Tropical Journal of Pharmaceutical Research, December 2008; 7 (4): 1143-1149
© Pharmacotherapy Group,
Faculty of Pharmacy, University of Benin,
Benin City, 300001 Nigeria.

All rights reserved.

Available online at http://www.tjpr.org

Research Article

In-vitro Antimicrobial and Antitumor Activities of *Stevia Rebaudiana* (Asteraceae) Leaf Extracts

Sathishkumar Jayaraman*, Muthu Saravanan Manoharan, Seethalakshmi Illanchezian
Life Teck Research Centre, Vadapalani, Chennai – 600026, India

Abstract

Purpose: *The purpose of the study is to evaluate the antimicrobial and antitumor activities of Stevia rebaudiana (Asteraceae) leaf extracts.*
Methods: *Four solvent extracts (ethyl acetate, acetone, chloroform and water) of Stevia rebaudiana leaves were investigated against Staphylococcus aureus, Salmonella typhi, Escherichia coli, Bacillus subtilis, Aeromonas hydrophila and Vibrio cholerae by using agar well diffusion method. Candida albicans, Cryptococcus neoformans, Trichophyton mentagrophytes and Epidermophyton species were used to test anti-yeast and antifungal activity. The cytotoxic effects of the extracts on Vero and HEp2 cells were assayed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT].*
Results: *Among the four extracts tested, acetone extract had effective antibacterial potential, followed by ethyl acetate extract. The acetone extract showed greater activity against Gram-positive than against Gram-negative organisms. All the extracts were active against Epidermophyton species and Candida albicans. The 1:8 dilution of the acetone extract was non-toxic to normal cells and also had both anticancer and anti-proliferative activities against cancerous cells.*
Conclusion: *The study confirms the antimicrobial and antitumor activities of Stevia rebaudiana leaves extracted using various solvents, and is therefore, a potential drug that requires further studies and development.*

Key words: *Stevia rebaudiana; Antibacterial; Antifungal; Antitumor; HEp2 cells; MTT assay*

Received: 02 July 2008 *Revised accepted: 26 August 2008*

***Corresponding author: E**-mail: jsathishkumar85@gmail.com Tel : +91 97101 68939.*

INTRODUCTION

There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action due to an alarming increase in the incidence of new and re-emerging infectious diseases and development of resistance to the antibiotics in current clinical use[1]. The screening of plant extracts has been of great interest to scientists in the search for new drugs for greater effective treatment of several diseases[2]. Therefore, plant extracts and phytochemicals with known antimicrobial properties can be of great significance in therapeutic treatments[3-5].

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compounds[6]. Many plant leaves have antimicrobial principles such as tannins, essential oils and other aromatic compounds[7,8]. In addition, many biological activities and antibacterial effects have been reported for plant tannins and flavanoids[9-11]. Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives[12]. These compounds protect the plant from microbial infection and deterioration[13]. Some of these phytochemicals can significantly reduce the risk of cancer due to polyphenol antioxidant and anti-inflammatory effects. Some preclinical studies suggest that phytochemicals can prevent colorectal cancer and other cancers[14-16].

One of the potent members of the Asteraceae family is *Stevia rebaudiana* (commonly referred to as Honey leaf, Candy leaf and Sweet leaf). It is rich in terpenes and flavanoids. The phytochemicals present in *Stevia rebaudiana* are austroinullin, β-carotene, dulcoside, nilacin, rebaudi oxides, riboflavin, steviol, stevioside and tiamin[17]. *Stevia* has important industrial uses in beverages, energizers as well as medicinal uses such as low uric acid treatment, vasodilator cardiotonic, anesthetic and anti-inflammatory.

The present study was carried out to evaluate the antimicrobial and antitumor activity of *Stevia rebaudiana* leaves extracted using various solvents.

MATERIALS AND METHODS

Test organisms

Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Salmonella typhi and *Vibrio cholerae* were used to test antibacterial activity while *Candida albicans, Cryptococcus neoformans, Trichophyton mentagrophytes, Epidermophyton species* were used to assess anti-yeast and antifungal activities. All the stock cultures were obtained from Microbial Type Cell Culture (IMTECH, India).

Plant material

Stevia rebaudiana leaves were obtained from Anna medicinal farm, Chennai. The leaves were washed with sterile water, dried in shade, finely powdered & stored in air tight bottles.

Preparation of plant extract

25 g of air-dried powder of *Stevia rebaudiana* leaves was immersed in 100 mL of organic solvent (ethyl acetate, acetone, chloroform) and water separately in a conical flask. It was incubated at room temperature for 48 hour at 150 rpm in an orbital shaker. The suspension was filtered and concentrated to dryness at 40 ^{o}C in hot air oven. The extract was dissolved in 0.25% Dimethyl Sulphoxide (DMSO, Merck) to a concentration of 100 mg/mL.

Assay for antibacterial activity

Preparation of inoculum

Stock cultures were maintained at 4 ^{o}C on nutrient agar (HiMedia) slants. Active cultures for experiments were prepared by transferring a loopful of culture to 10 mL of nutrient broth (HiMedia) and incubated at 37 ^{o}C for 24 hours for bacterial proliferation.

Agar-well diffusion method

Agar well bioassay was employed for testing antibacterial activity of *Stevia rebaudiana* leaves[18]. Each extracts were made to a final concentration of 50 mg/mL. 24 hour old cultures of test organisms (0.05 mL) were seeded onto Mueller Hinton agar (HiMedia)

plate and uniformly spread with a spreader. Wells (5mm) were made in the agar plate with a sterile cork borer. The plant extract was introduced into the well and the plates were incubated at 37 ^{o}C for 24 hours. The antibacterial activity of the plant extract was determined by measuring the diameter of the inhibition zone. Controls contained only DiMethyl Sulfoxide (DMSO). The antibacterial assay for each of the extracts against all microorganisms tested was performed in triplicates.

Assay for antifungal activity

Potato dextrose agar (HiMedia) was prepared and 1 mL (50 mg/mL) of plant extract was added to the medium. After solidification a loopful of culture was placed in the centre of the plate. Controls contained only DMSO. All the plates were incubated at 25 ^{o}C for 4 days[18]. The growth of the fungal cultures was measured and compared with the respective control plates. The antifungal assay for each of the extracts against all microorganisms tested was performed in triplicates.

Cell viability assay

Vero cells (African green monkey kidney cells) obtained from King Institute of Preventive Medicine, Chennai, India was used to determine the non-toxic dose of the plant extract. The cells were grown in a 24-well plate in Eagle's Minimum Essential Medium (HiMedia) supplemented with 10% fetal bovine serum (Gibco Laboratories) and antibiotics (streptomycin, penicillin-G, kanamycin, amphotericin B). About 1 mL cell suspension (10^5 cells/mL) was seeded in each well and incubated at 37 ^{o}C for 48 hour in 5% CO_2 for the formation of confluent monolayer. The monolayer of cells in the plate was exposed to various dilutions of the plant extract. The cell viability was measured using MTT assay[19] with MTT (5 mg/mL) and DMSO. This tetrazolium salt is metabolically reduced by viable cells to yield a blue formosan product measured at 540nm spectrophotometerically.[19] Controls were maintained throughout the experiment (untreated wells as cell control and diluent treated wells as diluent control). The assay was performed in triplicate for each of the extracts. The mean of the cell viability values was compared to the control to determine the effect of the extract on cells and % cell viability (Vero cells) was plotted against concentration of the plant extract.

Antitumor assay

The antitumor assay was performed on Human laryngeal epithiloma cells (HEp2) obtained from King Institute of Preventive Medicine, Chennai, India with non-toxic dose of the plant extract and its dilutions. The cell viability was measured using MTT assay as described above. Controls were maintained throughout the experiment (Untreated wells as cell control and diluent treated wells as diluent control). The assay was performed in triplicates for each of the extracts. The mean of the cell viability values was compared to the control to determine the effect of the extract on cells. A graph was plotted against the % cell viability (HEp2 cells) Vs dilution of the plant extract. The minimum concentration of plant extract that was non-toxic to Vero cells but toxic to HEp2 cells was recorded as the effective drug concentration.

RESULTS

The antibacterial activities of the solvent extracts of *Stevia rebaudiana* showed significant variations as shown in Table 1. Among the four extracts tested, acetone extract had greater antibacterial potential, followed by ethyl acetate extract and then the other extracts. The largest zones of inhibition were observed for acetone extract against *Staphylococcus aureus* (19 mm) and *Bacillus subtulis* (18 mm). Ethyl acetate extract was very effective against *Vibrio cholerae* (18 mm). Chloroform and water extracts were either slightly effective or ineffective against the test organisms, respectively.

The antifungal and anti-yeast activities of the solvent extracts of *Stevia rebaudiana* also varied significantly among the test organisms as shown in Table 2. All the four extracts inhibited the growth of *Epidermophyton species*. All the extracts had inhibitory effect on the growth of *Cryptococcus neoformans* except the water extract. All the extracts inhibited the growth of *Trichophyton*

Table 1: Antibacterial activity of the extracts of *Stevia rebaudiana* leaves

Test Organism	Zone of inhibition (mm)			
	Ethyl acetate	Acetone	Water	Chloroform
Staphylococcus aureus	10	19	-	-
Salmonella typhii	11	13	-	7
Escherichia coli	10	10	-	6
Bacillus subtilis	11	18	-	8
Aeromonas hydrophila	11	14	-	-
Vibrio cholerae	18	10	-	6

Table 2: Antifungal and anti-yeast activities of the extracts of *Stevia rebaudiana* leaves

Test Organism	Mycelial Growth (mm)				
	Control	Ethyl acetate	Acetone	Water	Chloroform
Epidermophyton species					
24 hour	6	2	2	2	5
48 hour	8	6	5	6	8
72 hour	14	9	7	7	10
96 hour	17	10	7	7	10
Trichophyton mentagrophytes					
24 hour	2	2	2	1	1
48 hour	4	2	2	2	2
72 hour	10	4	6	3	2
96 hour	12	9	11	11	11
Cryptococcus neoformans					
24 hour	4	2	2	3	2
48 hour	5	2	3	5	2
72 hour	6	3	4	5	3
96 hour	6	4	4	6	3
Candida albicans					
24 hour	3	1	2	2	2
48 hour	3	1	3	3	3
72 hour	4	2	3	3	3
96 hour	5	2	4	4	3

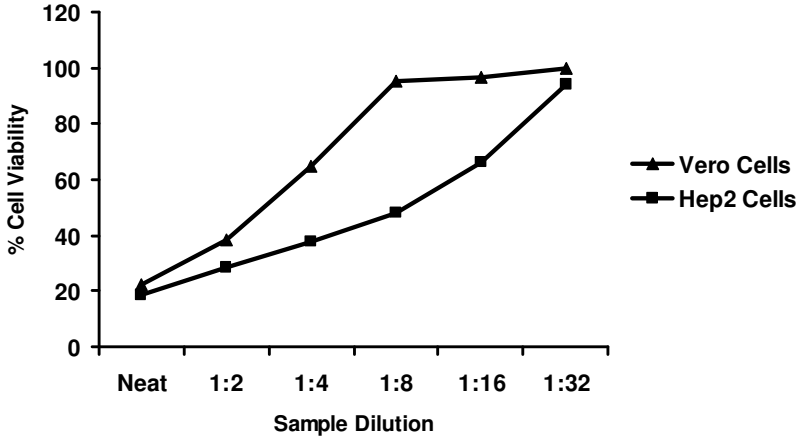

Fig. 1: MTT assay result showing plot of cell viability versus extract dilution for acetone extract**:** 1:8 dilution of the acetone extract of *Stevia rebaudiana* leaves is effective drug concentration (non-toxic to Vero cells but cytotoxic to more than 50% of HEp2 cells). ▲ – Vero cells ■ – HEp2 cells

mentagrophytes but as the incubation time was prolonged, the ethyl acetate extract showed higher inhibitory activity against *Trichophyton mentagrophytes* than the other extracts.

With regard to antitumor activity, the MTT assay for acetone extract of *Stevia rebaudiana* treated cells showed that 1:8 dilution was the effective drug concentration (Fig. 1. This extract concentration was non-toxic to Vero cells and also caused more than 50% cytotoxicity to HEp2 cells.

DISCUSSION

Stevia rebaudiana leaf extracts demonstrated antibacterial, antifungal, anti-yeast and antitumor activity. To the best of our knowledge, there is no previous reported work on the antimicrobial and antitumor activity of *Stevia rebaudiana*, except that of Tadhani and Subhash[20] who also studied antimicrobial activity of *Stevia rebaudiana* leaves.

The antibacterial activity of the acetone extract of *Stevia rebaudiana* leaves was higher than that of the other extracts. The acetone extract showed greater activity against Gram-positive organism than against Gram-negative organism. The higher antibacterial activity of the acetone and ethyl acetate extracts may be due to the greater solubility of the extract in these organic solvents[21]. The inhibitory activity (measured by zone of inhibition) of chloroform extract was not pronounced against *Bacillus subtulis* (8 mm), *Salmonella typhi* (7 mm), *Escherichia coli* (6 mm), respectively, and non-existent against *Staphylococcus aureus*. The water extract of *Stevia rebaudiana* leaves was practically ineffective against the test organisms. This finding is similar to that of Tadhani and Subhash[20] who also recorded very low antibacterial activity for water extracts of *Stevia rebaudiana* leaves. Several workers[22-24] have reported that water extracts do not have much activity against bacteria. It should be noted, however, that growth media also seems to play an important role in the determination of antibacterial activity[25].

All the extracts were active against *Epidermophyton species* and *Candida albicans.* The ethyl acetate extract showed high activity against *Trichophyton mentagrophytes* and *Epidermophyton species,* and this may be due to the greater stability of the active principles in the solvent over a longer period of time.

The aqueous extract of *Stevia rebaudiana* showed no pronounced antitumour activity but the acetone and ethyl acetate extracts of *Stevia rebaudiana* were more cytotoxic to HEp2 cells. Acetone extracts showed the highest cytotoxic activity followed by ethyl acetate and chloroform extracts. MTT assay was used to evaluate cytotoxicity based on metabolic reduction of MTT.

On treatment with Vero cells, 1:2 and 1:4 dilutions of the acetone extract showed cytotoxicity but there was no apparent cytotoxicity at 1:8 dilution. Further dilutions also had no toxic effects on Vero cells. 1:2 and 1:4 dilutions were cytotoxic to HEp2 cells, whereas 1:8 dilution caused more than 50% cytotoxicity and also cessation of cell growth. Further dilutions had less effect on the viability of the cancerous cells. Thus, the 1:8 dilution of the acetone extract of *Stevia rebaudiana* is non-toxic to the normal cells and also has both anticancer and anti-proliferative activities against the cancerous cells.

CONCLUSION

This study points to the probable antimicrobial and antitumor potentials of some solvent extracts of *Stevia rebaudiana* leaves. There is a need for further investigation of this plant in order to identify and isolate its active anticancer principle(s). The results of the study will also need to be confirmed using *in vivo* models.

ACKNOWLEDGEMENT

We thank Life Teck Research Centre, Chennai, for providing us the facilities and requisite support for this work. We also express our thanks to Dr. K. Rajagopal for his critical review of this paper.

REFERENCES

1. *Bauer J, Rojas R, Bustamante B. Antimicrobial activity of selected Peruvian medicinal plants. J Ethnopharmacol, 2003; 88: 199- 204.*
2. *Dimayuga RE, Garcia SK. Antimicrobial, screening of medicinal plants from Baja California sur, Mexico. J Ethnopharmacol, 1991; 31: 181-192.*
3. *Diallo D, Hveem B, Mahmoud MA, Betge G, Paulsen BS, Maiga A. An ethnobotanical survey of herbal drugs of Gourma district, Mali. Pharm Biol, 1999; 37: 80-91.*
4. *Rojas JJ, Ochoa VJ, Ocampo SA, Munoz JF. Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: A possible alternative in the treatment of non-nosocomial infections. BMC Complement Altern Med, 2006; 6: 2.*
5. *Erdogrul OT. Antibacterial activities of some plant extracts used in folk medicine. Pharm Biol, 2002; 40: 269-273.*
6. *Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. Afr J Biotechnol, 2005; 4: 685-688.*
7. *Kumar R, Singh M. Tannins, their adverse role in ruminant nutrition. J Agric Food Chem, 1984; 32: 447-453.*
8. *Kumar R, Singh NP. Effect of tannins in pala leaves (Zizyphus nummularia) on ruminal proteolysis. Indian J Anim Sci, 1984; 54: 881-884.*
9. *Haslam E. Plant Polyphenols. In: Haslam E (eds). Vegetable Tannins, Cambridge, England: Cambridge University Press, pp.15-89.*
10. *Scalbert A. Antimicrobial properties of tannin. Phytochemistry, 1991; 30: 3875-3883.*
11. *Chung KT, Wong TY, Wei Y, Huang YW, Lin Y. Tannins and human health. A review. Crit Rev Food Sci Nutr, 1998; 8: 421-464.*
12. *Geissman TA. Flavonoid compounds, tannins, lignins and related compounds. In: Florkin M and Stotz EH (eds). Pyrrole Pigments, Isoprenoid Compounds and Phenolic Plant Constituents, New York, USA: Elsevier Press, 1963, pp 265.*
13. *Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev, 1999; 12: 564-582.*
14. *Michaud DS, Feskanich D, Rimm EB. Intake of specific carotenoids and risk of lung cancer in two prospective U.S. cohorts. Am J Clin Nutr, 2000; 72: 990-997.*
15. *Greenberg ER, Baron JA, Tosteson TD. A clinical trial of antioxidant vitamins to prevent colorectal cancer. N Engl J Med, 1994; 331: 141-147.*
16. *Birt DF, Hendrich S, Wang WQ. Dietary agents in cancer prevention: flavanoids and isoflavonoids. Pharmacol Ther, 2001; 90: 157–177.*
17. *Crammer B, Ikan R. Sweet glycosides from the Stevia plant. Chem Br, 1986; 22: 915-917.*
18. *Linday EM. Practical Introduction to Microbiology, E & FN spon Ltd, 1962, pp 77.*
19. *Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983; 65: 55-63.*
20. *Tadhani BM, Subash R. In Vitro Antimicrobial Activity of Stevia Rebaudiana Bertoni Leaves. Trop J Pharm Res, 2006; 5 (1): 557-560.*
21. *De Boer HJ, Kool A, Broberg A, Mziray WR, Hedberg I, Levenfors JJ. Antifungal and antibacterial activity of some herbal remedies from Tanzania. J Ethnopharmacol, 2005; 96: 461-469.*
22. *Martin GJ. Ethnobotany: A Methods Manual. London, England, Chapman and Hall, 1995.*

23. Paz EA, Cerdeiras MP, Fernandez J, Ferreira F, Moyna P, Soubes M, Vázquez A, Vero S, Zunino L. Screening of Uruguayan medicinal plants for antimicrobial activity. J Ethnopharmacol, 1995; 45: 67- 70.
24. Vlietinck AJ, Van Hoof L, Totte J, Lasure A, Vanden Berghe D, Rwangabo PC, Mvukiyumwami J. Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. J Ethnopharmacol, 1995; 46: 31-47.
25. Lin J, Opoku AR, Geheeb-Keller M, Hutchings AD, Terblanche SE, Jager AK, van Staden J. Preliminary screening of some traditional zulu medicinal plants for anti-inflammatory and antimicrobial activities. J Ethnopharmacol, 1999; 68: 267-274.