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IN VITRO ANTIBACTERIAL ACTIVITIES OF ETHANOLIC EXTRACT OF *STEVIA REBAUDIANA* AGAINST *BACILLUS SUBTILIS* (ATCC No. 21332)

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ABSTRACT

Ethnomedicinal plants are considered recent resources for producing agents that could act as alternatives to antibiotics in remedy of antibiotic-resistant bacteria. As we know, there is no documented proof on antibacterial activities of *Stevia rebaudiana* (*S. rebaudiana*) alcoholic extract against *Bacillus subtilis* (*B. subtilis*) in west of Iran (in Kermanshah). As a screen test to detect antibacterial properties of the extract, agar disk and agar well diffusion methods were employed. Macro broth tube test was performed to specify MIC. The results of agar disk and agar well diffusion tests indicated SR have inhibited the growth of *B. subtilis*. Also in many of samples by increasing the concentration of SR, the inhibition zone increased. The MIC and MBC values were 0.125 g/ml for SR. Thus, the research represents the antibacterial effects of the medical herb against bacterium. The results indicate the fact that the extract from the plant can be useful as medicinal or preservatives composition.

KEYWORDS

Stevia rebaudiana, Alcoholic extract, Antibacterial activities, Macro-dilution method, Agar disk diffusion method and Agar well diffusion method.

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INTRODUCTION

A plant extract is a substance or an active with favorable effects that is removed from the tissue of a plant, to be used for a specific purpose. Plant extracts could be extracted from several parts like leaves, stems, flowers, roots including bushes and trees through distillation. They are efficacious on a

large numerous of Gram-negative and positive bacteria such as *Staphylococcus aureus*, *B.subtilis*, *Pseudomonas aeruginosa*, and *Escherichia coli O157:H7*¹. The use of plant extract compounds to treat bacterial disease is an old habit in a wide part of the world, especially in developing countries where there is dependence on traditional ethno medicine for a diversity of microbial diseases². Interest in plant extracts with antibacterial activities has revived as a result of common problems associated with the use of antibiotics³⁻⁵.

In Iranian medicine, plant in the different form are consumed by the population for the treatment of diseases including bacterial diseases⁶⁻¹⁴. In western states of Iran, a plant with the scientific name of *S. rebaudiana* has traditional medical usage. The genus *Stevia* of the family *Asteraceae* comprises about 154 species. *S. rebaudiana* is concentrated in Asia, Europe and South America¹⁵. *S. rebaudiana* have been used since ancient times in traditional medicines to treat diabetic, wounds, ulcers, cancer and microbial disease. Several extracts of the *S. rebaudiana* are traditionally used in treating bacterial diseases¹⁶.

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

MATERIAL AND METHODS

Plant sample collection

In this empirical-experimental study, medicine plant collected from Kermanshah.

Preparation of ethanolic extract

Successive solvent extraction was performed for *S.rebaudiana*. Plants powders were placed into the flask of the Soxhlet apparatus for extraction using 100% ethanol with increasing order of polarity to extract the plant compounds separately at 20°C for 4-5 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 desiccate the filtrates.

Source of microorganisms

Bacterial specie namely *B. subtilis* (ATCC No. 21332) 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 10⁸cfu/ml using Muller Hinton broth.

Culture media

Mueller-Hinton Agar was accumulated pursuant to the manufacturer's instruction (Oxoid, UK), autoclaved and dispensed at 20 ml per plate in 12 x 12cm Petri dishes.

Evaluation of antimicrobial activities

Agar disk and agar well diffusion were accomplished as diagnosis tests to assessment 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 accumulated. 60 µl of dilutions was splashed 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 Cefalexin was used as positive control in case of *B. subtilis*. The last can be demonstrated by pouring 60 µl of Minimum inhibitory concentration (MIC) tube and all dilutions before contents on agar plate. In this case, after incubation period, the lowest concentration which makes no growth shows Minimum bactericidal concentration (MBC). For specification of MIC value, macro broth dilution manner was used. Interpretation of the results was done due to national accepted letter¹⁷.

Statistical Analysis

Antibacterial effect was determined by One way variance analysis (ANOVA), using the SPSS 18

software package. Data were considered statistically significant at $p \leq 0.01$.

RESULTS AND DISCUSSION

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 23 mm in this dilution). There was no inhibition zone in *B. subtilis* due to 0.007 g/ml concentration. Growth inhibition zones due to different dilutions are listed in Figure No.1. No inhibition zone was observed due to distilled water.

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 distilled water. The data are discoverable in Figure No.2.

MIC and MBC determination

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

As the figures demonstrated, the inhabitation zone in many of samples have been increased when the extract amount has augmented. The results defined that in tested bacterium, there was a remarkable difference 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 23 mm in this dilution, and the value of growth inhibition zone of Cefalexin was 24 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 *B. subtilis* and has killed it. Thus, the research indicated the antibacterial effects of the ethno medical plant on *B. subtilis*. There are correspondences between this result and the similar studies. The antibacterial activities of the alcoholic extract of *S. rebaudiana* on *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *B. subtilis*, *Bacillus megaterium*, *Staphylococcus aureus* was studied and it was deduced that extract have significant antibacterial properties on *B. subtilis*¹⁸. In other study showed

alcoholic extract of *S. rebaudiana* have strong activities against a number of Gram-positive (*Staphylococcus albus*, *B. subtilis*) and Gram-negative (*E. coli*, *Klebsiella aerogenes*, *Enterobacter aerogenes*) bacteria¹⁹. In another study, the antibacterial effects of plant extracts was tested against two bacterial strains *B. subtilis* and *E. coli*, and it concluded that alcoholic extract of *S. rebaudiana* have substantial antibacterial effects on *B. subtilis*²⁰.

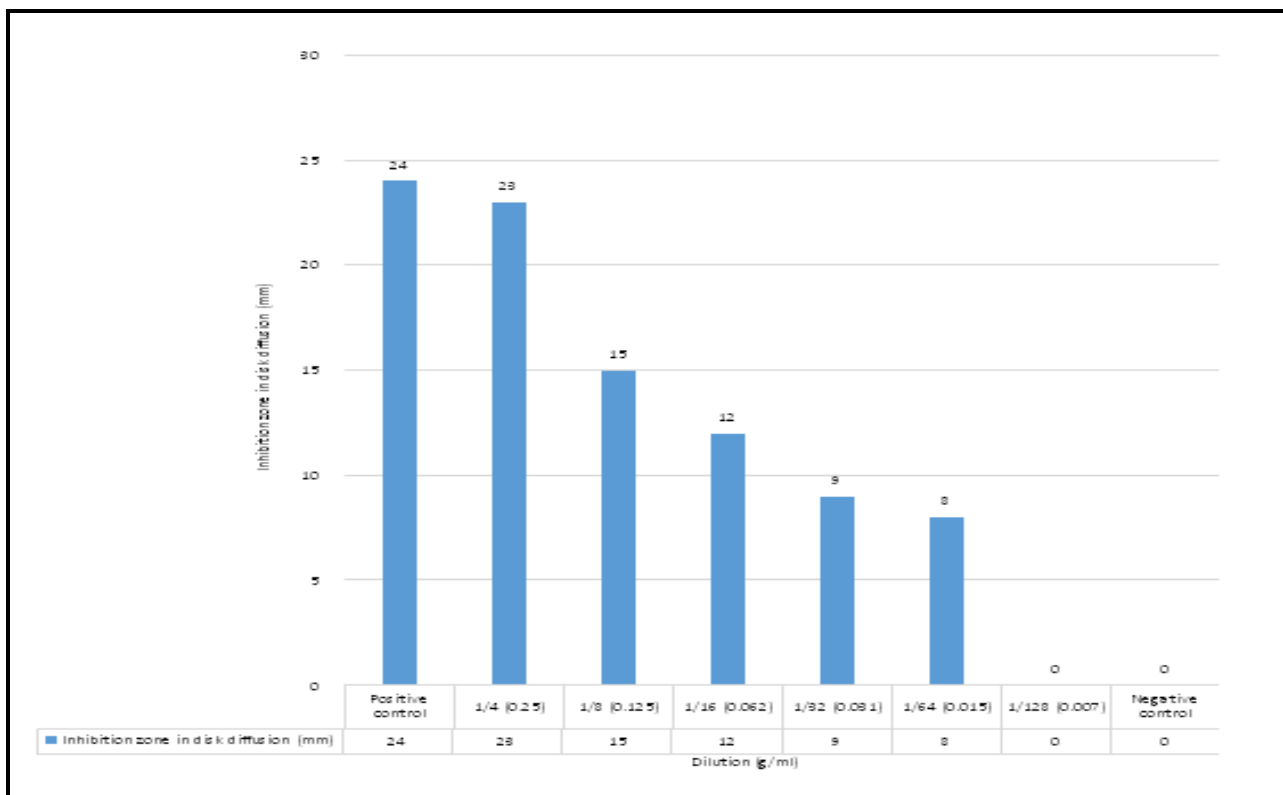


Figure No.1: The diameters of growth inhibition zones in agar disk diffusion test in different dilutions of *S. rebaudiana*

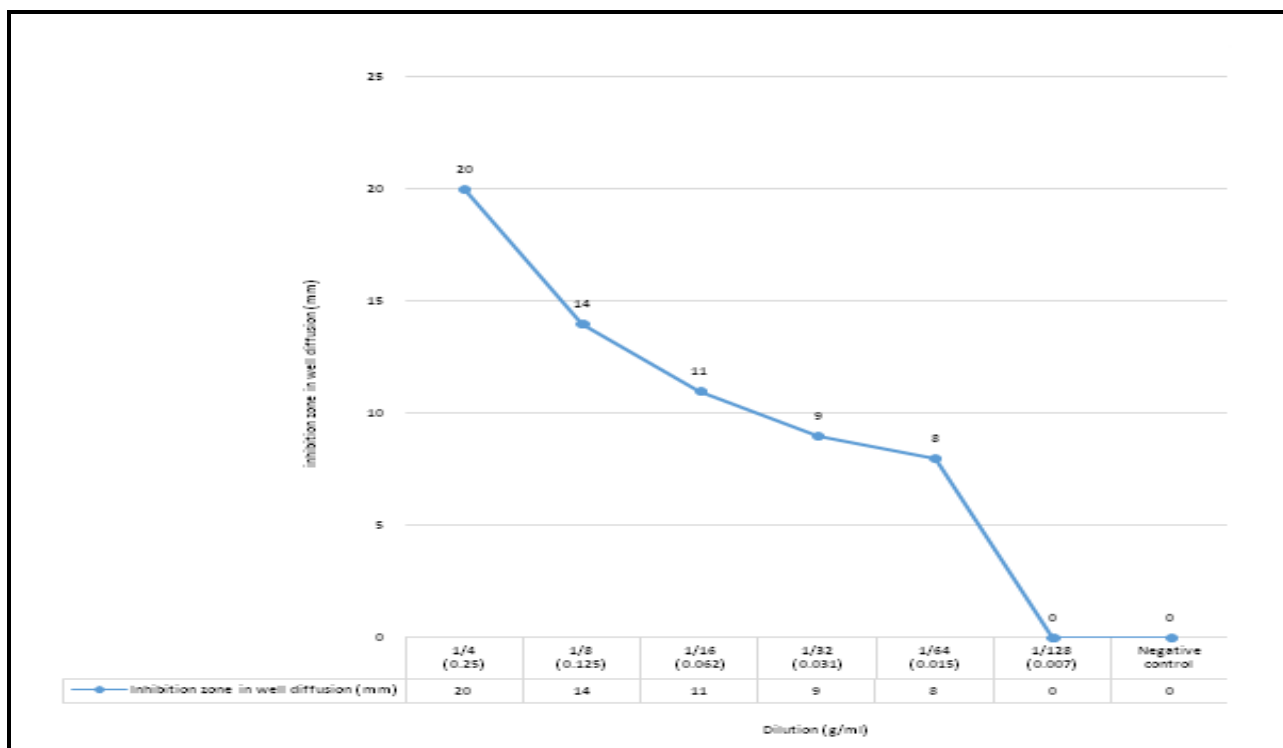


Figure No.2: The diameters of growth inhibition zones in agar well diffusion test in different dilutions of *S. rebaudiana*

CONCLUSION

From the study it can be concluded that the ethanolic extract of *S. rebaudiana* possess antibacterial activities. In fact *S. rebaudiana* have prevented the growth of *B. subtilis* and destroyed it. Also in many of samples by increasing the concentration of the extract, the inhibition zone increased. The results demonstrated that in tested bacterium, there was a considerable discrepancy in terms of sensitivity to *S. rebaudiana*. In other words, the most sensitivity was observed in agar disk diffusion method. Our results support the use of the plant in traditional medicine and suggest that ethanolic extract of *S. rebaudiana* possess good antibacterial properties. It can be used as antibacterial supplement in the developing countries towards the development of recent therapeutic 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 Mehrdad Poyanmehr, Mohammad Mahdi Zangeneh, Fariba Najafi, Reza Tahvilian, Lida Haghazari, Akram Zangeneh and Rohalah Moradi.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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