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Research Journal of Agricultural Sciences
An International Journal

P- ISSN: 0976-1675

E- ISSN: 2249-4538

Volume: 12

Issue: 06

Res. Jr. of Agril. Sci. (2021) 12: 2024–2028



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Anti-Microbial Assay of *Stevia rebaudiana* (Bertoni) Leaf Extracts Against Different Pathogens

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Received: 05 Jan 2021 | Revised accepted: 17 Oct 2021 | Published online: 12 Nov 2021
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ABSTRACT

Stevia rebaudiana (Bertoni) is considered as an economically important plant, belonging to *Asteraceae* family. The leaves have been noted to possess a high concentration level of sweet tasting secondary metabolites such as Steviosides and Rebaudiosides. It not only contains sweetness but also maintains the normal blood sugar level and hence it is also being used in homemade recipes and industries for diabetics. In the current study, chemical extracts from its leaves were subjected to microbial assay using three solvents (Hexane, ethylacetate, and ethanol) against four pathogenic bacteria (*Bacillus*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *E. coli*) and two food spoiling fungi (*Candida albicans* and *Aspergillus niger*) using a well diffusion method. 10 mg/ml of ethanoilic extract (minimum inhibitory concentration) was found sufficient enough to inhibit the growth of test microorganism (*Klebsiella pneumoniae* and *E. coli*) by well diffusion method. Amongst fungi (*Candida albicans* and *Aspergillus niger*) was found to have the highest resistance to Hexane and ethanol extract except ethyl acetate. The highest zone of inhibition (16mm) was obtained for *E. coli* against ethanolic extract. The highest zone of inhibition is (17mm) was obtained for *Candida albicans* against hexane extract. Ethyl acetate extract was found to have the least effective (lowest ZI). Invariably ethylacetate did not show any anti-microbial activity except demonstrating selective inhibition to specific organisms (*Klebsiella pneumoniae* and *E. coli*). This study clearly validated the use of *Stevia* metabolites as natural sweetener, with an additional anti-microbial property.

Key words: *Stevia rebaudiana*, Anti-microbial property, Plant extracts, *Klebsiella pneumoniae*, *Escherichia coli*

There are some few botanical discoveries which is quite so dramatics as the realization that the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (Compositae) are so highly sweet. The observation was brought to the attention of the scientific community about a hundred years ago [1-3]. *Stevia rebaudiana* (Bertoni) which is considered as an economically an important plant. This plant was first isolated in impure form in the first decade of the twentieth century [4], in 1970s, the group of professor Osamu Tanaka at the Hiroshima university in Japan isolated the rebaudioside A, by 1987 *Stevia rebaudiana* extracts containing stevio side occupied 41% of the high intensity sweetener in market place of Japan [5]. Wherea,s Koreans use this as a primary sweetening agent in the alcoholic beverages [6]. Stevioside is listed as an approved sweetener

in Brazil and other countries [7-8] behind it is well known for its application in treatment of many diseases like diabetes high blood pressure and weight loss in various traditional system of medicines. In recent times the extract has been subjected to various phyto-chemical, microbial, toxicological and bio chemical investigation and therapeutic applications have raised out. Therefore, the present study of this work is to investigate the anti-microbial activity of the plant extracts using three different solvents against selected micro-organisms including bacteria (*Bacillus*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *E. coli*) and fungi (*Candida albicans* and *Aspergillus niger*) by well diffusion method and clearly validated the use of *Stevia metabolites* as natural sweetener, with an additional anti-microbial property.

MATERIALS AND METHODS

Collection of plant sample

The plant of *Stevia rebaudiana* was collected from the Alandur nursery garden Chennai. The leaves of that plant were air dried at the room temperature. The air-dried leaves were finely powdered without any impurities mixed with them. The powdered leaves are stored at a room temperature for the further uses.

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Preparation of the extracts

Total of three types of extracts using three different solvents hexane, ethyl acetate and ethanol were collected from the samples of *Stevia rebaudiana*.

Hexane extract

100 gram of powdered leaves was mixed with 300 ml of hexane and kept in a conical flask. The mixture was well shaken and kept at a room temperature for 24 hours it was stirred 2-3 times a day after 24 hours the mixture was filtered thoroughly with a help of ordinary filter paper in an Mc bottle. The filtrate was allowed to evaporate for a week. The dried extract have a sticky appearance was stored in an effondoff at a room temperature for further use.

Ethyl acetate extract

The ethyl acetate extract was collected in the same way as a hexane extract. 100 gram of powdered leaves was mixed with 300 ml of hexane and kept in a conical flask. The mixture was well shaken and kept at a room temperature for 24 hours it was stirred 2-3 times a day after 24 hours the mixture was filtered thoroughly with a help of ordinary filter paper in an Mc bottle. The filtrate was allowed to evaporate for a week. The dried extract have a sticky appearance was stored in an effondoff at a room temperature for further use.

Ethanolic extract

The ethanolic extract was collected in the same way as the above two extract. 100 gram of powdered leaves was

mixed with 300 ml of hexane and kept in a conical flask. The mixture was well shaken and kept at a room temperature for 24 hours it was stirred 2-3 times a day after 24 hours the mixture was filtered thoroughly with a help of ordinary filter paper in an Mc bottle. The filtrate was allowed to evaporate for a week. The dried extract have a sticky appearance was stored in an effondoff at a room temperature for further use.

Anti-microbial assay

Anti-bacterial activity using agar well diffusion method

The samples were screened for antibacterial activity against following bacterial strains using agar diffusion method *Bacillus*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *E. coli*.

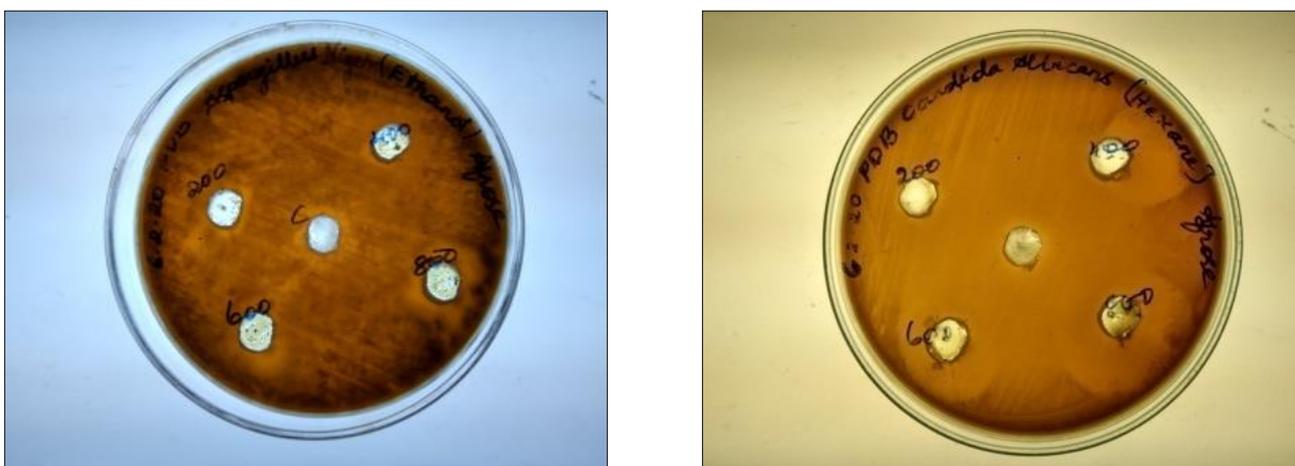
Sample preparation: The sample (10 mg/ml) was prepared in 10% DMSO.

Agar well diffusion method

Muller Hinton agar (MHA) plates were inoculated with the test organisms. The inoculums were evenly spread out. then wells were prepared in the plates with the sterile cork borer. Each well was located with 200, 400, 600, 800 µg/ml concentration of the sample and Tetracycline was used as a positive control for antibacterial activity. The plates were incubated for 24 hours 37°C. The development of inhibition zone around the well was measured (diameter) and recorded.



Klebsiella pneumoniae: In (Fig 1) Hexane, Ethyl acetate, and Ethanol shows the higher inhibitory activity against the bacterial pathogens
 Fig 1 The anti-bacterial activity of the sample



Aspergillus niger

Candida albicans

In (Fig 2) Hexane and ethanol shows a higher inhibitory activity against the fungal pathogens
 Fig 2 The anti-fungal activity of the sample

Anti-fungal activity using agar well diffusion method

The samples were screening for anti-fungal activity against human pathogens such as *Candida albicans* and

*Aspergillus niger.**Preparation of fungal spore*

The filamentous fungi were grown on potato dextrose agar slants at 28°C for 5 days the spores were collected using sterile double distilled water and stored in refrigerator.

Agar well diffusion method

Different concentrations of samples (200, 400, 600, 800 µg/ml) were used in this study Potato dextrose agar plates were inoculated with test organisms. The oplates were evenly spread out. The wells were prepared in the plates with the cork borer and Flucanazole was used as a positive

control. The plates were incubated for 48 hours at 37°C. The development of inhibition zone around the well was measured and recorded.

RESULTS AND DISCUSSION

The antimicrobial activity of *Stevia rebaudiana* was studied in different concentrations (200, 400, 600, and 800 in mg/ml) against four pathogenic bacterial strains (*Klebsiella pneumonia* and *E. coli*) and two fungal strains (*Aspergillus niger* and *Candida albicans*). These strains have selected for the basis of its application purpose for further formulation study. Antibacterial and anti-fungal potential of extracts were assessed in terms of zone of inhibition of bacterial and fungal growth. The results of antibacterial and anti-fungal activities are presented in (Table 1-3).

Table 1 Antibacterial activity of solvent extracts of *Stevia rebaudiana* leaves against bacterial test organisms (zone of inhibition in mm)

Organism	Extracts	Concentration (ug/mL)	Zone of inhibition (in mm)	Control
<i>Bacillus</i>	Hexane	200	-	8mm
		400	-	
		600	-	
		800	-	
	Ethyl acetate	200	5mm	9mm
		400	-	
		600	-	
		800	7mm	
	Ethanol	200	8mm	16mm
		400	11mm	
		600	11mm	
		800	11mm	
<i>Staphylococcus aureus</i>	Hexane	200	-	18mm
		400	-	
		600	-	
		800	-	
	Ethyl acetate	200	5mm	14mm
		400	-	
		600	10mm	
		800	10mm	
	Ethanol	200	10mm	17mm
		400	11mm	
		600	12mm	
		800	13mm	

It is clearly proved in (Table 1-2) among the four bacterial pathogens selected for this study the highest rate of susceptibility was exhibited by *Klebsiella pneumonia* hexane (13mm), ethyl acetate (14 mm), ethanol (13mm) and *E. coli* hexane (11mm), ethyl acetate (13 mm), ethanol (16mm) in all tree extracts. The present investigation

concludes with the basic information about the non anti biotic drug molecules of a plant origin especially ethanol extract of *stevia rebaudiana* leaves which is found to be potent enough in exhibiting substantial anti-microbial activity against the dreaded animal pathogens like *Klebsiella pneumonia* and *E. coli* bacteria [9].

Table 2 Antibacterial activity of solvent extracts of *Stevia rebaudiana* leaves against bacterial test organisms (zone of inhibition in mm)

Organism	Extracts	Concentration (ug/mL)	Zone of inhibition (in mm)	Control
<i>Klebsiella pneumonia</i>	Hexane	200	10mm	18mm
		400	11mm	
		600	12mm	
		800	13mm	
	Ethyl acetate	200	11mm	19mm
		400	12mm	
		600	12mm	
		800	14mm	
	Ethanol	200	10mm	17mm

<i>Escherichia coli</i>	Hexane	400	11mm	18mm	
		600	12mm		
		800	13mm		
		200	4mm		
	Ethyl acetate	Hexane	400	8mm	14mm
			600	10mm	
			800	11mm	
			200	10mm	
		Ethanol	400	11mm	17mm
			600	13mm	
			800	13mm	
			200	17mm	
		400	12mm		
		600	13mm		
		800	16mm		
		800	16mm		

Possession of sizeable antimicrobial activity against food spoiling fungi like *Candida albicans* and *Aspergillus niger* may be explored as a natural food preservative to sugar substituting property. Now a days *Stevia rebaudiana* are used for diet restricted package food products. Therefore, these molecules could be proved as a future potential. It is clearly proved in (Table 3) among the two

fungal pathogens (*Candida albicans* and *Aspergillus niger*) and three solvent extractions selected for this study the highest rate of susceptibility was exhibited by both pathogens in only hexane and ethanolic extract of *Stevia rebaudiana*. Whereas, the ethyl acetate does not show any activity against the two fungal strains [10].

Table 3 Antifungal activity of solvent extracts of *Stevia rebaudiana* leaves against fungal pathogens (zone of inhibition in mm)

Fungi	Extracts	Concentration (ug/mL)	Zone of inhibition (in mm)	Control
<i>Candida albicans</i>	Hexane	200	7mm	9mm
		400	10mm	
		600	16mm	
		800	17mm	
	Ethyl acetate	200	-	-
		400	-	
		600	-	
		800	-	
	Ethanol	200	10mm	10mm
		400	11mm	
		600	12mm	
		800	13mm	
<i>Aspergillus niger</i>	Hexane	200	11mm	11mm
		400	12mm	
		600	12mm	
		800	13mm	
	Ethyl acetate	200	-	-
		400	-	
		600	-	
		800	-	
	Ethanol	400	11mm	10mm
		600	13mm	
		800	14mm	
		800	14mm	

The result of the present work indicates *Stevia rebaudiana* leaf extract can be an ideal candidate either as a non anti- biotic pharmaceuticals or food preservatives or plant micro biocides after proper toxicity study in plant and animal models and clinical trials are addressed. Antimicrobial properties of medicinal plants have been increasingly reported throughout the world. In the present work, the extracts obtained from *Stevia rebaudiana* shows a strong activity against the bacterial and fungal pathogens. The above result shows that the ethanolic extract of *stevia rebaudiana* shows a significant antibacterial and anti-fungal activity. the higher antibacterial activity of the ethanolic extract of *Stevia rebaudiana* may be due to the greater the solubility of the extracts [11]. The inhibitory activity of

ethanolic extract was more active against the bacterial strains (*Klebsiella pneumonia* and *E. coli*) and non-existence against the (*Staphylococcus aureus* and *Bacillus*). The ethyl acetate extract of stevia was ineffective against the two fungal strains (*Candida albicans* and *Aspergillus niger*). This shows the low anti-fungal activity against the ethyl acetate extract. Where as the other two extracts hexane and ethanol shows a higher inhibitory activity against the fungal pathogens [12].

CONCLUSION

This study points to be probable antibacterial and anti-fungal potentials of some solvents extract of *Stevia*

rebaudiana leaves. There is a need for further investigation of this plant in order to identify and isolate the active compounds for the cytotoxicity studies. The result of the study will also need to be confirmed using in vivo models.

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