SCREENING AND EVALUATION OF ANTI-MICROBIAL ACTIVITY IN *COLEUS FORSKOHLII* AND *STEVIA REBAUDIANA*

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Abstract: The results showed that aqueous and alcoholic extracts of leaf of parent plant of *Coleus forskohlii* showed antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermidis* and *Bacillus* species. Alcoholic extract of stem of of parent plant of *Coleus forskohlii* showed significant activity against *Staphylococcus aureus* and *Staphylococcus epidermidis.* Antibacterial activity shown by the alcoholic as well as aqueous extracts of *in vitro* raised plant of *Coleus forskohlii* against the gram-positive bacteria was tested. The significant amount of activity against *Bacillus* species only was shown by the alcoholic leaf extract but the alcoholic stem extract exhibited mild but statistically insignificant ($P<0.05$) activity against *Enterococcus faecalis* and *Bacillus* species. The aqueous leaf extract of parent plant of *Stevia rebaudiana* exhibited significant activity against only *Staphylococcus epidermidis* and the alcoholic leaf extract showed significant activity against *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis* and *Bacillus* species. MIC values of the alcoholic leaf extract of parent plant of *Coleus forskohlii* against the tested gram-positive bacterial species ranged from 9.43×10^{-3} to 18.65×10^{-3} µg/ml and MIC values of the aqueous leaf extract of parent plant of *Coleus forskohlii* against the tested bacterial species ranged from 3.15×10^{-3} to 4.65×10^{-3} µg/ml. MIC values of the alcoholic leaf extract of parent plant of *Stevia rebaudiana* against the tested gram-positive bacterial species ranged from 9.43×10^{-3} to 25.15×10^{-3} µg/ml and aqueous leaf extract of parent plant of *Stevia rebaudiana* against *Staphylococcus epidermidis* was found to be 12.5×10^{-3} µg/ml.

Keyword: Antimicrobial activity, MIC, *Coleus forskohlii, Stevia rebaudiana*

INTRODUCTION

Plants have been used as a valuable source for obtaining a variety of natural products that are useful for maintaining healthy life. More than 50% of the drugs in clinical use in the world are natural products and their derivatives (Sofowora, 1984). These plants, producing some active substances which cause certain reactions and involved in cure of diseases on human organisms are called as medicinal plants (Junior *et al.,* 1994). The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (Janovská *et al.,* 2003). In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions (Ahmad *et al.,* 1998). Parekh and Chanda, (2008) suggested that there is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and reemerging infectious diseases. Medicinal plants are not only effective antimicrobials but also are less toxic to humans and possibly having a novel mechanism of action (Parekh and Chanda, 2007). In the ancient traditional Indian Ayurvedic Medicine *Coleus forskohlii* have been used for treating heart diseases, convulsions, spasmodic pain and painful urination (Dubey *et al*, 1981). *Coleus* has been found useful in accelerating the breakdown of existing fat stores and promotes healthy cardiovascular function, also lowering the elevated blood pressure (Dubey *et al.,* 1981; Kramer *et al.,* 1987; Schlepper *et al.,* 1989). *Stevia rebaudiana* is a wild plant native to South America (Katayma *et al.,* 1976). *Stevia rebaudiana* is used for being anesthetic, anti-inflammatory, vasodilator cardiotonic as well as for lowering the uric acid levels (Jayaraman *et al.,* 2008) and ailments including diabetes, candidacies, high blood pressure, obesity (Ghosh *et. al*., 2008). Aim of present study is screening and evaluation of anti-microbial activity in *Coleus forskohlii* and *Stevia rebaudiana*.

MATERIALS AND METHODS

Collection of plant material: Fresh leaf, stem and nodal segments were collected from 2 years old plant of *Coleus forskohlii* and 6 months old plant of *Stevia rebaudiana* grown in botanical garden, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India.

***In vitro* shoot regeneration (for *in vitro* plant extract):** The cut ends of the explants started callusing after 4 weeks of incubation when the leaf explants were cultured on medium comprised of MS+BA(6-Benzyladenine; 5µM). The induced callus was initially yellow in colour but later on it turned green in colour and organogenic. The induction of shoot bud occurred in 6 weeks old culture. The transformation of shoot buds into elongated shoots occurred after second subculture passage in the fresh medium of the same composition. These microshoots (3-5 cm long) were then transferred to root induction medium containing MS + IBA (Indole-3-butyric Acid; 2.5µM).Within 2 weeks of transfer, the healthy roots were induced. These root plantlets were initially acclimatized in culture room conditions by their transfer in soilrite containing thermocole cups. After the hardening of one month these were then transferred to green house conditions. The plants thus obtained were then used for antimicrobial studies by using various parts of the plant.

Plant Extraction: The antibacterial activity was tested in both aqueous and alcoholic extracts of these plants. By following the method of Singh and Singh (2000) the extraction was done with few modifications mentioned by Shahid *et al.*, (2007).

(a) Aqueous Extraction: 15 g each of fresh plants parts (leaves, stem, fruit and flower head) were taken from the parent plant as well as *in-vitro* cultivated plants for aqueous extraction. First of all these parts were sterilized in 70% ethyl alcohol for 1 minute. After this, washing was done thrice with sterilized DDW at an interval of 5 minute. All plant materials were grounded with sterile pestle and mortar in 150 ml sterilized DDW. These homogenized tissues were then subjected to centrifugation at 5000rpm for 15 minutes. The supernatant was decanted as aqueous extract.

(b) Alcoholic Extraction: The fresh plant materials (15 g each) were taken and subjected to homogenization in 150 ml of absolute alcohol (100% ethanol). The homogenized tissues were centrifuged at 5000 rpm for 15 minutes. The supernatant was decanted as the alcoholic extract.

These aqueous and alcoholic extracts were used immediately for experimentation.

Testing of Bacterial Strains: The bacterial strains tested are *Staphylococcus aureus* ATCC 25923, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Bacillus species*.

Antimicrobial Susceptibility Test: Muellar-Hinton Agar (M 173; Hi media, India) was employed for ascertaining antimicrobial susceptibility test. For fastidious organisms like *Streptococci*, the blood agar used is composed of 5% defibrinated sheep blood. Agar well diffusion method was used for determining antimicrobial activity (Shahid *et al*., 2007).

The stock cultures (both standard and clinical) were first thawed and 2-3 identical colonies were immediately suspended into nutrient broth. It was kept in an incubator at 37^0 C. The turbidity of the bacterial suspension was adjusted to that of 0.5 McFarland Barium Sulphate tube. Then with the help of sterile swabs, two sets of Muellar-Hinton Agar plates (one for aqueous and the other for alcoholic extracts) were then lawn cultured with respective bacterial suspensions.

In each of the plates seven wells of 5 mm diameter were made with the help of sterile borer.20 µl of the plant extracts (aqueous as well as alcoholic extracts) were poured into the wells with the help of micropipette. The blank controls for the aqueous and alcoholic extracts used were sterile DDW and ethanol respectively. These plates were kept in the upright position for 5-10 minutes so that the solution diffuses completely into the medium. After this the plates were incubated aerobically at 37^0C overnight. After overnight incubation period, the streptococci cultures were suspended into Brain-Heart infusion (BHI) broth and lawn cultured on two sets of 5% sheep blood agar (one for aqueous and the other for alcoholic extracts). With the help of sterile borer, seven wells of 5 mm diameter were done in each plate. 20µl of plant extracts (both aqueous and alcoholic) were poured in these wells with the

help of micropipette. For about 5-10 minutes, these plates were kept in upright position so that the poured solution (extracts) diffuses into the medium. These plates were incubated in atmosphere enriched with 10% CO_2 at temperature of 37^0C. The diameter of the zone of inhibition was recorded as the result of activity. Each of these experiments were performed in triplicate.

Minimal Inhibitory Concentration (MIC) Determination for Bacterial Species Tested: In order to determine the MICs, broth micro-dilution testing was done according to the method defined by Clinical and Laboratory Standards Institute (CLSI) (Formerely National Committee for Clinical Laboratory Standards, NCCLS, 2000). Some modifications in this testing were employed as observed by Shahid *et.al*. 2007. By using RPMI-1640 (Hi Media Lab Ltd., India) broth supplemented with 0.3g L-glutamine (Hi Media Lab Ltd., India), 0.165 M MOPS buffer (Hi Media Lab Ltd., India) (33.54g/l) and 0.01% DMSO (Qualigens Fine Chemicals, India), the doubling dilution of extracts was prepared. Firstly the exracts were diluted in pure 100% DMSO then they were diluted 1:50 in RPMI-1640 medium and each resulting solution was used for doubling dilution series. The microtitre plates were prepared having 20µl of undiluted extracts in the first well followed by doubling dilutions of the extracts from the second well. After this, 20µl of the standardized inoculums of each bacterial species was added into the respective dilution wells (including the first well). The extract concentration ranges from 1×10^{-1} µg/ml (in the first well) to 7.81×10^{-4} µg/ml (in the last dilution well). For each of the test, there was a sterility control well having only RPMI-1640(40 µl), two sets of growth control wells (one containing RPMI-1640 broth plus the bacterial growth while the other one having RPMI-1640 broth plus the bacterial growth and DMSO, each 40 µl in volume) and an extract control (40 µl).

These microtitre plates were incubated for 24 hours at 37^0C (upper surface covered with sterile sealers).The lowest concentration not showing any visible growth was considered MIC of that extract for the bacterial species tested. The values are expressed in µg/ml. The duplicates were run for all the MIC experiments.

STATISTICAL ANALYSIS:

The data were statistically analyzed by using one way analysis of variance (ANOVA) followed by Turkey's multiple analysis test used to compare the antibacterial effects of different explants extracts (SPSS Software, Chicago III, Version 10). P values were calculated by the one-sample T-test and $P<0.05$ is taken as statistically significant.

RESULT AND DISCUSSION

Antibacterial activity present in the aqueous and alcoholic extracts of parent plant of *Coleus forskohlii* against the gram-positive bacteria tested. Against these tested gram-positive bacteria the significant amount of activity was found in both the aqueous and alcoholic extracts. Significant ($P<0.05$) amount of antibacterial activity was exhibited by the leaf extract against *Staphylococcus aureus, Staphylococcus epidermidis* and *Bacillus* species. The alcoholic extract of stem showed significant activity against *Staphylococcus aureus* and *Staphylococcus epidermidis.* The aqueous extracts of both leaf and stem exhibited a significant amount of antibacterial activity against *Staphylococcus aureus* and *Staphylo-coccus epidermidis* (Table 1). Antibacterial activity shown by the alcoholic as well as aqueous extracts of *in vitro* raised plant of *Coleus forskohlii* against the gram-positive bacteria tested. The significant amount of activity against *Bacillus* species only was shown by the alcoholic leaf extract but the alcoholic stem extract exhibited mild but statistically insignificant ($P>0.05$) activity against *Enterococcus faecalis* and *Bacillus* species. (Table 2). Antibacterial activity present in the alcoholic as well as aqueous extracts derived from the parent plant of *Stevia rebaudiana* against the Gram-positive bacteria. The aqueous leaf extract exhibited significant activity against only *Staphylococcus epidermidis.* The alcoholic leaf extract showed significant activity against *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis* and *Bacillus* species (Table 3).

MIC values of the alcoholic leaf extract of parent plant of *Coleus forskohlii* against the tested gram-positive bacterial species ranged from 9.43×10^{-3} to 18.65×10^{-3} µg/ml and MIC values of the aqueous leaf extract of parent plant of *Coleus forskohlii* against the tested bacterial species ranged from 3.15×10^{-3} to 4.65×10^{-3}

µg/ml. MIC values of the alcoholic leaf extract of parent plant of *Stevia rebaudiana* against the tested gram-positive bacterial species ranged from 9.43×10^{-3} to 25.15×10^{-3} µg/ml and aqueous leaf extract of parent plant of *Stevia rebaudiana* against *Staphylococcus epidermidis* was found to be 12.5×10^{-3} µg/ml (Table 4).

Ekwenye and Elegalam (2005) reported that ethanolic extract of *ginger* (*Zingiber Officinale*) inhibited growth of *S. typhi*. Akinpelu and Onakoya, (2006) reported that methanolic extracts of *P. guajava* (20 mg/ml) and *M. indica* (20 mg/ml) produced inhibition zone against *Bacillus cereus, Bacillus suitilis, Corynbacterium pyogenes, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas fluorescens, Shigella dysenteriae, Staphylococcus aureus* and *Streptococcus faecalis*. Nair and Chanda (2007) reported that ethanolic and aqueous extract of *Emblica officinalis* showed strong activity against *Staphylococcus aureus*, *Bacillus cereus* and also concluded that ethanol plant extracts were more potent than aqueous plant extracts. Elekwa *et al*. (2009) studied the antimicrobial activity of extracts (ethanol, methanol and aqueous) of the leaves and stem barks of *Psidum guajava L* and reported that only the aqueous extract inhibited *Bacillus subtilis*. Our study concluded that medicinal plants have capability to inhibit the growth of pathogenic microorganism and *Coleus forskohlii* and *Stevia rebaudiana* can be used as substitute of antibiotics in future.

Table 1. Antibacterial Activity present in the Aqueous and Ethanolic extracts of parent plant of *Coleus forskohlii* against the tested Gram-positive bacteria

Organisms	Zone of inhibition(mm)±SE					
	Ethanol (Control for Alcoholic extracts)	DDW (Control for Aqueous extracts)	Parent Plant extract			
			Leaf aqueous	Leaf alcoholic	Stem Aqueous	Stem alcoholic
Streptococcus pyogenes	8.67±0.33^{bc}	5.00±0.00^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{b}
Strptococcus agalactiae	8.67±0.33^{bc}	5.00±0.00^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{b}
Staphylococcus aureus ATCC 25923	9.33±0.33^{ab}	5.00±0.00^{a}	11.00±0.58^{b} (P=0.009)	11.67±0.33^{ab} (P=0.020)	11.33±0.67^{a} (P=0.011)	11.67±0.33^{a} (P=0.020)
Staphylococcus epidermidis	8.67±0.33^{ab}	5.00±0.00^{a}	12.67±0.33^{a} (P=0.002)	13.00±0.58^{a} (P=0.024)	11.67±0.67^{a} (P=0.010)	12.33±0.33^{a} (P=0.012)
Enterococcus faecalis	7.67±0.33^{c}	5.00±0.00^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{b}
Bacillus species	9.33±0.33^{ab}	5.00±0.00^{a}	0.00±0.00^{c}	12.00±0.33^{b} (P=0.027)	0.00±0.00^{b}	0.00±0.00^{b}

Table 2. Antibacterial Activity of Aqueous and Alcoholic extracts of *in vitro* raised plant of *Coleus forskohlii* against the tested pathogenic Gram-positive bacteria

Organisms	Zone of inhibition(mm)±SE					
	Ethanol (Control for alcoholic extracts	DDW (Control of Aqueous extracts	*In vitro* plant extract			
			Leaf Aqueous	Leaf Alcoholic	Stem Aqueous	Stem Alcoholic
Streptococcus pyogenes	7.67±0.33^{bc}	5.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{c}
Streptococcus agalactiae	7.67±0.33^{bc}	5.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{c}
Staphylococcus aureus ATCC 25923	8.33±0.33^{ab}	5.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{c}
Staphylococcus epidemidis	8.33±0.33^{ab}	5.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{c}
Enterococcus faecalis	9.33±0.33^{c}	5.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{a}	8.33±0.33^{b} (P=0.185)
Bacillus species	10.33±0.33^{a}	5.00±0.00^{a}	0.00±0.00^{a}	12.33±0.33^{a} (P=0.027)	0.00±0.00^{a}	11.00±0.58^{a} (P=0.366)

Table 3. Antibacterial Activity of Aqueous and Alcoholic extracts of parent plant of *Stevia rebaudiana* against the tested pathogenic Gram-positive bacteria

Organisms	Zone of inhibition(mm)±SE			
	Ethanol(Control for alcoholic extracts	DDW(Control of Aqueous extracts	Parent plant extract	
			Leaf Aqueous	Leaf Alcoholic
Streptococcus pyogenes	8.33 ± 0.33^{bc}	5.00 ± 0.00^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}
Streptococcus agalactiae	7.67 ± 0.33^{bc}	5.00 ± 0.00^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}
Staphylococcus aureus ATCC 25923	9.33 ± 0.33^{ab}	5.00 ± 0.00^{a}	0.00 ± 0.00^{b}	12.00 ± 0.58^{ab} (P=0.044)
Staphylococcus epidemidis	8.33 ± 0.33^{ab}	5.00 ± 0.00^{a}	9.67 ± 0.33^{a} (P=0.005)	11.33 ± 0.33^{ab} (P=0.027)
Enterococcus faecalis	8.33 ± 0.33^{c}	5.00 ± 0.00^{a}	0.00 ± 0.00^{b}	10.67 ± 0.33^{b} (P=0.012)
Bacillus species	10.33 ± 0.33^{a}	5.00 ± 0.00^{a}	0.00 ± 0.00^{b}	12.33 ± 0.33^{a} (P=0.027)

Table 4. Minimal Inhibitory Concentration (MIC) determination for the leaf extracts of parent plant of *Coleus forskohlii* and *Stevia rebaudiana* against the tested bacterial species

Bacterial species tested	MIC values(µg/ml)			
	parent plant of *Coleus forskohlii*		parent plant of *Stevia rebaudiana*	
	alcoholic leaf extract	aqueous leaf extract	alcoholic leaf extract	aqueous leaf extract
Staphylococcus aureus ATCC 25923	18.65×10^{-3}	4.65×10^{-3}	18.55×10^{-3}	-
Staphylococcus epidermidis	9.43×10^{-3}	3.15×10^{-3}	12.35×10^{-3}	12.50×10^{-3}
Bacillus species	12.25×10^{-3}	-	9.43×10^{-3}	-
Enterococcus faecalis	-	-	25.15×10^{-3}	-

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