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Studies on Antibacterial Activity, Phytochemical Analysis of *Stevia rebaudiana* (Bert.) - An Important Calorie Free Biosweetner

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Abstract

Stevia rebaudiana is an outstanding herb bearing leaves of very refreshing sweet taste and remarkable health promoting activities. The herb is nutrient rich, containing substantial amounts of protein, calcium, phosphorous, sodium, magnesium, zinc, rutin, vitamin A, vitamin C and other nutrients, yet has no caloric value. Six solvent extracts from leaf, three solvent extracts from flower of stevia were assayed for *in vitro* antibacterial activity against pathogenic bacteria such as *Bacillus subtilis, Klebsiella pneumonia, Proteus vulgaris, Streptococcus pneumoniae, Staphylococcus aureus* and *Pseudomonas fluorescence,* the zone of inhibition were compared with different standard antibiotics. Phytochemical screening of the crude extracts revealed the presence of secondary compounds such as alkaloids, flavonids, steroids, tannins and phenols. The organic solvent extracts of flowers are very active against the tested bacterial stains when compared with leaf extracts. Among the entire extracts petroleum ether flower extract showed high activity (11-13 mm zone of inhibition) on all the tested organisms. Petroleum ether leaf and flower extracts gave very low MIC values. Petroleum ether flower extract gave lowest MIC values (0.390 to 1.562 µg/ml) against all the bacterial isolates tested.

Key Words: *Stevia rebaudiana*, Flower, Leaf extracts, Antibacterial activity, Phytochemical analysis

Introduction

Medical plants constitute an effective source of both traditional and modern medicines, herbal medicine has been shown to have genuine utility and about 80% of rural population depends on it as primary health care. Over the years the World Health Organization (WHO) advocated that countries should interact with traditional medicine with a view to identify and exploiting aspects that provide safe and effective remedies for aliments of both microbial and non-microbial origins [1].

Plant based drugs are being increasingly preferred in medical science. The curative parts of a medicinal plant are not simply its woody stem or its leaves, but the number of chemical compounds (phytochemicals) produced and uses for its own growth and development. The therapeutic value and pharmacological action of a drug is due to the presence of certain chemical constituents such as carbohydrates, derivatives of carbohydrates, gums, mucilages, pectins, various forms of glycosides, tannins, phenolic compounds, lipids, fixed and volatile oils, resins, various kinds of alkaloids etc. These phytochemicals are of immense importance to mankind. Phytochemical investigation of plants is an interesting area of research, leading to the isolation of several new compounds. Though voluminous literature has accumulated on secondary products of plant, very little information is available on their presence and biosynthetic pathways in plants growing in arid zones. Knowledge of chemical constituents of plants is desirable, not only for the discovery of therapeutic agents, but also because such information may be of value in discovering new sources such as tannins, oils, gums, precursors for the synthesis of complex chemical substances etc. In addition, knowledge of the chemical constituents of plants would be valuable in discovering the actual value of folkloric remedies [2]. The development of bacterial resistance to presently available antibiotics has necessitated the search for new antibacterial agents. Initial screening of potential antibacterial and antifungal compounds from plants may be performed with pure substances or crude extracts [3, 4]. The isolation and characterisation of flavonoids from *Indigofera tinctoria* plant parts and callus culture was carried out earlier [5].

The present study mainly aims at phytochemical screening for secondary compounds and antibacterial activity of *Stevia rebaudiana*

Materials and Methods

Collection of plant material and identification

Stevia rebaudiana plant material was collected from rural villages of Tirupathi, A.P, India and grown under nursery shade conditions in Department of Biotechnology, S.V. University, Tirupathi, India.

Preparation of plant extracts

The plant materials (leaves and flowers) were dried in shade and powdered by mechanical grinder. The leaves and flowers were powdered and extracted following the published procedure with slight modifications [6]. The powdered material was isolated in ethanol, methanol, chloroform, hexane, ethyl acetate, diethyl ether by keeping them in a shaker for 3 days. The extracts were reduced to 10% of its original volume and filtered. The filtered organic solvents were concentrated in vacuum using a rotator evaporator, while aqueous extracts were subjected to antimicrobial activity and phytochemical analysis.

Phytochemical analysis

Phytochemical analysis of all the evaporated solvent extras was conducted following the procedure of Indian Pharmacopoeia [7]. By this analysis, the presence of several phytochemicals listed in table-1 was tested. To test for alkaloids (200mg plant material in 10 ml methanol, filtered); a 2ml filtrate +1% HCl +steam, 1 ml filtrate+6drops of Mayor's reagents/Wagner's regent/Dragendroff reagent, creamish precipitate/brownish-red precipitate/orange precipitate respectively indicated the presence of respective alkaloids. For tannins (200mg plant material in 10 ml distilled water, filtered); a 2ml filtrate+2ml Fecl3, blue-black precipitate indicated the presence of tannins. For flavonoids (200 mg plant material in 10ml ethanol, filtered); a 2ml filtrate + conc. HCl + magnesium ribbon pink-tomato red colour indicated the presence of flavonoids. Steroids (Liebermann-Burchard reaction: 200 mg plant material in 10 ml chloroform, filtered); a 2 ml filtrate+2ml acetic anhydride +conc.H_2SO_4. Blue-green ring indicated the presence of steroids. For phenols, 1ml of each solvent extracts dissolved in alcohol or water was separately treated with a few ml of neutral ferric chloride solution. The change in colour indicated the presence of phenols.

Bacterial strains and growth conditions

The following cultures of bacteria such as *Bacillus subtilis, Proteus vulgaris, Staphylococcus aureus, Klebsiella pneumonia, Staphylococcus pneumonia and Pseudomonas fluorescence were* used for screening antibacterial activity. All the cultures were obtained from Microbiology department, S.V. University, Tirupati. The bacterial cultures were maintained in nutrient agar slants at 2 – 8^0C.

Inoculum preparation

The bacterial strains were inoculated on nutrient broth (0.5 % peptone, 0.5% Nacl, 0.15% yeast extract, P^H 7.4) and incubated at 37^0C for overnight. The bacterial cells were harvested by centrifuging at 5000g for 15 minutes. The pellet formed was washed twice with PBS (phosphate buffer saline, 10 mM sodium chloride, P^H 7.4) and the cells were counted by heamocytometer. The bacterial cells were diluted to approximately 10^5 cfu /ml before use [8].

Determination of antibacterial activity

The antibacterial activity of leaves, flower extracts was determined by using agar well diffusion method with slight modification of published procedure [9]. Nutrient agar slants after solidification was inoculated with the test microorganisms, by spreading the bacterial inoculums under aseptic conditions. Wells of 5mm diameter were punched in the agar medium with sterile cork borer and filled with 100 ml of plant extract. The antibiotics such as penicillin, kanamycin, tetracycline and cefotoxine at 100 µg/ml concentration were used in the test system as positive controls. The plates were incubated at 37^0C for 24 hrs. The negative control was added without adding the cultures to know the sterile conditions. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition for the respective plant extract and antibiotic.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined by macro broth dilution method (10). The reconstituted extract was serially diluted two-fold in nutrient broth medium. Duplicate tubes of each dilution were inoculated with 5 x 10^5 cells (cfu) of the test bacterial strain and cultures incubated at 37^0C for 18 hours. Two-fold serial dilutions of penicillin were included in each experiment as controls. MIC was taken as the highest dilution (least concentration) of controls. MIC was taken as the highest dilution (least concentration) of extract showing no detectable growth in the macro-broth assay.

Results and Discussion

Phytochemical analysis

The preliminary phytochemical analysis of crude extracts of leaves and flowers of *S. rebaudiana* revealed the presence of various phytochemicals such as alkaloids, flavonoids, phenols, steroids and tannins (Table-1). Alkaloids were presented in high concentration in chloroform and hexane leaf extracts and a moderate amount of flavonoids were present in methanol, chloroform and petroleum ether flower extracts and in ethanol leaf extracts. Flavonoids were present in high concentrations in ethanol leaf extract. Higher concentrations of phenols were also present in the ethanol leaf extract when compared to the other extracts. Fewer amounts of phenols are present in the flower extracts. The preliminary phytochemical studies received pronounced importance, because the crude drugs possess varied composition of secondary metabolites [11].

Higher concentrations of steroids were present in ethanol and hexane leaf extracts and in chloroform flower extract. Less amount of steroids are present in the methanol and petroleum ether extracts of flower. Ethanol leaf extract contains high concentrations of tannins when compared with the other extracts. Methanol and petroleum ether flower extracts contain fewer amounts of tannins.

Antibacterial Activity

In vitro determination of antibacterial activity of leaf and flower extracts of *Stevia rebaudiana.* Antibacterial activity of the crude extracts of *Stevia rebaudiana* against *Psedomonas fluorescence*, *Proteus vulgaris, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia* and *Streptococcus pneumonia* were represented. Standard antibiotics such as kanamycin, penicillin, cefotaxime and tetracycline were also tested for comparing with the crude extracts.

A total of thirteen plant extracts, representing leaf and flower of *Stevia rebaudiana* obtained by various organic solvents were investigated for antibacterial effects using the agar well diffusion method. The plant parts used, extracts tested, standard antibiotics and the results of the bacterial

sensitivity were tabulated. All the nine crude extracts showed good antibacterial activity. Among all the extracts, petroleum ether flower extract showed high activity (11-13 mm of zone of inhibition) on all the organisms. All the flower extracts (methanol, chloroform and petroleum ether) showed high activity (10-13 mm) when compared to the leaf extracts (ethanol, methanol, ethyl acetate, chloroform, hexane and petroleum ether), which showed a moderate activity (6-10 mm). The obtained results of the crude extracts are comparable with the standard antibiotics such as kanamycin, penicillin, cefotaxime and tetracycline. All the tested organisms showed more or less similar sensitivity to the petroleum ether flower extract of *S. rebaudiana* (11-13 mm) like the standard penicillin, cefotaxime and tetracycline antibiotics (Table-2; Figure-1 & 2).

On overall, flower extracts are very active against all the tested bacterial strains when compared with leaf extracts. From these studies it is obvious that the active principles are confined to the aerial parts of this plant especially in flowers. The presence of some of the secondary metabolites reported by the earlier workers from the leaves such as *Eudesmanoids* [12], *isoflavone glycosides* [13] and essential oils [14] may be the cause of antibacterial activity of this plant. It is evident that from the literature, the phenols [15], tannins [16, 17], terpenoids [18].

Table: 1. Qualitative analysis of phytochemicals such as alkaloids, flavonoids, phenols, steroids, tannins in different plant parts of *S. rebaudiana*

Types of extract	Metabolites present in the material				
	Alkaloids	Flavonoids	Phenols	Steroids	Tannins
Leaf					
Ethanol	+++	++++	++++	++++	++++
Methanol	++	+++	+++	++	++
Ethylacetate	++++	++	++	++	++
Chloroform	++++	++	++	++	++
Hexane	++++	++	++	++++	++
Petroleum ether	++	++	+	++	++
Flower					
Methanol	+++	++	++	+	+
Chloroform	+++	++	++	++++	++
Petroleum ether	+++	++	+	+	+

Table: 2. Susceptibility of test bacterial strains to leaf, flower and root extracts of *S. rebaudiana* and standard antibiotics

Types of extract / antibiotic used	Zone of inhibition or antibacterial activity (in mm)					
	Pseudomonas fluorescence	*Proteus vulgaris*	*Bacillus subtilis*	*Stayphylococcus aureus*	*Klebsiella pneumonia*	*Streptococcus pneumonia*
Leaf						
Ethanol	7.0	6.5	9.0	9.0	8.0	9.0
Methanol	9.0	9.0	10.0	9.0	10.0	10.5
Ethylacetate	7.5	8.0	9.0	8.0	9.0	8.0
Chloroform	9.0	9.0	10.0	8.5	8.0	9.5
Hexane	8.0	9.0	8.5	8.0	9.5	9.0
Petroleum ether	8.0	8.5	9.0	9.0	8.0	9.0
Flower						
Methanol	10.5	10.0	11.0	10.5	11.0	11.0
Chloroform	10.0	11.0	10.5	10.0	12.0	12.5
Petroleum ether	12.0	13.5	12.0	13.0	12.0	13.0
Standard antibiotics						
Kanamycin	11.0	12.0	22.0	11.0	13.0	11.5
Penicillin	9.0	7.5	12.0	4.5	15.0	5.0
Tetracycline	8.0	14.0	14.0	10.0	12.0	13.0
Cefotaxime	10.5	12.0	9.0	12.0	10.0	12.0

Table: 3. Minimum inhibitory concentrations (MIC) of the crude extract of *Stevia rebaudiana* against the test bacterial strains.

Type of extract used	MIC (in µg/ml)					
	Pseudomonas fluorescence	*Proteus vulgaris*	*Bacillus subtilis*	*Stayphylococcus aureus*	*Klebsiella pneumonia*	*Streptococcus pneumonia*
Leaf						
Ethanol	25	50	12.5	12.5	25	6.25
Methanol	6.25	12.5	3.125	6.25	3.125	3.125
Ethylaceate	25	6.25	6.25	12.5	3.125	12.5
Chloroform	12.5	12.5	3.125	6.25	6.25	3.125
Hexane	6.25	3.125	3.125	12.5	6.25	6.25

Petroleum ether	3.12	6.25	1.562	1.562	6.25	3.125
Flower						
Methanol	1.562	3.125	1.562	6.25	1.562	0.781
Chloroform	3.125	1.562	1.562	3.125	0.781	0.781
Petroleum ether	1.562	0.390	0.390	0.781	3.125	0.390

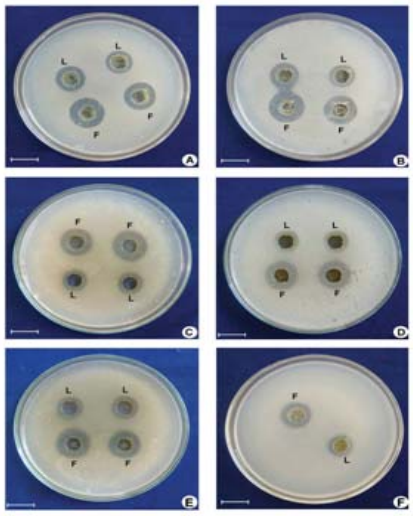

Figure: 1: Antibacterial activity of leaf and flower extracts of field grown *S.rebaudiana* plants
A. Antibacterial activity of petroleum ether leaf and flower extracts against *Klebsiella pneumonia.*
B. Antibacterial activity of petroleum ether leaf and flower extracts against *Proteus vulgaris.*
C. Plate tested petroleum ether leaf and flower extracts against *Pseudomonas fluorescence.*
D. Petroleum ether leaf and flower extracts showing activity against *Bacillus subtilis.*
E. Plate tested chloroform leaf and flower extracts against *Pseudomonas fluorescence.*
F. Chloroform leaf and flower extracts showing activity against *Proteus vulgaris.*
'L' Leaf extract 'F' Flower extract

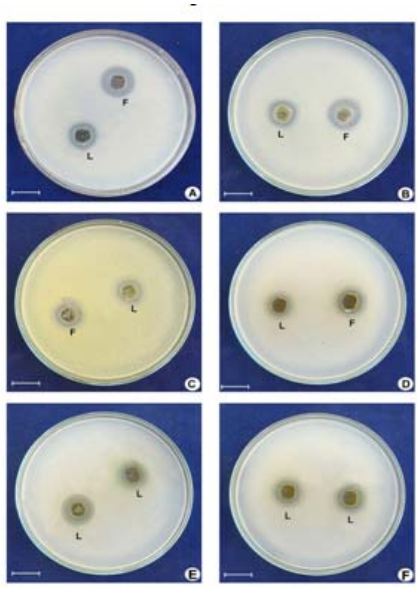

Figure: 2: Antibacterial activity of leaf and flower extracts of field grown *S.rebaudiana* plants
A. Plate tested methanol leaf and flower extracts against *Streptococcus pneumonia.*
B. Plate tested methanol leaf and flower extracts against *Bacillus subtilis.*
C. Methanol leaf and flower extracts showing activity against *Klebsiella pneumonia.*
D. Methanol leaf and flower extracts showing activity against *Proteus vulgaris.*
E. Antibacterial activity of ethanol leaf extracts against *Bacillus subtilis.*
F. Antibacterial activity of petroleum ether leaf extracts against *Pseudomonas fluorescence*
'L' Leaf extract 'F' Flower extract

Minimum inhibitory concentrations (MIC)

The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tubes, was regarded as MIC.

The results of this analysis are shown in table-3. Some of the extracts, like the petroleum ether leaf and flower extracts gave very low MIC values. Petroleum ether flower extract gave lowest MIC values (0.390 to 1.562 µg/ml) against all the bacterial isolates tested. A lowest MIC value (0.390 µg/ml) was recorded against *Bacillus subtilis, Pseudomonas vulgaris* and *Streptococcus pneumoniae*. Methanol flower extract showed the MIC values in the range of 0.781 to 6.25 µg/ml whereas leaf methanol extract showed 3.125 and 12.5 µg/ml values respectively for all the organisms. Of all the extracts tested, lowest MIC values were recorded on all organisms for the flower extracts than leaf extracts.

The effects of herbal compounds and phytochemicals on pathogenic and economically important bacteria have been well studied [19]. Antimicrobial activity of the essential oil of *Cestrum diurnum* was studied [20], antibacterial activity of the flower extracts of *Cassia alata* [21], leaf extracts of *Adhatoda vasica* [22] rose petal extracts, [23]; leaves of *Mucuna pruriens* [24] were also studied. Phytochemical screening of 55 Iranian plants belonging to 21 families was carried out by other research workers [25]. Quantitative analysis of total phenolics was carried out in callus, leaf, stem and roots of both normal and tissue cultured plants of *Bacopa monnieri* [26].

Conclusion

In the present day scenario, increase in antibiotic resistance in a wide range of bacterial species is the major problem around the world. Medicinal plant or herbal drugs are the major resources to counteract this problem. In the present study, antibacterial screening was carried out with various organic crude extracts and found all the bacterial strains tested are highly sensitive to the petroleum ether flower extracts. Hence further research in this line is very useful to develop potential drugs from this valuable medicinal plant.

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