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Comparative antimicrobial potential of different extracts of leaves of *Stevia rebaudiana* Bert.

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Abstract

The present investigation was evaluated for potential antimicrobial activity of *Stevia rebaudiana* leaf extracts, procured from Indian acidic and basic soil zones. Separately *Stevia* leaves were extracted with aqueous, methanol and ethanol solvents and their microbiocides were compared against few selected gram positive (*Bacillus substilis* and *Staphylococcus aureus*) and gram negative bacteria (*Escherichia coli*, *Salmonella typhi*) by disc diffusion technique. The study revealed the potential antimicrobial activity of different leaf extracts of *Stevia rebaudiana* Bert., determined with zone of inhibition against standard amphicilin. However among all the extracts, aqueous leaf extract of *Stevia* plant having concentration dependent significant antimicrobial activity ($P<0.001$) compared to extracts collected from basic soil zone. Such results variation may be due to the effect of rich organic carbon content in acidic soil that increase the amount of glycoside content in *Stevia* leaves. This proved *Stevia* is a potential antimicrobial agent as non antibiotics sources.

Key words: Acidic soil, antimicrobial, basic soil, *Stevia*.

INTRODUCTION

Stevia rebaudiana Bert. is a plant of recent focus in world wide as supplement of sugar. The plant was originated from South America (Paraguay and Brazil), belongs to the family Compositae. The plant is also recognize as a medicinal plant in India with its versatile medicinal applications viz. treatment in diabetic condition (acts as non caloric), candidacies, against high blood pressure, weight loss, skin tonner and in other various uses in traditional system of medicine. The leaf of this plant is the main attention for the economic and commercial application due to its extreme sweetness nature particularly with the presence of the main active constituents stevioside and rebaudioside A [1, 2]. In the recent year, the extract of *Stevia* leaves has been subjected to various pharmacological, clinical and toxicological investigations and results revealed interesting therapeutic applications [3, 4]. It also has been used in developing broiler embryos [5]. In addition there has been an immense interest in utilization of natural plant extracts as antimicrobial activity due to the increase in out break of food borne diseases and to minimize the health causing diseases over synthetic drugs. Few of the literatures have been described antimicrobial activity of *Stevia rebaudiana* leave extracts using different methods [6, 7, 8] but there was no literature reveals the comparative antimicrobial activity of *Stevia rebaudiana* collected from different soil nature. It was reported that active constituent of *Stevia* plant varied with the agronomic practices [9, 10] and that may causes variations in therapeutic activities. Keeping these in view a part of PhD research work was undertaken to investigate the antimicrobial activity of *Stevia* leaves collected from acidic and basic soil environments and compared the same.

MATERIALS AND METHODS

Plant material collection:

A six month period of *Stevia* field experiment was conducted in two different soil zone of Karnataka (Acidic soil zone in Shimoga, pH 6.10 and basic soil zone in Aravavi, pH 8.20), India. Control *Stevia* leaves were periodically collected in month intervals from both the zones separately and oven dried at 60° C for 6 hours. Further the dried leaves were stored at 4°C and were used for further antimicrobial investigation.

Microorganisms Used:

The bacterial strains used were obtained from stock culture of the department of Microbiology, Al-Ameen College of Pharmacy, Bangalore, India. Two gram positive and two gram negative organisms viz. *Bacillus substilis* NCIM 2708, Straphyloccus aureus NCIM 2079 and Escherichia coli NCIM 2685, *Salmonella typhimurium* NCIM 2242 respectively were used for the present study, were grown and maintained on nutrient agar medium.

Preparation of extracts and antibiotic solution:

25 g of dried *Stevia* leaves were extracted with various solvents viz. distilled water, HPLC grade methanol and ethanol. Two separate methods were used for extraction of *Stevia* leaves likely soxhalation for methanolic and ethanolic extracts during 4 hours and refluxation for aqueous extract during 6 hours after standardization of method. Oven temperature was maintained at 45°C. Extracts were collected and filtered using Whatman No 1 filter paper and the filtrates were then subjected to evaporation under reduced pressure to get soft extract and stored in labeled sterile screw capped bottles at -15° C. The yield of aqueous, methanolic and ethanolic extracts of the leaf (from acidic

soil zone) were found to be 28 g%, 26.2 g% and 24.8 g % respectively and 26.4 g%, 25.8 g% and 18.9 g % respectively (collected from alkaline zone) on dry weight basis.

Stock solution of broad-spectrum antibiotic (Ampicilin as standard) was prepared as 30 mcg/ ml (w/v) concentration in sterile distilled water. The concentration of 0.1 ml ampicillin was used for the antibacterial assay in this study.

Preparation of inoculum:

The suspension of organism was prepared as per Mac-Farland nephlometer standard [11]. A 24-hour-old culture was used for the preparation of bacterial suspension. A suspension of organism was made in a sterile isotonic solution of sodium chloride and the turbidity was adjusted such that it contained approximately $1.5x10^6$ cells/ml. It was obtained by adjusting the optical density (650 nm) equal to 0.5ml of 1.175-% barium chloride in 100ml of 1.0 % sulphuric acid.

Determination of minimum inhibitory concentration (MIC)

Plate dilution method was followed to determine MIC of all the above mentioned extracts. Different concentrations were used (100, 200 and 300 mcg/ml) against 0.1 ml of 10^{-4} inoculum, dilution prepared from 24 hours incubated culture of *E. coil*, *S. typhi*, *B. substilis*, S. aureus into different sterile Petri plates followed by pouring of 20 ml autoclaved nutrient agar media so as to understand the minimum concentration needed to prevent the growth of the microbial strain and use the obtained MIC from this test for evaluation of zone of inhibition for all extracts. The plates were prepared in triplicates and were incubated at 37°C for 48 hours and the growth was observed.

Antimicrobial Assay:

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All the extracts were subjected to antimicrobial assay by measuring the diameter of zone of inhibition (IZD) using disc diffusion technique. The Petri dishes were washed and sterilized in hot air oven at 160°C for one and half hour and then 1.0% of the inoculum was added to the sterilized nutrient agar medium at 45°C. Three bores were made on the medium using sterile borer (diameter of borer was 9 mm). 0.2 ml of 10^{-4} dilution of 24 hours old bacterial cultures were used so as to ensure the concentration of these organisms to contain approximately $1x\ 10^6$ CFU/ ml. All the extracts were taken at different concentration of 100, 200 and 300 mcg/ml.

Statistical analysis:

The experimental results were repeated thrice and zone of inhibition were determined in mm. All the results were statistically expressed as the mean ± standard error of mean (SEM). Values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The in vitro antimicrobial activity of aqueous, methanol and ethanol extracts of dried *Stevia* leaves (collected from acidic and basic soil field), were tabulated separately in table 1 & 2 respectively. In Table-1, all the *Stevia* extracts showed high significant activities ($p< 0.001$) against B. subtilis and S. aureus whereas no activities found against E. coil and *S. typhi*. Among the extracts, only aqueous extract shows higher activities against B. subtilis and S. aureus (10.5 mm and 11.5 mm respectively) than methanolic and ethanolic extracts. However methanolic extract showed little higher activity against S. aureus (10.5 mm) than ethanolic extract (10.2 mm) at 300 mcg/ml concentration, whereas reverse activity shown against B. subtilis (9.9 mm for ethanolic extract and 9.8 mm for methanolic extract) at 300 mcg/ml concentration, but there were no significant variation in methanolic and ethanolic extracts against B. subtilis and S. aureus.

In other way, the extracts obtained from the basic soil zone (Table-2), the same trend followed as earlier where aqueous extract gave significant high activity than other two extracts. But interestingly, aqueous and ethanolic extracts showed activities against *S. typhi*, B. subtilis and S. aureus, whereas no activity shown against *S. typhi* with none of the former extracts collected from the acidic soil zone (Table-1). Related to significant activity against *S. typhi*, Table-2 clearly indicated that aqueous extract was significantly active against *S. typhi* (10.03 mm) at 300 mcg/ml concentration where as ethanolic extract was active significantly at 200 mcg/ml concentration (9.43 mm) but as per zone of inhibition measured, aqueous extract showed 9.7 mm where as ethanolic extract showed 9.43 mm at 200 mcg/ml concentration. These observations clearly highlighted that among the extracts, aqueous extract shown higher activities ($p<0.05$) against *S. typhi* (10.03 mm), S. aureus (11.23 mm) and B. subtilis (10.3 mm) followed by methanolic and ethanolic extracts. Methanolic extract showed higher activities against S. aureus and B. substilis (10.06 mm and 9.96 mm respectively) than ethanolic extracts for the same (9.49 mm and 9.68 mm respectively). Totally negative activity showed against E. coli with all the extracts collected from both the zones.

Antimicrobial activity of various plant parts have been reported by the many researchers [12, 13] but it is worthwhile to focus on the area where no literatures investigated comparative antimicrobial activity of different extracts of *Stevia* leaves collected from different soil zones. Keeping this, different *Stevia* extracts were prepared (procured from acidic and basic soil zone) and were evaluated for antimicrobial study and compared among them for higher microbial activity. The present investigations endow with the basic information about plant extract especially aqueous extracts of *Stevia rebaudiana* leaves which were found to be potent substantial antimicrobial activity against pathogens like *S. typhi, B. subtilis* and *S. aureus* bacteria but no such activities were found with extracts obtained from both the soil zones against *E. coil,* which also shows same results reported by earlier paper [6, 14]. In general however, both extracts showed a concentration dependent inhibitory effect on all the bacteria species. This finding also

correlated with the literatures earlier reported [15, 16, 17] who independently found that various plant extracts inhibits the growth of some bacteria isolates.

Acidic soil contains more organic carbon, which is responsible for more accumulation of glycoside in *Stevia* leaves, which helps for the antimicrobial activity.

Table – 1 Antimicrobial activity of three different extracts of Stevia rebaudiana (From acidic soil, pH 6.10) (Mean of three readings; Mean ± SEM)

Name of the extracts	Conc. (mcg/ml)	*E. coli*	*S. typhi*	*B. subtilis*	*S. aureus*
Aqueous extract	100	NA	NA	9.5 ± 0.09**	9.74 ± 0.09**
	200	NA	NA	9.8 ± 0.09**	10.4 ± 0.15**
	300	NA	NA	10.5 ± 0.11**	11.5 ± 0.09**
Methanolic extract	100	NA	NA	9.3 ± 0.09**	9.4 ± 0.09**
	200	NA	NA	9.7 ± 0.09**	9.6 ± 0.07**
	300	NA	NA	9.8 ± 0.09**	10.5 ± 0.09**
Ethanolic extract	100	NA	NA	9.3 ± 0.06**	9.3 ± 0.11**
	200	NA	NA	9.4 ± 0.09**	9.8 ± 0.06**
	300	NA	NA	9.9 ± 0.09**	10.2 ± 0.11**
Standard (Ampicilin)	30mcg/100ml	22.5	22.5	22.5	22.5

** P < 0.001; Each value represents the mean ± SEM (n =3); NA = Not active

Table 2. Antimicrobial activity of three different extracts of Stevia rebaudiana (From basic soil, pH 8.2) (Mean of three readings; Mean ± SEM)

Name of the extracts	Conc. (mcg/ml)	*E. coli*	*S. typhi*	*B. subtilis*	*S. aureus*
Aqueous extract	100	NA	9.23±0.0881*	9.16±0.0333**	9.4±0.0577*
	200	NA	9.7±0.1155*	9.93±0.08819*	9.63±0.1453*
	300	NA	10.03±0.1764**	10.3±0.1155**	11.23±0.1764**
Methanolic extract	100	NA	NA	9.16±0.0333**	9.36±0.1202*
	200	NA	NA	9.36±0.08819*	9.3±0.0577*
	300	NA	NA	9.96±0.1202*	10.06±0.08819*
Ethanolic extract	100	NA	9.2±0.0574*	9.5±0.1155*	9.16±0.06667*
	200	NA	9.43±0.1453**	9.53±0.2028*	9.63±0.1453*
	300	NA	9.73±0.08819*	9.68±0.1764**	9.49±0.1764*
Standard (Ampicilin)	30mcg/100ml	20.3	20.3	20.3	20.3

P > 0.05 and ** P < 0.05; Each value represents the mean ± SEM (n =3); NA = Not active

Even though the activities were lower than standard ampicilin but it is clearly indicates that the antimicrobial activities also depends on soil nature of the cultivated plants.

In our present study, *Stevia* leave extracts collected from acidic soil zone shown high significant results than the extract procured from basic soil zone, determined by zone of inhibition.

On same concept the work also carried out earlier and reported acidic soil shown good anti microbial effect [18, 19].

CONCLUSION

Although all the individual extracts (obtained from cultivated *Stevia* plant in different soil zones) show potential antimicrobial activity as compared to standard ampicilin but the activities were lesser than standard. Aqueous extract show statistically high significant antimicrobial activity in both the separate experiments but the concentration dependent higher activity shown by the aqueous extract of *Stevia* leaves collected from acidic soil zone due to high range of organic carbon content in soil. This further concluded that *Stevia* plant could be proved as future potential antimicrobial agent as non antibiotics sources.

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