International Journal of Basic Medical Sciences and Pharmacy (IJBMSP)
Vol. 6, No. 1, June 2016, ISSN: 2049-4963

www.ijbmsp.org

Comparative Anti-Microbial Study of Different Parts of *Stevia Rebaudiana Bert* against Different Microorganisms

Muhammad Hussain[1], Sulaiman Bahadar*[2], Malik Mujaddad Ur Rehman[2,] Fahad Ali [4], Tariq Mahmood[1], Syed Wajid Ali Shah[1], Zarnab Zainab[3], Komal Habib[2], Azam hayat[2], Muhammad Ayub Jadoon[2], Najeebullah[1], Umar Ali[5]

[1]Department of Microbiology, Hazara University, Mansehra, KPK, Pakistan.

[2]Department of Microbiology, Abbottabad University of Science & Technology, KPK, Pakistan.

[3]FCPS Part 1 General Medicine, Ayub Medical collage Abbottabad, KPK, Pakistan.

[4]Department of Genetics, Hazara University, Mansehra, Pakistan.

[5]Department of Zoology, Kohat University of Science and Technology, Kohat,

Email: salupakhtoon@gmail.com

Abstract – A major global therapeutic problem of multi drug resistant *Escherichia coli* and many other bacteria which produce ß-lactamase has emerged. The need to explore novel antimicrobial drugs in light of the facts of fast worldwide spread of drug resistant microbes is of supreme significance. Four types' extracts were prepared for each plant sample by using ethanol, acetone, hexane and water as solvents. These extract were assayed against nine human pathogenic bacterial strains and a single fungus. Antimicrobial assay was done by disc diffusion method. Leaves extract were more active than stem extracts against tested specific microbes. The highest activity among all extracts was showed by water extract of leaf against *staphylococcus* aurous (21-25 mm in 6 and 12 micro letter conc respectively) followed by ethanol (20-25), Acetone (25-20 mm) extracts of leaves and hexane extract of stem against *Pseudomonas aeruginosa* (15-22 mm) in concern concentration. This study showed that *Stevia rebaudiana bert* have remarkable anti-bacterial and anti-fungal activities and comparatively, leaves have high antimicrobial activity than stem.

Keywords – *Stevia rebaudiana Bert, Microorganisms, Antimicrobial activity, Disc Diffusion method, Controls*

1. Introduction

Stevia rebaudiana belongs to genus Stevia which have 190 species [14]. Locally, it is called as honey leaf sweet weed, sweet leaf and sweet herb. It is a natural herbal sweetener [1]. *Stevia* rebaudiana is a small perennial herb belongs to asteraceae (compositeae) family which grows up to 65 cm with a maximum height up to 180 cm. The leaves are green, sessile, and lanceolate to oblanceolate which are arranging oppositely. The leaves are also serrated above the middle and are 2-3 cm long. Stevia rebaudiana is a short day plant and blooms from January to April [2, 18]. Stem is brittle and flowers are small (15-17 mm) and white in colors. Flowers of *Stevia* rebaudiana are hermaphrodite having both male and female organs, present in small coryumb of two to six small flowers [5]. The androcium consist of small Anthers. Anthers are five in number and the pollen is highly allergenic. Gynecium consist of bi-lobed Stigma and anthers are present around the style. The length of the seeds is 3 mm and is present in cylindrical achenes. There are twenty permanent pappus with short hairs in every achenes [5].The plant has extensive root system. The plant is cultivated in well-drained and sandy mud soil with a in the pH range 6.5-7.5. To cultivate this plant, saline soil should be avoided. *Stevia* Seeds have a very low germination power [4]. Steviosides level in the leaves of the plant and plant growth when grown from stem cuttings or vegetatively growing or tissue culture were more homogeneous than the seeds grown plant. Vegetatively grown plant has high number of roots, shoot biomass and stevioside contents [17]. Stevia grows in areas with up to 1375mm of rain a year. To use as an alternative for sugar, it is one of the most important medicinal plant and widely grown for its sweet taste [9].

Stevia leaves extracts has recognized properties against bacteria, fungi, viruses and yeasts. It also has anti-inflammatory, anti-histaminic cardiovascular, diuretic and hypoglycemic activities [3]. It is a single natural sweetner which is used for diabetes [12]. Stevia rebaudiana is also used for management of other ailments such as hypertension and obesity. For many years in traditional South American treatment of diabetes, extracts of the leaves have been used [7].

2. Material and Methods

The present research work "Comparative antimicrobial study of different parts of Stevia Rebaudiana against

specific microbes" was performed in Pakistan Council of Scientific and Industrial research (PCSIR), laboratories complex, Peshawar, Pakistan.

2.1. Collection of Plant

The plant, *Stevia rebaudiana* was collected in the month of October, from PCSIR Peshawar. Only leaves and stem were collected.

2.2. Processing of plant

To eliminate dust, and any other impurities, the plant material was rinsed thoroughly by water properly. Then the plant sample was kept in shaded condition over a big paper for drying at room temperature. After complete drying, both samples were grinded into powder. Then1 kg of each sample was taken and divided into four equal portions. Each powder portion was added to borosilicate glass conical flask. To each flask, 400 ml of solvent was added. Four solvents like ethanol, acetone, hexane and water were used. The flasks were shaked regularly. All the flasks were plugged in with cotton swab to prevent evaporation of the organic solvent and entrance of any contamination. All the flasks were kept at room temperature for 24 hours. Each portion of the soaking samples were filtered through Whatmann filter paper. All the flasks were shaking gently to enhance extraction and filtration. To get maximum crude extract, the filtration was repeated twicely. The different viscous crude extracts were obtained through vacuum rotary evaporator at specified conditions, weighted and were preserved for anti-microbial assay. 0.6 gram of every sample crude extract was dissolved in 3.6 ml of dimethyl sulphoxide (DMSO) solvent in vials for antimicrobial activity and kept on hot plate for complete dissolution.

2.3. Disc Diffusion Method for Antimicrobial Activity

Kirby-Bauer disc diffusion method was used for the study of anti-microbial activity of the various extracts of *Stevia rebaudiana* [15]. After incubation, streaking was done in aseptic condition of laminar flow hood. With the help of micro pipette, 15 micro liters of broth media was taken from every broth tubes and poured into labeled and already sterilized Petri plates in Laminar flow Hood. Then through cotton swab, vertical and horizontal uniform streaking was done gently. The inoculums were takes 5 minutes for drying. *Salmonella typhi (ATTC # 14028), Erwinia carotovora (ATTC No. 672), Escherichia coli (ATTC No. 29922) ,Pseudomonas aeruginosa (ATTC No. 9721) ,Bacillus subtilis (ATTC No. 6051), Bacillus atrophaeus (ATTC No. 9372) Staphylococcus aurous (ATTC # 6538), Klebsiella Pneumoniaeee (ATTCC No.1705), Agrobacterium tumufaciens (ATTC No.33970)* and *Candida albicans (ATTC No.10231).* The double disc was placed on the top while single disc on the bottom of agar Petri plates. On single disc, 6 µl extract was applied while 12 micro litters on double disc.For Positive control, levofloxacin was used for Gram negative and Azithromycine for Gram positive bacteria while Clotrimazole for Candida albican. For negative control DMSO was used. Same methodology was applied for Candida albican. The process was repeated three times. After 24 hour, zones of inhibition were formed around the discs. These were measured carefully in millimeter with the help of ruler.

3. Results and Discussion

3.1. Ethanolic Extract of Leaves Sample of the Stevia Rebaudiana

In Table 1, the anti-microbial activities of Ethanolic extract of leaves sample showed remarkable activity against *Staphylococcus aurous* followed by *Klebsiella pneumoniae* in 6 micro liter concentration (21 mm) and Erwinia carotovora (19 mm) while in 12 micro liter, it shown same activity against *Klebsiella pneumoniae* and *pseudomonas aeruginosa* (20mm). Ethanolic extract also have activity against *Candida albican* while no activity against *S.typhi.*

Table 1: Antimicrobial activity of Ethanolic extract of leaves sample

Leaves Sample	**Bacterial Strains + Fungus**	**Average Zone of inhibition in various concentration in millimeters**			
		6 µl	**12 µl**	**Positive control**	**Negative control**
Ethanolic Extract	*Escherichia coli*	19	14	31	00
	Salmonella typhi	NA	NA	36	00
	Klebsiella pneumoniae	21	20	32	00
	Bacillus subtilis	15	17	34	00
	Bacillus atrophoeus	12	13	33	00
	Erwinia carotovora	19	23	27	00
	Staphylococcus aurous	20	25	32	00
	Pseudomonas aeruginosa	13	20	31	00
	A.tumefaciens	10	13	32	00
	Candida albican	17	19	33	00

Table 2: Antimicrobial activity of acetone fraction of leaves sample

Leaves Sample	**Bacterial Strains + Fungus**	**Average Zone of inhibition of the extracts In various concentration in mili meters**			
		6 µl	**12 µl**	**Positive control**	**Negative control**
Acetone Extract	*Escherichia coli*	11	14	31	00
	Salmonella typhi	NA	NA	36	00
	Klebsiella pneumoniae	18	18	32	00
	Bacillus subtilis,	20	22	34	00
	Bacillus atrophoeus	13	13	33	00
	Erwinia carotovora	20	22	27	00
	Staphylococcus aurous	25	20	32	00
	Pseudomonas aeruginosa	14	18	31	00
	A. tumefaciens	12	14	32	00
	Candida albican	14	18	33	00

Table 3: Antimicrobial activity of hexane extract of leaves sample

Leaves Sample	**Bacterial strains + Fungi**	**Average Zone of inhibition of the extracts in mili meter in various concentration**			
		6 µl	**12 µl**	**Positive control**	**Negative control**
Hexane Extracts	*Escherichia coli*	14	09	31	00
	Salmonella typhi	NA	NA	36	00
	Klebsiella pneumoniae	09	13	32	00
	Bacillus subtilis,	17	20	34	00
	Bacillus atrophoeus	15	20	33	00
	Erwinia carotovora	10	21	27	00
	Staphylococcus aurous	21	19	32	00
	Pseudomonas aeruginosa	15	18	31	00
	A.tumefaciens	18	19	32	00
	Candida albican	18	19	33	00

3.4. Acetone Based Extract of Leaves Sample of the Stevia Rebaudiana

Table no 2, the acetone extracts of leaves showed inhibitory activity almost against all microbes.

Highest zone of inhibition was found against *Staphylococcus aurous* which is 25 and 20 mm while smallest against *Escherichia coli* which was 11 and 14 mm in 6 and 12 micro liter concentrations respectively while no activity against *Salmonella typhi*.

3.5. Extract of Leaves Sample of the Stevia Rebaudiana In Hexane

The hexane extract showed remarkable activity for staphylococcus aurous (21 and 19 mm in 6 and 12 micro liter respectively) (Table 3). It has same activity for *Agrobacterium tumefaciens* and *Candida albican* (18-19) mm in each concentration). In 12 micro liter concentrations, it has shown high activity for *Erwinia carotovora* which is 21 mm.

Table 4: Antimicrobial activity of aqueous extract of leaves sample

Extracts Sample	**Bacterial Strains + Fungus**	**Average Zone of inhibition in various concentration in mili meters**			
		6 µl	**12 µl**	**Positive control**	**Negative control**
Leaves Water Extract	*Escherichia coli*	NA	NA	31	00
	Salmonella typhi	NA	NA	36	00
	Klebsiella pneumoniae	NA	NA	32	00
	Bacillus subtilis	17	24	34	00
	Bacillus atrophoeus	12	13	33	00
	Erwinia carotovora	09	15	27	00
	Staphylococcus aurous	21	25	32	00
	Pseudomonas aeruginosa	13	16	31	00
	A.tumefaciens	15	14	32	00
	Candida albican	15	10	33	00

Table 5: Anti-microbial activity of water extract of stem sample

Stem Sample	**Bacterial strains + Fungi**	**Average Zone of inhibition In various concentration in mili meters**			
		6 µl	**12 µl**	**Positive control**	**Negative control**
Stem Water Extract	*Escherichia coli*	NA	NA	31	00
	Salmonella typhi	NA	NA	36	00
	Klebsiella pneumoniae	NA	NA	32	00
	Bacillus subtilis	10	20	34	00
	Bacillus atrophoeus	NA	NA	33	00
	Erwinia carotovora	NA	NA	27	00
	Staphylococcus aurous	10	21	32	00
	Pseudomonas aeruginosa	NA	NA	31	00
	A.tumefaciens	NA	NA	32	00
	Candida albican	NA	NA	33	00

3.6. Aqueous Extract of Leaves Sample of the Stevia Rebaudiana

The water extracts of leaves sample showed highest anti-bacterial activity against *Staphylococcus aurous* (21-25 mm) followed by *Bacillus subtilis* (17-24 mm) while smallest activity against *Bacillus atrophoeus* (12-13 mm in both concentration) (Table 4).

3.7. Aqueous Extract of Stem Sample of the Stevia Rebaudiana

Table 5 showed antimicrobial activity of stem sample of water extract. It showed activity against very limited microbes. These were *Staphylococcus aurous* (10-21 mm) and *Bacillus subtilis* (10-20 mm).

3.8. Extract of Stem Sample of the Stevia Rebaudiana in Acetone

Table 6, showed the antimicrobial activity of acetone extract of stem sample. The acetone extracts of stem sample showed a slightly uniform activity. In 6 micro liter concentrations, It showed high activity against *Bacillus subtilis* (15 mm) followed by *staphylococcus aurous (14mm)* While in concentration of 12 micro liters, this extract has more activity against *Erwinia carotovor*a (20 mm), followed by *B. subtilis* (18 mm), *S. aurous* and *Candida albican* (17 mm).

Table 6: Antimicrobial activity of acetone extract of stem sample

Stem Extracts	**Bacterial Strains + Fungus**	**Average Zone of inhibition in various concentration in mili meters**			
		6 µl	**12 µl**	**Positive control**	**Negative control**
Acetone Extract	*Escherichia coli*	NA	NA	31	00
	Salmonella typhi	NA	NA	36	00
	Klebsiella pneumoniae	14	14	32	00
	Bacillus subtilis	15	18	34	00
	Bacillus atrophoeus	13	20	33	00
	Erwinia carotovora	12	14	27	00
	Staphylococcus aurous	14	17	32	00
	Pseudomonas aeruginosa,	10	13	31	00
	Agro-bacterium tumefaciens	11	14	32	00
	Candida albican	13	17	33	00

Table 7: Antimicrobial action of Ethanolic extract of stem sample

Stem Sample	**Bacterial Strains + Fungus**	**Average Zone of inhibition in various concentration in mili meters**			
		6 µl	**12 µl**	**Positive control**	**Negative control**
Ethanolic Extract	*Escherichia coli*	NA	NA	31	00
	Salmonella typhi	NA	NA	36	00
	Klebsiella pneumoniae	15	20	32	00
	Bacillus subtilis,	16	18	34	00
	Bacillus atrophoeus	NA	NA	33	00
	Erwinia carotovora	NA	NA	27	00
	Staphylococcus aurous	7	11	32	00
	Pseudomonas aeruginosa	10	10	31	00
	Agro-bacterium tumefaciens	NA	NA	32	00
	Candida albican	11	12	33	00

3.9. Extract of Stem Sample of the Stevia Rebaudiana in Ethanol

Table 7, showed ethanol extract of stem sample has highest inhibitory effect in 12 micro liter concentrations against *K.pneumoniae* with the zone of 20 mm and *B.subtilis* 18 mm respectively, while in 6 micro liters, it has the zone of inhibition 15 and 16 mm against the same strains respectively.

3.10. Extract of Stem Sample of the Stevia Rebaudiana in Hexane

Hexane extracts showed maximum effects at 12 µl concentrations. Maximum activity was showed against *P,aeruginosa* (22 mm), followed by *K.pneumoniae* 15 mm. Similarly at 6 µl concentrations, highest inhibitory effect was showed for *K.*pneumoniae 18 mm.

Table 8: Antimicrobial activity of hexane extract of stem sample

Stem Sample	**Bacterial Strains + Fungus**	**Average Zone of inhibition in various concentration in mili meters**			
		6µl	**12 µl**	**Positive control**	**Negative control**
Hexane Extract	*Escherichia coli*	NA	NA	31	00
	Salmonella typhi	NA	NA	36	00
	Klebsiella pneumoniae	18	15	32	00
	Bacillus subtilis,	13	14	34	00
	Bacillus atrophoeus	10	13	33	00
	Erwinia carotovora	12	15	27	00
	Staphylococcus aurous	7	11	32	00
	Pseudomonas aeruginosa	15	22	31	00
	Agro-bacterium tumefaciens	NA	09	32	00
	Candida albican	09	14	33	00

Principally, when the zone of inhibition of a plant is higher than 6 mm (size of the disc), it is assumed that it has anti-microbial activity [10]. The anti-microbial activities of Ethanolic extract of leaves in a concentration of 6 and 12 micro liters showed remarkable activity against staphylococcus aurous which was 20 and 25 mm in used concentrations respectively which is near to standard (32 mm) (Table, 5). These zone of inhibition were somewhat high than showed by Pugalvendhan [13]. This may be due to environmental condition like soil nature, its pH value, cultivation procedure and high susceptibility of the tested microbes. It is reported that plant collected from acidic soil shows high anti-microbial activity [8]. The acetone extracts of leaves showed highest activity against staphylococcus aurous which is 25 and 20 mm while smallest against *Escherichia coli* which was 11 and 14 mm in 6 and 12 micro liter concentrations respectively which was near to the value of Jayyaraman research work [6]. The remarkable activity was shown by hexane extract of leaves sample for *staphylococcus aurous* (21 and 19 mm in 6 and 12 micro liter respectively). Zone of inhibition formed by Hexane against *Staphylococcus aurous* was more than from the work of [11]. Similarly, the water extracts of leaves sample showed highest anti-bacterial activity against *Staphylococcus aurous* (21 and 25 mm) followed by *Bacillus subtili*s (17 and 24 mm) and *Agrobacterium tumefaciens* (15 and 14 mm). This result is much high than previously work done by [16]. In case of stem sample of water extracts, it has showed activity against very limited microbes. The possible reason for this restricted activity is the low or negligible concentration of antimicrobial substances in water based extract due to inorganic nature. The acetone extracts of stem sample has showed a slightly uniform activity. The Ethanol extract of stem sample has highest inhibitory effect in 12 micro liter concentrations. Hexane extracts showed maximum effects at 12 µl concentration.

4. Conclusion

Currently all over the world, various bacterial strains shows high resistance to antibiotics. This problem can be minimized through herbal medicines. The present research work shows that both leaves and stem samples of Stevia rebaudiana have strong antibacterial as well as anti-fungal activity. The growth of both Gram positive and Gram negative are well inhibited. Comparatively, leaves shows high antimicrobial activity than stem. Almost, all the extracts showed no activity against S.typhi.

References

[1] H. Ahmad, “Capacity building for cultivation and sustainable harvesting of medicinal and aromatic plants”. In: H. Ahmad, A.A. Khan, editors. Proceeding of international workshop on conservation and sustainable uses of medicinal and aromatic plants in Pakistan, WWF, 2003

[2] S. Bhosle, “Commercial cultivation of Stevia rebaudiana”, Agrobios Newslett., 3:43-45, 2004

[3] R. Curi, M. Alvarex and R.B. Bazotte, “Effect of Stevia rebaudiana on glucose tolerance in normal adult humans, Braz. J. Med. Biol. Res., 19: 771-774.1986.

[4] G.M.Felippe, and N.M.C Lucas, “Estudo daviabilidabe dos frutos de Stevia rebaudiana”, Hoehnea., 1: 95-105, 1971.

[5] J. Goettemoeller and A. Ching, “Seed germination in Stevia rebaudiana: in J. Janick, (ed). Perspectiveson new crops and new uses. ASHS Press, Alexandria, VA., 510-511, 1999.

[6] S. Jayyaraman and M.S. Manoharan, “Invitro antimicrobial and anti tumour activities of Stevia rebaudiana leaf extracts”, Tropical Journal of pharmaceutical research., 7: 1143-1149, 2008.

[7] P.B.Jeppensen, S. Gregerson and K. Hermansen, “ Stevioside and steviol stimulate insulin secretion from isolated mouse islets”, Diabetologia., 125: 472-475, 1996.

[8] D. Kuntal, D. Raman and G. Nilesh, ‘Comparative antimicrobial potential of different extracts of leaves of Stevia rebaudiana Bert”, International Jounal of Natural and Engineering Sciences., 3: 65-68, 2009.

[9] H. Miyagawa and N. Fujioka, “Studies on the tissue culture of Stevia rebaudiana and its components: II. Induction of shoot primordial”, Planta Medica., 4: 321-323,1986.

[10] H.S.Muhammad, “The use of Lawsoniam Inermis Linn. (Henna) in the management of burns wound infections”, Biotechnology., 4:934-937,2005.

[11] D. Pareethi, T.M.Sridhar, P. Joshtha and C.V. Naidu, "Studies on antibacterial activity, phytochemical analysis of Stevia rebaudiana (Bert) - An important calorie free biosweetner Preliminary screening of some folklore medicinal plants", Journal of Eco-biotechnology., 3: 5-10,2011.
[12] G.M.Planas and J.Kuc, "Contraceptive properties of Stevia rebaudiana", Science., 162: 1007-1012,1968.
[13] R. Pugalvendhan and G. Prabakaran, "Study of antibacterial activity of Stevia rebaudiana against Wound infection causing bacteria", International Journal of Recent scientific Research., 3:285, 2012
[14] B. L. Robinson, "Contributions from the Grey herbarium of Harvard University. The Grey herbarium of Harvard University, Cambridge, 1930
[15] H. Rosnani, "The effects of Processing Parameter on the on the Antibacterial Activity of Zingiber Zerumbet Oleoresin", Msc thesis, University Teknologi Malaysia,2007.
[16] M.Tandhani and Subhash, 'Preliminary studies on Stevia rebaudiana leaves; proximal composition, Mineral Analysis and phytochemical Screening", J.Med Sci., 6: 321-326,2006.
[17] T.T.Truong and P.Valicek, "Verification of growth and stevioside content of Stevia plants propagated by vegetative and generative method", Agric. Trop. Subtrop., 32:7984,1999.
[18] S.Q.Zang, O.Kutowy and K.Ashwani, "Stevia rebaudiana leaves- A low calories source of sweeteners", Canadian Chemical News., 5:22-23,1999.