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## Antimicrobial assay of *Stevia rebaudiana* Bertoni leaf extracts against 10 pathogens

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### Abstract

With an objective of understanding the antimicrobial potential of *Stevia rebaudiana* (popularly called as Stevia and synonymously known as 'sugar substitute' belongs to family Asteraceae), chemical extracts from its leaves were subjected to microbial assay using six solvents against ten selected pathogenic as well as food spoiling fungal (*Alternaria solani*, *Helminthosporium solani*, *Aspergillus niger*, *Penicillium chrysogenum*) and pathogenic bacterial (*Escherichia coli*, *Bacillus subtilis*, *Enterococcus faecalis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) isolates. 250 $\mu$ g/ml of petroleum ether extract (minimum inhibitory concentration) was found sufficient enough to inhibit the growth of test microorganism *E.coli* completely in petriplates (by plate dilution method). *S. aureus* amongst bacteria and *P. chrysogenum* amongst fungi exhibited highest range of susceptibility against four extracts namely; water, petroleum ether, cyclo-hexane, and chloroform but *B. subtilis* was found to have highest resistance to all except petroleum ether and acetone extract. Highest antifungal index (A<sub>f</sub>-15mm) and antibacterial index (A<sub>b</sub>-11.2mm) was obtained for petroleum ether extract against all pathogens signifying its best antimicrobial potentiality but ethanol and cyclo-hexane extracts were proved to be least effective (lowest A<sub>b</sub>I and A<sub>f</sub>I). Invariably cyclo-hexane, acetone and ethanol did not show anti fungal activity except demonstrating selective inhibition to specific microorganisms *P. chrysogenum* 8.0mm), *A. solani* (7.0mm) and *A. niger* (9.0mm) respectively. The above findings support the idea that plant extracts of *Stevia rebaudiana* Bertoni leaves may have a role to be used as pharmaceuticals and/or preservatives.

**Keywords:** *Stevia rebaudiana* Bertoni, Antimicrobial property, Plant extracts, Anti bacterial Index, Anti fungal index.

## INTRODUCTION

*Stevia rebaudiana*, a natural alternative to artificial sweetener found to contain over 100 phytochemicals including well characterized stevioside and rebaudioside A (Komissarenko *et al.*, 1994). Besides, it is well known for its application in treatment of many diseases like diabetes, candidacies, high blood pressure and weight loss in various traditional systems of medicine. In recent times, the extract has been subjected to rigorous chemical, bio-chemical, pharmacological, clinical and toxicological investigations and many new therapeutic

applications have been emerged out (Pinheiro, 1987; and Takahashi, 2001). Thus with time traditional uses have been rationalized on the basis of modern scientific approaches. In addition, there has been a great interest in exploration and use of natural antimicrobial compounds of plant origin to treat diseases because of development of resistance by pathogens, expensive treatment regimen of synthetic drugs already in practice and their gross side effects due to indiscriminate use (Nychas, 1995; Tauxe, 1997; Cowan, 1999; Smid and Gorris, 1999; Sharif, 2001; Tomoko *et al.*, 2002). Microbiological investigation of stevia plant extract against pathogenic species using an array of solvents has neither been attempted earlier by many workers seriously nor reported much (Takaki, 1985; Tomita, 1997; Tadhani *et al.*, 2006). Therefore, efforts are taken in this present investigation on antimicrobial

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assay of a range of six solvents by using *Stevia rebaudiana* leaves against a host of 10 selected disease causing and food spoiling test organisms. The present work exclusively deals with a greater scope of analysis using four more solvents; acetone, chloroform, cyclohexane and petroleum ether which was not being undertaken by earlier workers.

## MATERIALS AND METHODS

### Plant Materials and Microorganisms

Fresh leaves collected from the young plantlets grown in Silviculture station of Department of Forest, Orissa were packed in polythene bags and stored at -18°C until its use.

Six bacterial (*Escherichia coli* MTCC1089, *Bacillus subtilis* MTCC 441, *Enterococcus faecalis* MTCC 2729, *Proteus mirabilis* MTCC 3310, *Pseudomonas aeruginosa* MTCC 647, *Staphylococcus aureus* MTCC3160) and four fungal (*Alternaria solani* MTCC 2101, *Aspergillus niger* MTCC 1344, *Helminthosporium solani* MTCC 2075, *Penicillium chrysogenum* MTCC 161) isolates obtained from Microbial type culture collection & gene bank (MTCC), Chandigarh, India were stored at -20°C. These microorganisms were selected for microbial assay study as these are common pathogens either to plants or animals or cause food spoilage. The pure cultures were maintained by routine sub-culturing at one week interval in nutrient agar and potato dextrose agar slants for bacteria and fungi respectively (Hi-Media laboratories private limited, Mumbai, India).

### Preparation of extracts

*Stevia rebaudiana* (popularly called as Stevia and synonymously known as 'sugar substitute' belongs to family Asteraceae) leaves were washed, air dried for 7-8 days, grinded into powder and 100 g weighed powder was taken for extraction in the flask of Soxhelet apparatus using six different solvents viz; water, ethanol, petroleum ether, cyclo-hexane, acetone and chloroform (all solvents used were HPLC grade) separately at temperature 20°C for 3-4 hours except in case of chloroform extraction where the leaf sample was submerged in 10%

chloroform for 2-3 days at room temperature. The extracts were filtered using Whatman No.1 filter paper and the filtrates were then evaporated to dryness under reduced pressure. The residual yield of water, ethanol, petroleum ether, cyclo-hexane, acetone and chloroform extracts were found to be 50 g %, 30 g %, 10 g %, 25 g %, 20 g % and 20 g % respectively. The extracts were stored in labeled sterile screw capped bottles at -20 °C until further analysis.

### Determination of minimum inhibitory concentration (MIC)

Plate dilution method was followed to determine MIC of petroleum ether extract taking different concentrations (100, 250, 500, 750 µg/ml) against 0.1 ml of  $10^{-4}$  inoculum dilution prepared from 24 hours incubated culture of *E.coli* into a sterile petriplate followed by pouring of 20ml 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 inhibition zone diameter for all other extracts against ten test microorganisms. The seeded plates were incubated at 37°C for 48 hours and the growth was noted down for different volumes of extract separately. All the experiments were done in triplicates and positive, negatives controls were run parallel along with sample analysis. The main objective of the present work being comparative evaluation of anti microbial potential of several extracts along with standard antibiotics by measuring diameter of inhibition zone (Janssen et al., 1986, Baratta et al., 1998), MIC was obtained for one extract using *E. coli*.

### Antimicrobial Assay

All extracts were subjected to antimicrobial assay by measuring the diameter of zone of inhibition (IZD) using disc diffusion technique. Nutrient agar and potato dextrose agar plates were prepared by pouring 20ml each in sterile Petri dishes for bacterial and fungal assay respectively and allowed to solidify. 0.2 ml of  $10^{-4}$  dilution of 24 hours old bacterial and 48 hours old fungal cultures were used so as to ensure the concentration of these organisms to contain approximately  $1 \times 10^6$  CFU/ml. Sterilized cotton swabs dipped in respective cultures were swabbed on solidified agar surface. Pre-sterilized filter paper discs of 5mm diameter which absorbs 10-12 µg sample/disc were dipped into individual

extract of 250 $\mu$ g/ml concentration separately and placed on the swabbed agar plates before incubation. Similar process is followed for controls using streptomycin and cotrimazole discs (10 $\mu$ g drug/disc), obtained from Hi-Media laboratories private limited, Mumbai, India as standard against bacteria and fungi respectively. At the end of incubation period diameter of inhibition zones formed in all three replicates were measured in mm using measuring scale and the average of the three was determined (Barry *et al.*, 1985).

### **Antimicrobial Activity Index**

Antibacterial index ( $A_b$ I) and antifungal index ( $A_f$ I) for individual chemical extract of stevia were calculated as the mean value of zone of inhibition obtained against all individual bacterial and fungal test strains respectively (Saikia *et al.*, 2001).

### **Statistical Analysis**

Antimicrobial activity evaluated in terms of IZD was compared statistically (ANOVA) to understand the level of significance between two treatments, the most potent extract showing highest activity and one of the least potent extracts in terms of F-values using statistical package PAST.

## **RESULTS AND DISCUSSION**

A concentration of 250 $\mu$ g/ml of petroleum ether extract was found sufficient enough to inhibit the growth of test microorganism *E.coli* completely in Petri plates. This indicates better effectiveness of the extract at lower concentration level preventing the growth of microorganisms.

It is clearly depicted in table 1 that among the bacterial pathogens selected for this study, highest rate of susceptibility was exhibited by *S. aureus* invariably by all four extracts petroleum ether (16.3mm), cyclohexane (9.0mm), chloroform (11.0mm), water extracts (9.3mm) except acetone (5.0mm) and ethanol (5.0mm) which were not able to inhibit this specific microorganism at all. *P. aeruginosa* and *P. mirabilis* showed highest resistance to different extracts except against petroleum ether (showing IZD 11mm, 10mm) and ethanol (8mm, 10.8mm). Among fungal strains used higher susceptibility was observed by *P. chrysogenum*, *A. niger* and

least susceptible fungus was found to be *A. solani* against the extracts in general.

Of all the extracts petroleum ether has highest  $A_b$ I with highest antimicrobial activity against 3 bacterial species *S. aureus*, *E. faecalis*, *P. aeruginosa* forming an IZD of 16.3mm, 13mm, 11mm respectively. The  $A_b$ I indicates petroleum ether has got the total best antimicrobial property against all six bacterial species. Against other three bacterial species *E.coli*, *P. mirabilis*, *B. subtilis*, the best activity was shown by water, ethanol and acetone extract with an IZD of 11mm, 10.6mm and 10.3mm respectively. The water extract has got the antimicrobial activity only against *E. coli*, *S. aureus* and *B. subtilis*. Similar results were reported by M.B. Tadhani and R. Subhash (2006) that water extract of Stevia leaf showed activity against *B. subtilis* and *S. aureus* only. IZD obtained by the ethanol extract in the present study also coincides with the response got by same workers using methanol extract which gave the highest zone of inhibition against *P. aeruginosa* and minimum against *S. aureus* and yeast. Smaller anti bacterial index as shown in the table indicates less activity as observed by few extracts like acetone against *E.coli*, *B. subtilis*, by ethanol against *P. mirabilis*, by chloroform against *S. aureus*, by cyclohexane against *S. aureus* and by ethanol against *P. mirabilis* when compared to standard antibiotics ( $A_b$ I 22.2). No activity was found in few extracts of stevia against selected pathogens which are represented as 5.0 mm IZD in the table.1.

The highest anti fungal index was found in case of petroleum ether extract ( $A_f$ I 15) irrespective of fungal species used, showing the highest activity forming (IZD of 16mm,14mm,16mm,14mm) followed by water extract (8.75  $A_f$ I) with IZD of (11mm, 10mm, 6mm, 8mm) against those fungal species namely *A. niger*, *P. chrysogenum*, *A. solani*, *H. solani* as depicted in table 1. Very less activity was observed by ethanol, acetone, chloroform and cyclohexane extracts of  $A_f$ I 5,5,5,5.75 and 6.5 respectively except some specific activity against organisms like *H. solani* (9.0mm), *A. niger* (9.0mm), *A. solani* (7.0mm), *P. chrysogenum* (8.0mm).

It can be concluded that the calculated F-value (22.33) between IZD obtained form the treatment of petroleum ether extract showing the highest antimicrobial activity and cyclohexane, one of the least potent extract was found to be greater

**TABLE 1:** Antifungal and Antibacterial Activity of Solvent Extracts Of Stevia (IZD in mm)

| Microorganism                          | Extract | Petroleum ether | Cyclo hexane | Chloroform | Water | Acetone | Ethanol | Standard |
|----------------------------------------|---------|-----------------|--------------|------------|-------|---------|---------|----------|
| <i>A. niger</i>                        |         | 16              | 5            | 5          | 11    | 5       | 9       | 23       |
| <i>P. chrysogenum</i>                  |         | 14              | 8            | 7          | 10    | 5       | 5       | 22       |
| <i>A. solani</i>                       |         | 16              | 5            | 5          | 6     | 7       | 5       | 11       |
| <i>H. solani</i>                       |         | 14              | 5            | 9          | 8     | 5       | 5       | 10       |
| Antifungal index (A <sub>fI</sub> )    |         | 15              | 5.75         | 6.5        | 8.75  | 5.5     | 5       | 16.5     |
| <i>E. coli</i>                         |         | 7               | 5            | 7          | 11    | 10      | 5       | 22       |
| <i>P. aeruginosa</i>                   |         | 11              | 7            | 5          | 5     | 6       | 8       | 25       |
| <i>P. mirabilis</i>                    |         | 10              | 5            | 7          | 5     | 5       | 10.6    | 23       |
| <i>E. faecalis</i>                     |         | 13              | 6            | 5          | 5     | 8.3     | 5       | 18       |
| <i>S. aureus</i>                       |         | 16.3            | 9            | 11         | 9.3   | 5       | 5       | 23       |
| <i>B subtilis</i>                      |         | 10              | 5            | 5          | 9     | 10.3    | 5       | 22.5     |
| Antibacterial index (A <sub>bI</sub> ) |         | 11.2            | 6.1          | 6.6        | 7.4   | 7.4     | 6.4     | 22.2     |

than the tabulated F-value (4.8) with P≤ 0.001 (for degrees of freedom n<sub>1</sub>=5 and n<sub>2</sub>=54). Thus the deviation due to different treatments of petroleum ether and cyclohexane was found to be highly significant.

The present investigations endow with the basic information about new non antibiotic drug molecules of plant origin, especially petroleum ether extract of stevia leaves which is found to be potent enough in exhibiting substantial antimicrobial activity against dreaded animal pathogens like *S. aureus*, *E. faecalis* bacteria. Possession of sizeable antimicrobial activity against food spoiling fungi like *A. niger*, *P. chrysogenum* and bacteria *S. aureus* may be explored for a value addition as natural food preservative to sugar substituting property of stevia used now a days for diet restricted package food products. Extraordinary anti fungal activity higher than the standard fungicide used against plant pathogens *H. solani* and *A. solani* may be substituted as potent bio-fungicide. Therefore, these molecules could be proved as future potential candidates either as non

antibiotic pharmaceuticals or food preservatives and/or plant micro-biocides after proper toxicity study in plant and animal models and clinical trials are addressed.

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