13	13	11	–	–	17	312	312	312	–	–	20
St	14	15	–	–	14	24	312	625	–	–	20	10
Sf	14	13	11	11	23	18	312	625	625	625	20	20
Ss	16	15	12	14	–	26	312	625	312	625	–	10
Sa	18	16	10	11	11	24	156	312	312	625	20	10
Vc	17	14	14	–	–	22	312	625	312	–	–	20

EPEC: enteropathogenic *Escherichia* coli; ETEC: enterotoxigenic *E. coli*; Pa: *Pseudomonas aeruginosa*; St: *Salmonella typhimurium*; Sf: *Shigella flexneri*; Ss: S. *sonnei*; Sa: *Staphylococcus aureus*; Vc: *Vibrio cholerae*; A: Ampicillin; C: Ciprofloxacin

Table 2: Synergy study among aqueous extract of *Croton roxburghii* leaf

Name of the Strain	Aqueous extract					
	MIC Ak alone (μg/ml)	MICAk combination (μg/ml)	MIC extract alone (μg/ml)	MIC extract combination (μg/ml)	FIC	Type of interaction
EPEC	≥1500	192	2500	192	0.2	Synergy
ETEC	≥1500	192	≥5000	192	0.16	Synergy
S. typhimurium	≥1500	96	≥5000	96	0.08	Synergy
S. aureus	≥1500	48	2500	48	0.05	Synergy
V. cholerae	≥1500	96	≥5000	96	0.08	Synergy

Table 3: List of the bacteria used to assess the antibacterial activity

Name of the organism	Relevant properties		MAR %	Sources
	Resistant to	Sensitive to		
EPEC	Ak, Ap, B, Ctn, E, Aug, Ce, Nal	A, C, Ch, Caz, G, Gf, Lvx, Ofl, Pb, St, Te, Vn	40	MTCC 1098, Chandigarh
ETEC	Ak, Ap, B, Pb, Lvx	A, Aug, C, Ce, Caz, Ch, Ctn, E, G, Gf, Nal, Ofl, St, Te, Vn	25	NICED, Kolkata
Pa	A, Ak, Ap, B, Ctn, E, Aug, Ce, Nal, Pb	C, Ch, Caz, G, Gf, Lvx, Ofl, St, Te, Vn	50	MTCC 1034, Chandigarh
St	Ak, Ap, B, Ctn, E, Aug, Ce, Nal, Pb	A, C, Ch, Caz, G, Gf, Lvx, Ofl, St, Te, Vn	45	MTCC 3216, Chandigarh
Sf	Ak, Ap, B	A, Aug, C, G, Ce, Cez, Ctn, E, Gt, Olf, Lvx, Nal, Pb, St, Vn, Te, Ch	15	RMRC, Bhubaneswar
Ss	A, Ak, Ap, B, Gt, Ch, Nal	C, G, Ce, Cez, Ctn, Olf, Lvx, Pb, St, Vn, E, Aug, Te	35	RMRC, Bhubaneswar
Sa	Ak, Ap, B, Aug, Ctn, G, Ce, Nal, Pb	A, C, Cez, Ch, Olf, Te, Lvx, E, Gt, St, Vn	45	MTCC 1144, Chandigarh
Vc	A, Ak, Ap, Aug, B, E, Gt, G, St, Vn	C, Ce, Cez, Ctn, Ch, Nal, Olf, Pb, Te, Lvx	50	MTCC 3904, Chandigarh

Ak: Amikacin (30 μg); Aug: Amoxicillin (10 μg); Ap: Amphotericin (100 units); A: Ampicillin (10 μg); B: Bacitracin (10 units); Ctn: Cefoxitin (10 μg); Cez: Ceftriaxone (10 μg); Ce: Cephotaxime (30 μg); Ch: Chloroamphinecol (10 μg); C: Ciprofloxacin (10 μg); E: Erythromycin (15 μg); Gf: Gatifloxacin (30 μg); G: Gentamycin (10 μg); Lvx: Levofloxacin (5 μg); Nal: Naladixic acid (30 μg); Ofl: Ofloxacin (5 μg); Pb: Polymyxin-B (300 units); St: Streptomycin (10 μg); Te: Tetracycline (10 μg); Vn: Vancomycin (30 μg) (Hi Media Pvt. Ltd., Mumbai, India)

strain multiplied at a constant rate and it started dying out rapidly at the end 12 h. However, by the combination of aqueous extract and Amikacin went into lag phase much earlier as compared to the individual treatment of antibiotic or aqueous extract against EPEC [Figure 1].

Antimicrobial resistance has been reported among pathogenic microorganisms like *S. aureus, E. coli, P. aeruginosa,* and *S. typhimurium.*[10] In the present experiment strains viz. *P. aeruginosa* and *V. cholerae* showed 50% MAR index, *S. aureus* and *S. typhimurium* 45% MAR index, EPEC 40% MAR index, *S. sonnei* showed 35%, ETEC 25%, and *S. flexneri* showed 15% MAR index [Table 3].

The combined action of Amikacin with the water extract of leaf of *C. roxburghii* showed a different mode of action. The prime reason for this selection is that higher plants are the rich source of antimicrobials and bioactive substances while traditional medicinal plants are the basic part of health care. A synergistic effect was observed for EPEC, ETEC, *S. typhimurium, S. aureus,* and *V. cholerae* which showed resistance to different antibiotics.

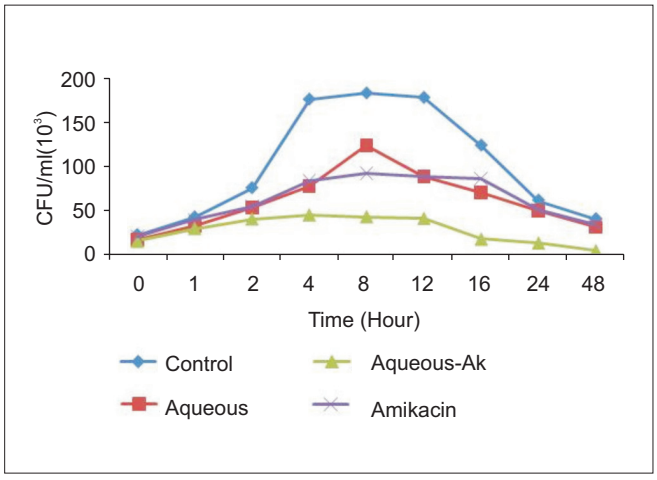

Figure 1: Time kill kinetics of the aqueous extract against EPEC

At this point, the exact mechanism of synergy is unknown but several hypothesis can be put forward to explain. First, water extract may disrupt the lipopolysaccharide layer of EPEC and helps in restoring protein channels thus facilitating the flow of antibiotic-Amikacin to target

sites. Second, it may have negative effects on the efflux mechanism and lead to a sufficient concentration of Amikacin to remain in the bacterium, thus supporting its inhibitory activity. Third, *C. roxburghii* may be inhibiting protein synthesis in combination with Amikacin which may not possible to inhibit alone the antibiotic or the extract. Fourth, *C. roxburghii* may be blocking the inhibitory effects of the enzymes or additional inhibitory effects of the plant material.

Considerable work has been carried out on the chemistry and biological activity of the genus *Croton* which has been reviewed by several workers and is reported to have chiefly diterpenoids such as phorbol esters, clerodane, labdane, kaurane, trachylobane, pimarane, etc.[11] The antibacterial activity of the bark and leaf of *C. roxburghii* against the enteric pathogens has not been documented so far in the literature. The present work authenticates the scientific use of both aqueous and alcoholic extracts of the bark and leaf of *C. roxburghii* against a battery of enteric pathogens, causative agents of diarrhea and urinary tract infection.

CONCLUSION

Further studies are required to isolate the active compounds, from the aqueous and alcoholic extracts of *C. roxburghii* bark and leaf, responsible for the antibacterial property which may lead to compound(s) in the field of antimicrobial.

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