Study on the in vitro antibacterial properties of alcoholic extract of *Stevia rebaudiana* in west of Iran

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Abstract: Stevia rebaudiana (S. rebaudiana) is a native plant in Iran, which the plant has been used as an antidiabetic, antioxidant, antifungal, antiviral, and antiflammatory agent in Iran. As we know, there is documented proof on antibacterial properties of S. rebaudiana alcoholic extract against Pseudomonas aeruginosa (P. aeruginosa) in west of Iran (in Kermanshah). As a screen test to discover antibacterial activities of the extract, agar disk and agar well diffusion methods were employed. Macrobroth tube test was performed to specify MIC. The results of agar disk and agar well diffusion tests demonstrated S. rebaudiana have inhibited the growth of P. aeruginosa. Also in many of samples by increasing the concentration of S. rebaudiana, the inhibition zone increased. The MIC and MBC values were 0.125 g/ml. Thus, the present research indicates the antibacterial effects of the medical plant on P. aeruginosa. In vivo studies and clinical trials would be needed to justify and further evaluate the potential of the plant as an antibacterial agent in topical or oral applications.

Keywords: Stevia rebaudiana; alcoholic extract; Antibacterial properties; Macro-dilution method; Agar disk diffusion method; Agar well diffusion method.

1. Introduction

Infection is the invasion of an organism's body tissues by disease-causing agents such as virus, bacterium, and fungus. Infections disease due to bacterial species also stay a crucial clinical problem. Antibiotics are types of antibacterial drugs used in the treatment and prevention of bacterial infections. They may either kill or inhibit the growth of bacteria. Since the detection of these antibiotics and their use as chemotherapeutic agents, there was a belief in the medical fraternity that this would cause to the presumptive eradication of infectious diseases. But overuse of antibiotics has become the main factor for the emergence and dissemination of multi-drug resistant strains of different groups of microorganisms [1-3]. The emergence of resistance of bacteria to antibiotics is a common phenomenon. Emergence of resistance often reflects evolutionary processes that take place during antibiotic therapy. Down the ages plants have evoked interest as sources of innate products. They have been screened for their potential uses as alternative remedies for the treatment of several infectious diseases [4-9]. Plant-derived products have a major variety of phytochemicals such as phenolic acids, flavonoids, tannins, and other small compounds. Some medicinal

plants used in traditional Iranian medicine are efficient in treating diverse ailments caused by bacterial and oxidative stress [10-15]. A plant extract is a substance or an active with desirable properties that is removed from the tissue of a plant, to be used for a particular purpose. There are reports of the active principles of extracts from different plants with antifungal or antibacterial effects. The original benefit of plant extracts is that they do not increase the antibiotic resistance [16]. Herbal extract compounds have antimicrobial activities on a wide number of bacteria, and most of these compounds have phenolic groups in their structure [17]. In Iranian medicine, plant extracts in the form of infusion, decoction, tincture or herbal extract are consumed by the population for the treatment of diseases including infectious diseases. *S. rebaudiana* is a plant species in the genus *Stevia* of the *Asteraceae* family. *S. rebaudiana* is one of the edible plants which have produced a lot of interest throughout human history as a medicinal panacea. This plant is cultivated and used to sweeten food elsewhere in Asia [18]. The historical tradition of *S. rebaudiana* use in medicine is substantial. *S. rebaudiana* is known to have beneficial effects on a wide range of diseases, antimalarial, antiasthmatic, antiviral, antimicrobial, anti-inflammatory, gastroprotective, antihypertensive, antidiabetic, protective and antioxidant, nutritional and anti-cholesterol [19].

Based on knowledge of authors, in comparison to many other pharmaceutical-industrial plants, there is a very little data about antibacterial activities of alcoholic extract of *S. rebaudiana* collected from Kermanshah province, west of Iran. Hence, the aim of the current study was evaluation of antibacterial properties of the alcoholic extract of plant against *P. aeruginosa* with broth macro-dilution and agar disk and agar well diffusion methods.

2. Material and Methods

2.1. Source of microorganisms

Bacterial specie namely *P. aeruginosa* (PTCC No. 1707) was procured from Iranian Research Organization for Science and Technology as lyophilized. Bacterial strain was activated on Tryptic Soy broth, constant at 37°C for 18 h. Then 60 µl of the broth was transferred to Nutrient agar and incubated at 37°C for another 24 h; cell concentration was then adjusted to obtain final concentration of 10^8 cfu/ml using Muller Hinton broth.

2.2. Culture media

Mueller-Hinton Agar (Müller-Hinton agar is a microbiological growth medium that is commonly used for antibiotic susceptibility testing) was prepared according to the manufacturer's instruction (Oxoid, UK), autoclaved and dispensed at 20 ml per plate in 12 x 12cm Petri dishes. Set plates were incubated overnight to ensure sterility before use.

2.3. Plant sample collection

In this empirical-experimental study, medicine plant collected from Kermanshah. The sample was cleaned from any strange, plants, dust, or any other contaminants.

2.4. Preparation of alcoholic extract

Successive solvent extraction was performed for *S. rebaudiana*. Plants were washed, air dried for 7-8 days, and ground into powder before they were placed into the flask of the Soxhlet apparatus for extraction using 100% ethanol with increasing order of polarity to extract the phytoconstituents separately at 20ºC for 3-4 h (The ethanol used was HPLC grade obtained from Sigma-Aldrich, Germany). Whitman filter papers No.1 were then applied to filter the extract. After that, reduced pressure was applied to evaporate and dry the filtrates which were stored at -20ºC in labeled, sterile, screw capped bottles.

2.5. Evaluation of antimicrobial activities

Agar disk and agar well diffusion were used as screen tests to evaluate antibacterial property of *S. rebaudiana* based on standard protocol. The solution of the *S. rebaudiana* was yielded in 1g/ml from which six fold serial dilutions (v/v) were prepared. 60 µl of each dilution was poured on each disk and well in order. After a period of 24 hours incubation, the diameters of growth inhibition zones around the disks and wells were measured. Distillated water was used as negative control whereas Kanamycin was used as positive control in case of *P. aeruginosa*. Minimum inhibitory concentration (MIC) means the lowest concentration of the probable antimicrobial agent which prevents growing of bacteria (regardless of killing the bacteria or stopping the growth of them). The lowest dilution which no gross microbial growth has been seen indicates MIC. Minimum bactericidal concentration (MBC) means the lowest concentration of the agent which causes death to test bacteria. The last can be revealed by pouring 60 µl of MIC tube and six dilutions before contents on agar plate. In this case, after incubation period, the lowest concentration which makes no growth indicates MBC. For determination of MIC value, macrobroth dilution method was applied. Interpretation of the results was done due to national accepted letter [20].

2.6. Statistical Analysis

Antibacterial effects was determined by One way variance analysis (ANOVA), using the SPSS 18 software package. Data were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Agar disk diffusion test

About *S. rebaudiana*, the widest zone was seen in 0.25 g/ml concentration (The value of growth inhibition zone was 22 mm in this dilution). Growth inhibition zones due to different dilutions are listed in figure 1. No inhibition zone was observed due to distillated water.

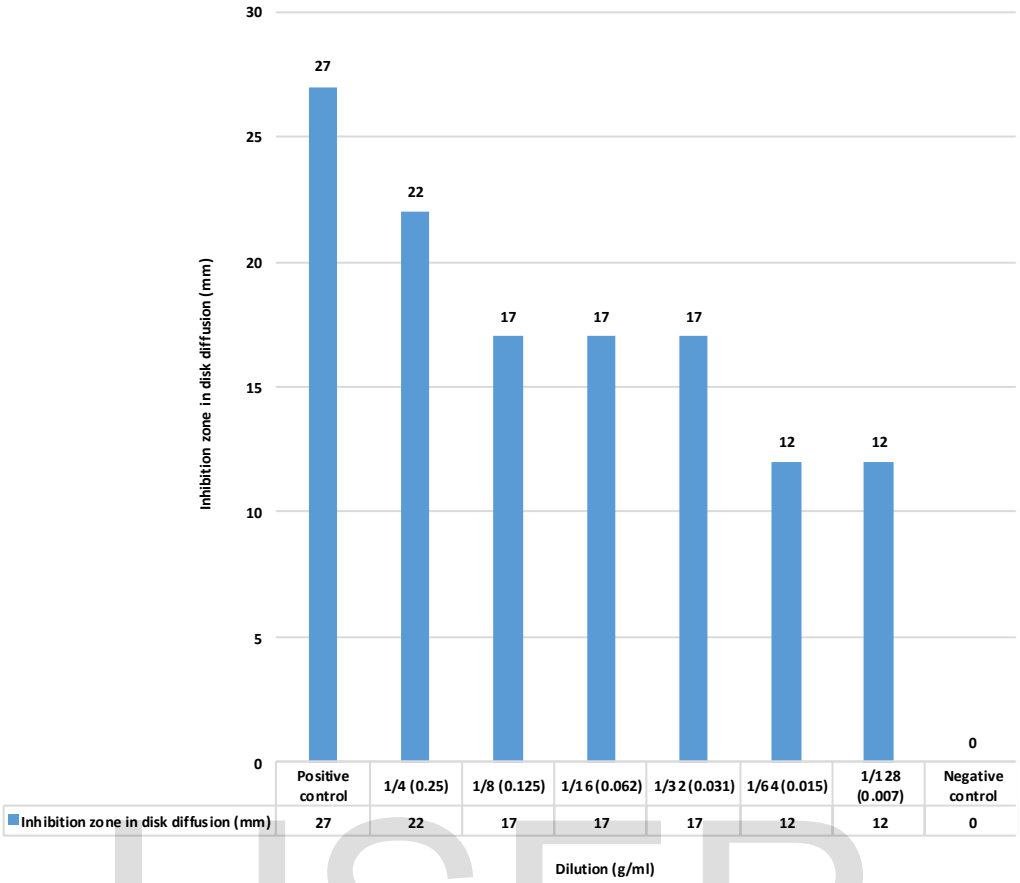

Figure 1. The diameters of growth inhibition zones in agar disk diffusion test in different dilutions of *S. rebaudiana*.

3.2. Agar well diffusion test

In regard to *S. rebaudiana*, the widest zone was seen in 0.25 g/ml concentration (The diameter of growth inhibition zone was 20 mm in this dilution). No inhibition zone was observed due to distillated water. The data are discoverable in figure 2.

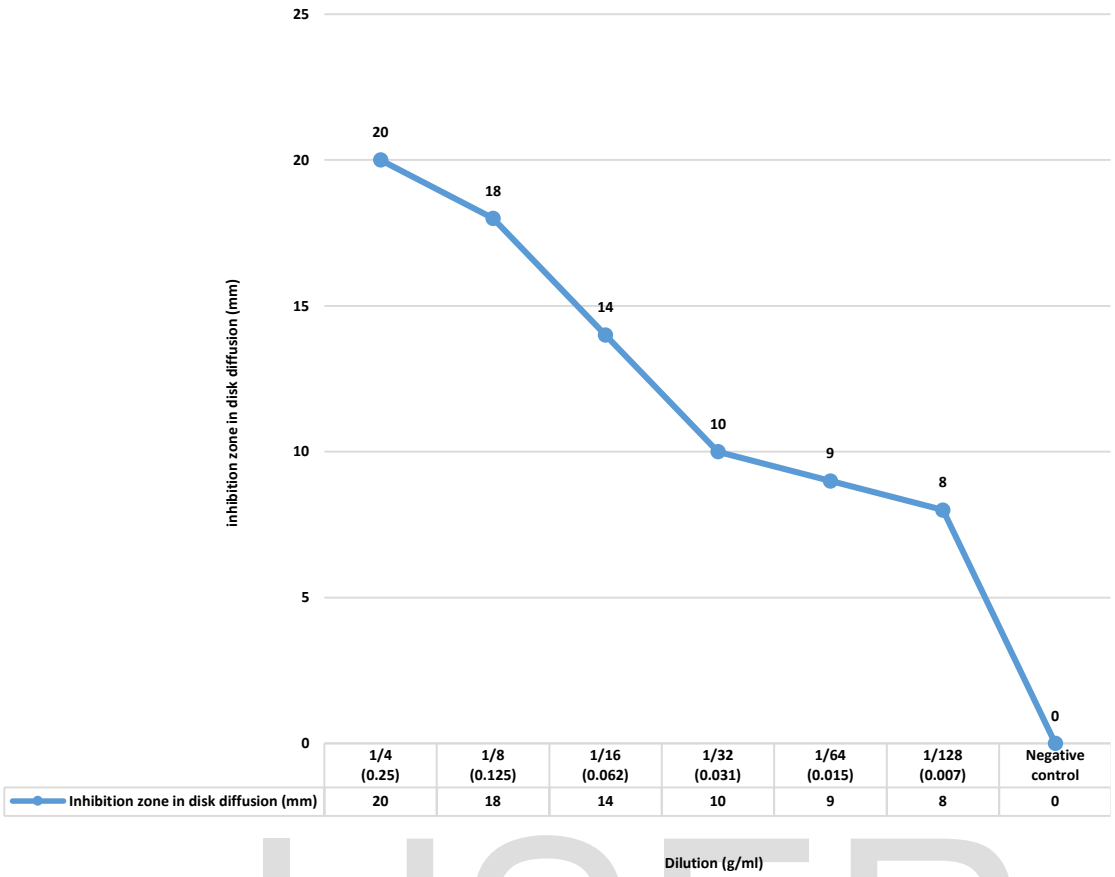

Figure 2. The diameters of growth inhibition zones in agar well diffusion test in different dilutions of *S. rebaudiana*.

3.3. MIC and MBC determination

The values for MIC and MBC were 0.125 g/ml.

4. Discussion

*P. aeruginosa*as a gram negative bacteria has been the main cause of serious illnesses recently. In body this bacterium is becoming resistance to certain type of antibiotics (such as kanamycin). Kanamycin is an aminoglycoside bactericidal antibiotic that used to treat a wide range of infections. Kanamycin is mainly effective against Gram-negative aerobic bacteria. Serious side effects of Kanamycin include tinnitus, toxicity to kidneys, and allergic reactions to the drug [21]. It has become a great concern for finding an eligible substitution (such as herbal extracts) for curing them. The antibacterial activities of plant extracts from a wide number of plants have been appraised and reviewed [22, 23]. *S. rebaudiana* is well known plant in Iran and different parts of this plant have long been used in traditional medicines of Iran. It is also used as a spice and food additive. It has been applied for treating different inflammatory and bacterial diseases [18, 19].

As the figures showed, the inhabitation zone in many of samples have been increased when the extract amount has increased. The results defined that in tested bacterium, there was a considerable discrepancy in terms of sensitivity to *S. rebaudiana*. In agar disk diffusion test, the widest inhibition zone was seen in 0.25 g/ml concentration (The value of growth inhibition zone was 22 mm in this dilution, and the value of growth inhibition zone of Kanamycin was 27 mm). In agar well diffusion test, the widest zone was seen in 0.25 g/ml concentration (20 mm). *S. rebaudiana* with 0.125 g/ml concentration has prevented the growth of *P. aeruginosa* and has destroyed it. Thus, the research represents the antibacterial effects of the medical herb on *P. aeruginosa*. There are correspondences between this result and the similar studies. In a study showed moderate antibacterial activities of alcoholic extract of *S. rebaudiana* against *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, P. aeruginosa, Staphylococcus aureus* and indicated that gram negative bacteria were more sensitive than gram positive bacteria in the selected plant extract [24]. The antibacterial effects of the alcoholic extract of *S. rebaudiana* on *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Micrococcus luteus, P. aeruginosa, Bacillus subtilis, Bacillus megaterium, Staphylococcus aureus* was studied and it was concluded that extract have considerable antibacterial activities on *P. aeruginosa* [25].

5. Conclusion

The result indicated alcoholic extract of *S. rebaudiana* have antibacterial properties. In fact *S. rebaudiana* have inhibited the growth of *P. aeruginosa* and eradicated it in 0.125 g/ml concentration. Also in many of samples by increasing the concentration of the extract, the inhibition zone increased. It can be used as antibacterial supplement in the developing countries towards the development of new remedial agent.

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Authors' Contribution

The core idea of this work came from Mohammad Mahdi Zangeneh and Akram Zangeneh, also the experiments, evaluation and Statistical Analysis of antimicrobial activities done by Mohammad Mahdi Zangeneh, Fariba Najafi, Reza Tahvilian, Lida Haghnazari, Akram Zangeneh, Mohammad Abiari, and Rohalah Moradi.

References

[1] Luria SE, Delbrück M. 1943. Mutations of Bacteria from Virus Sensitivity to Virus Resistance. *Genetics*. 28 (6): 491–511.

[2] Nychas, G.J.E. 1995. Natural Antimicrobials from Plants. In New Methods of Food Preservation; Gould, G.W., Ed.; *Blackie Academic Professional: London, UK*, 58-89.

[3] Tahvilian R, Moradi R, Zhale H, Zangeneh MM, Zangeneh A, Yazdani H, Hajialiani M. 2016. Ethnomedicinal Plants: In vitro antibacterial effect of essential oil of *Pistacia khinjuk*. *International Journal of Scientific & Engineering Research.* 7(10): 437-447.

[4] Kirbag S, Zengin F, Kursat M, et al. 2009. Antimicrobial Activities of Extracts of some Plants. *Pakistan J. of Botany*. 41(4): 2067-2070.

[5] Najafi F, Tahvilian R, Zangeneh MM, Zangeneh A, Moradi R. 2016. Screening of essential oil of *Allium sativum* for antibacterial effects against Bacillus subtilis. *International Journal of Recent Scientific Research.* 7(11): 14172-14176.

[6] Foroughi A, Zangeneh MM, Kazemi N, Zangeneh A. 2016. An in vitro study on antimicrobial properties of *Allium noeanum reut ex regel*: An ethnomedicinal plant. *Iranian J Publ Health.* 45 (2).

[7] Foroughi A, Pournaghi P, Tahvilian R, Zangeneh MM, Zangeneh A, Moradi R. 2016. Assessment of chemical composition and antibacterial effects of Anethole-rich hydroalcoholic extract of *Pimpinella anisum*. *International Journal of Pharmaceutical and Clinical Research.* 8(11): 1459-1463.

[8] Foroughi A, Pournaghi P, Najafi F, Zangeneh A, Zangeneh MM, Moradi R. 2016. Evaluation of antibacterial activity and phytochemical screening of *Pimpinella anisem's* essential oil. *International Journal of Pharmacognosy and Phytochemical Research*. 8(11); 1886-1890.

[9] Hemalatha M, Arirudran B, Thenmozhi A, Mahadeva Rao US. 2011. Antimicrobial Effect of Separate Extract of Acetone, Ethyl Acetate, Methanol and Aqueous from Leaf of *Milkweed (Calotropis gigantea L.)*. *Asian Journal of Pharmaceutical Research*. 1(4): 102-107.

[10] Rangarajan N, Sathiyamoorthy M. 2016. Phytochemical Screening and Antioxidant Studies in the Pulp Extracts of Cucurbita maxima. *South Asian Journal of Engineering and Technology*. 2(24):131-140.

[11] Nascimento G, Locatelli P, Freitas C et al. 2000. Antibacterial Activity of Plant Extracts and Phytochemicals on Antibiotic resistant Bacteria. *Brazilian J. of Microbiology*. 31: 247-256.

[12] Madhuri S, Pandey G. 2009. Some anticancer medicinal plants of foreign origin. *Current Science*. 96(6): 25.

[13] Shakeri A, Hazeri N, Vlizadeh J, et al. 2012. Photochemical Screening, Antimicrobial and Antioxidant Activity of Anabasis aphylla L. Extracts. *Kragujevac J. of Science.* 34: 71-78.

[14] Foroughi A, Pournaghi P, Zhaleh M, Zangeneh A, Zangeneh MM, Moradi R. 2016. Antibacterial activity and phytochemical screening of essential oil of *Foeniculum vulgare*. *International Journal of Pharmaceutical and Clinical Research*. 8(11): 1505-1509.

[15] Foroughi A, Pournaghi P, Najafi F, Zangeneh MM, Zangeneh A, Moradi R. 2016. Chemical composition and antibacterial properties of *Chenopodium botrys L*. essential oil. *International Journal of Pharmacognosy and Phytochemical Research*. 8(11); 1881-1885.

[16] Patil SB, Lende MY, Thakur VS, Naikwade NS, Magdum CS, Chavan GM. 2012. Protective effect from UV rays by Medicinal flowers. *Asian J. Res. Pharm*. 2(1): 24-25.

[17] Samydurai P, M. Saradha. 2016. Effects of Various Solvent on the Extraction of Antimicrobial, Antioxidant Phenolics from the Stem Bark of Decalepis hamiltonii Wight and Arn. *Asian J. Res. Pharm*. 6(2): 129-134.

[18] Misra H, Soni M, Silawat N, Mehta D, Mehta BK, Jain DC. 2011. Antidiabetic activity of medium-polar extract from the leaves of *Stevia rebaudiana Bert. (Bertoni)* on alloxan-induced diabetic rats. *J Pharm Bioallied Sci*. 3 (2): 242–248.

[19] Goyal SK, Samsher, Goyal RK. 2010. Stevia (*Stevia rebaudiana*) a biosweetener: a review. *Int. J. Food Sci. Nutr*, 61: 1-10.

[20] Clinical and laboratory standards institute (CLSI). 2006. M7-A7, 26(2).

[21] Garrod LP, Livingstone C. 1981. Antibiotic and Chemotherapy. 131.

[22] Foroughi A, Zangeneh MM, Zangeneh A, Kazemi N. 2016. A survey on antibacterial activities of *Allium eriophyllum* alcoholic extract: An ethnomedicinal plant. *Iranian J Publ Health*, 45 (2).

[23] Zangeneh MM, Tahvilian R, Najafi F, Zangeneh A, Souri N, Moeini Arya M, Zhaleh S. 2016. Evaluation of the in vitro antibacterial effect of the hydroalcoholic extract of *Scrophularia striata. International Journal of Scientific & Engineering Research.* 2016. 7(10): 1693-1702.

[24] Sunitha V, Irene wilsy J, Reginold.M. 2015. Antibacterial activity in medicinal plant (*Stevia rebaudiana*) using twosolvents. *International Journal of Recent Scientific Research*. 6 (7): 5070-5071.

[25] Manish B. Tadhani, Rema Subhash. 2006. In Vitro Antimicrobial Activity of *Stevia Rebaudiana Bertoni* Leaves. *Tropical Journal of Pharmaceutical Research*. 5 (1): 557-560.