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Original Article

A Comparative and Dose-dependent Evaluation on *Stevia rebaudiana* Antimicrobial Activity; A Challengeable Study

Vahid Ganjiani[1], Mohammad Tabatabaei[2*] and Mohammad-Sadegh Golvajouei[3]

[1]*Clinical Sciences Department, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*
[2]*School of Veterinary Medicine, Shiraz University, Shiraz, Iran*
[3]*Biotechnology Center, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*

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Abstract

Comparative and dose-dependent evaluation of antimicrobial potential of *Stevia rebaudiana* (Bertoni) Bertoni was aim of current study. Six solvents were used for extraction of *S. rebaudiana* leaves to compare the effect of different solvents. These extracts were confronted against six selected pathogens. To determine the effect of dose on *S. rebaudiana* antimicrobial activity, nine concentrations were prepared for every extract to check against every mentioned pathogen using spot diffusion technique which taking of different concentrations is a prominent feature of present study in comparison with similar studies. Results were recorded by measuring the diameter zone of inhibition (DZI). Broth dilution method was also used to determine the minimum inhibitory concentration (MIC). Among six extracts, acetone extract revealed the best results against all pathogens except *A. hydrophila*. Furthermore, acetone extract showed better antimicrobial potential against G^+ microorganisms than G^- ones. Ethanol extract was most effective extract to inhibit *A. hydrophila*. No or a little activity was revealed by water and hexane extracts. Among six microorganisms, the most susceptible one was *B. cereus* exhibiting largest DZIs by all extracts. In MIC results, acetone extract revealed better results than ethanol and methanol extracts against all microorganisms, but this excellence was significant just against *S. aureus* and *E. coli* ($p<0.05$). Present study greatly proved *S. rebaudiana* has powerful antimicrobial potential and this feature is highly affected by solvent type and dose. *S. rebaudiana* can be used in food and pharmaceutic industries for either prevention or treatment.

Keywords: *Stevia rebaudiana*, Antimicrobial activity, Acetone extract

Introduction

There is always a concern about new and reemerging infections when they become incident, due to hardness of their control and also many problems that arisen in their treatment [1]. One of these problems is antibiotic resistance which is growing severely, and the outlook for using antimicrobial drugs in future is still uncertain [2]. In fact, bacteria have this potential to acquire and even transmit the ability of resistance to drugs specially antibiotics (which are utilized as therapeutic agents), which this origins from their genetic [3]. Regarding to recent increase in number of antibiotic resistance cases, attention is focused on alternative candidates [4,5]. It is worth noting that, although the antibiotic resistance level is critically alarming, but the progress in discovery of new antibiotics with different modes of actions has been significantly limited [6].With all these problems, some approaches must be taken for reduction of this problem, like controlling use of antibiotics, develop researches to better understand the genetical mechanisms of resistance, and continue studies to develop new drugs, either synthetic or natural [2]. Due to all these problems, tendency for discovering of herbal antibiotics increased. Having lesser side effects and being cheaper than synthetic antibiotics are another forcefully reasons for this tendency [7-9]. Hence, more studies are needed to emphasize use of plants as therapeutic agents; especially

*Corresponding author: School of Veterinary Medicine, Shiraz University, Shiraz, Iran
Email Address: Tabatabaie@shirazu.ac.ir

those are related to control of antibiotic-resistant microbes.

In past few years, a plant which has attracted world's attention is *Stevia rebaundiana* (Bertoni) Bertoni. Although it has been used by local people in several countries, but recent researches on this plant caused its new introduction to the world. Phytochemical analysis of *S. rebaundiana* showed existing of some bioactive constituents in its leaves like polyphenols, tannins, and alkaloids and also several glycosides mainly containing Stevioside (uses as non-caloric bio-sweetener in some countries), rebaudioside A, steviole, and isosteviol; these contents give several applications to *S. rebaudiana* [10,11]. *S. rebaudiana* has numerous therapeutic benefits including anti-hyperglycemic, anti-inflammatory, antiviral, and usages in treatment of diarrhea [12-15]. Antioxidant feature of *S. rebaudiana* is a key property among its therapeutic benefits which is caused by having great amounts of polyphenols [16-18]. One of prominent features of *S. rebaudiana* is antimicrobial activity. *S. rebaudiana* antimicrobial potential had been assayed in low number researches with limited concentrations [19-23].

The aim of current study was to evaluate the antimicrobial activity of *S. rebaudiana* from *Asteraceae* family using different solvents and concentrations to determine how different solvents and concentrations may affect this potential of *S. rebaudiana*. To the best of our knowledge, none has investigated *S. rebaudiana* antimicrobial activity with taking of different concentrations (which is a prominent feature of present study in comparison with similar studies) in Iran at this potential till now. Finally, a comparative discussion with similar researches was carried out.

Material and Methods

Plant Preparation

Fresh leaves of *S. rebaudiana* had been collected in summer time from farms of Zargiah Company (Fars province, Iran) and verified by department of agronomy and plant breeding, school of agriculture, Shiraz University, Shiraz, Iran. These fresh leaves were washed with distilled water, dried in shade, and finally grounded into powder.

Microorganisms Preparation

Six pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus lentus*, *Aeromonas hydrophila*, and *Yersinia pseudotuberculosis*) were chosen to evaluate the antimicrobial potential of *S. rebaudiana*. Pathogens isolates were obtained from department of pathobiology, school of veterinary, Shiraz University, Shiraz, Iran and stored in -18°C. These selected pathogens are causing important and high prevalence diseases in humans, animals, and even in plants.

Extract Preparation

Six solvents (methanol, ethanol, acetone, water, ether, and hexane) were opted for extraction of *S. rebaudiana* leaves. About 100gr of *S. rebaudiana* leaves powder was immersed with 500ml of every solvent separately. Each suspension was shook at room temperature (25°C) for 24 hours using an orbital shaker. All extracts were filtered separately by using of Whatman No.1 filter papers and the filtrates were evaporated with rotary evaporator under reduced pressure. Final stage was freeze drying; the extracts were dried and grinded into powder. Then all extracts were kept in labeled sterile falcon tubes and stored in -80°C until used.

Antimicrobial Assay

Six different extracts were prepared for the objective of understanding the antimicrobial activity of *S. rebaudiana* by measuring the diameter zone of inhibition (DZI) using spot diffusion technique on Muller Hinton agar plates. Then, 100 µl of every pathogen suspension with concentration of 1.8×10^8 CFU/ml (which prepared from 24 hours culture) were separately spread on the surface of culture media. For preparation of concentrations for every extract a sufficient amount of every extract lyophilized powder was dissolved into 5% DMSO to making doses. Nine concentrations were made for each extract (100, 50, 25, 12.5, 6.25, 3.125, 1.565, 0.781, and 0.390 mg/ml). 10 µl of every extract concentration was spotted on culture media and similar process was followed for all six extracts (In other words, the amount of every extract that was spotted on the surface of media from highest to lowest concentration were 1000, 500, 250, 125, 62.5, 31.75, 15.78, 7.81, and 3.9µg, respectively). Control that only had 5% DMSO was checked out. Beside 5% DMSO, gentamycin (10 µg/disc) and ampicillin (10 µg/disc) standard discs were utilized as the reference in present disquisition. Petri dishes were incubated for 24 h at 37°C. After incubation, DZIs were measured using measuring scale. The solvents used for the extract were also tested against bacteria.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of methanol, ethanol, and acetone extracts of *S. rebaudiana* was determined using broth dilution method against six selected pathogens taking different concentrations. First, 100 µl Muller Hinton broth was poured into all wells of microtitration plate. 10 µl of each bacterial suspension (adjusted to 1.8×10^8 CFU/ml) was added to intended wells. Each above mentioned extract was first diluted in a volume of 100 µl at a concentration of 5% and then was diluted for four times (2.5%, 1.25%, 0.625%, and

0.3125%). All the experiments were done in triplicates; positive and negative controls were run parallel along with sample analysis. Optical density of each well was measured at a wavelength of 620 nm at zero and 24 hours after incubation at 37°C. The percentage of growth inhibition was calculated by the formulation below:

Percentage of growth inhibition: $\frac{C-E}{O} \times 100$

O= OD of positive control at hour 24 – OD at zero

E= OD of sample containing extract and bacterium at hour 24 – OD at zero

Statistical Analysis

For statistical analysis, one-way ANOVA and Tukey test were performed using SPSS package.

Results

Antimicrobial Assay Outcomes

Antimicrobial activity of all concentrations of *S. rebaudiana* various extracts is shown in Table1.

Among all extracts, acetone extract demonstrated highest potential of antimicrobial activity against all selected microorganisms except *A. hydrophila*. In other words, largest zones of inhibition were observed for acetone extract. DZIs for acetone extract in concentration of 100 mg/ml were 17 mm (*S. aureus*), 15 mm (*E. coli*), 17 mm (*B. lentus*), 18 mm (*B. cereus*), 17 mm (*Y. pseudotuberculosis*), and 13 mm (*A. hydrophila*) that whatever obviously can be understood is acetone extract has greater potential to inhibit the growth of Gram-positive microorganisms than Gram-negative ones. A notable point to mention is which the acetone extract revealed a much powerful potential to inhibit the microorganisms, so that its lowest concentration (0.39 mg/ml) even had better results than highest concentration of some other extracts. About *A. hydrophila*, this microorganism was inhibited greatly by ethanol extract in comparison to other extracts. Among six selected microorganisms, the most susceptible one was *B. cereus* which exhibited largest zones of inhibition by all extracts. Among the microorganisms, *B. cereus* revealed largest DZI by ether extract in 100 mg/ml concentration, but with reducing in concentration, this microorganism showed greater reducing in DZI than other microorganisms. In this investigation, no evidence of antimicrobial activity was observed by water and hexane extracts, but only limited effects against *B. cereus* was observed by both extracts just in high concentrations (just 100 mg/ml, 50 mg/ml, 25 mg/ml, and 12.5mg/ml). A momentous point to mention is that the growth of *B. cereus*, as most susceptible microorganism, was not inhibited by ampicillin standard discs while all extracts, even water and hexane ones, revealed notable effects on growth of this bacterium which this can help us in treatment of microorganisms with antibiotic resistance (like *B. cereus* toward ampicillin). 5% DMSO had not any effect on all microorganisms. Besides, two of solvents just had limited effects on two of microorganisms.

Minimum Inhibitory Concentration Results

The results of broth dilution method used for identifying the MIC of *S. rebaudiana* at concentration of 3.125 mg/ml are shown in table 2.

Acetone extract showed significantly better effects than methanol and ethanol extracts against *S. aureus* and *E. coli* ($p<0.05$) and there wasn't any significant difference between ethanol and methanol extracts about *E. coli*, but for *S. aureus*, ethanol extract significantly differs with methanol one ($p<0.05$).

Discussion

Development and spread of antibiotic resistance in bacteria is an alarming danger to both humans and animals [24]. This problem is mainly due to over-prescribing of antibiotics, poor infection control in hospitals and clinics, patients not finishing their treatment, and over-use of antibiotics in livestock and fish farms. Antibiotic resistance is a threat for life in same sense as cancer, either in prevalence or in outcomes, so some actions can highly be effective on prevention of this danger development: public education, investigation on new and also old antibiotics, control of antibiotics use, and alternatives to antibiotics and etc. [24]. Medicinal plants have been being used for new drugs discovery. Plants synthesize special components for their defense against environmental threats. Moreover, secondary antimicrobial metabolites may be produced as a part of their normal growth or in response to stress [25]. *S. rebaudiana* is one of these plants with numerous medical features like anti-hypertension, immunomodulatory, antifungal, and anti-tumor and etc. [20,26-28].

Acetone and ethanol extracts had significantly better results against *B. lentus*than methanol extract, but about *B. cereus*, there was no significant difference among three extracts ($p<0.05$).

In present study, the effect of extraction solvent and different concentrations on antimicrobial potential of *S. rebaudiana* was studied. For this purpose, six different solvents (methanol, ethanol, ether, water, acetone, and hexane) were used to extract from *S. rebaudiana* to assess the effect of solvent type on its antimicrobial activity. These six extracts were checked against six prevalent microorganisms (*E. coli*, *S. aureus*, *B. cereus*, *B. lentus*, *A. hydrophila*, and *Y. pseudotuberculosis*).

Table 1 Antimicrobial activity of extracts of *Stevia rebaudiana (Bertoni) Bertoni* (DZI in cm)

Extracts		Pathogens					
Type of extract	Concentration (µg)	*S. aureus*	*E. coli*	*B. lentus*	*B. cereus*	*Y. pseudotuberculosis*	*A. hydrophila*
Methanol extract	1000	1.1	1.1	1.1	1.2	1.2	1
	500	1.1	1.1	1.1	1	1.1	1
	250	1	1.1	1	0.9	1.1	1
	125	1	1.1	1	0.9	1	1
	62.5	1	1.1	1	0.8	1	1
	31.75	1	1.1	1	0.7	1	1
	15.78	1	1	1	0.7	1	1
	7.81	1	1	1	0.7	1	1
	3.9	1	1	1	0.6	1	1
Ethanol extract	1000	1.4	1.4	1.4	1.5	1.5	1.4
	500	1.3	1.4	1.4	1.4	1.5	1.3
	250	1.2	1.4	1.4	1.3	1.5	1.2
	125	1.2	1.3	1.3	1	1.4	1.2
	62.5	1.2	1.2	1.3	1	1.4	1.1
	31.75	1.2	1.2	1.3	1	1.4	1.1
	15.78	1.1	1.2	1.3	1	1.3	1.1
	7.81	1	1.2	1.2	1	1.2	1.1
	3.9	1	1.2	1.1	1	1.1	1.1
Acetone extract	1000	1.7	1.5	1.7	1.8	1.7	1.3
	500	1.6	1.5	1.5	1.8	1.5	1.3
	250	1.5	1.5	1.4	1.6	1.3	1.2
	125	1.4	1.5	1.4	1.5	1.3	1.2
	62.5	1.2	1.4	1.3	1.2	1.2	1.2
	31.75	1.2	1.4	1.3	1	1.2	1.2
	15.78	1.2	1.3	1.3	1	1.2	1.2
	7.81	1.2	1.3	1.2	1	1.1	1.2
	3.9	1.1	1.3	1.2	1	1.1	1.2
Ether extract	1000	1.2	1.2	1.2	1.3	1.2	1.1
	500	1.2	1.2	1.2	1	1.2	1
	250	1.2	1.2	1.2	0.9	1.1	1
	125	1	1.1	1.1	0.9	1.1	1
	62.5	1	1.1	1	0.8	1	1
	31.75	1	1.1	1	0.8	1	1
	15.78	1	1	1	0.7	1	1
	7.81	1	1	1	0.7	1	0.9
	3.9	1	0.9	1	0.5	0.9	0.9
Water extract	1000	0	0	0	0.5	0	0
	500	0	0	0	0.4	0	0
	250	0	0	0	0.2	0	0
	125	0	0	0	0.1	0	0
	62.5	0	0	0	0	0	0
	31.75	0	0	0	0	0	0
	15.78	0	0	0	0	0	0
	7.81	0	0	0	0	0	0
	3.9	0	0	0	0	0	0
Hexane extract	1000	0	0	0	0.6	0	0
	500	0	0	0	0.5	0	0
	250	0	0	0	0.4	0	0
	125	0	0	0	0.2	0	0
	62.5	0	0	0	0	0	0
	31.75	0	0	0	0	0	0
	15.78	0	0	0	0	0	0
	7.81	0	0	0	0	0	0
	3.9	0	0	0	0	0	0
Antibiotic discs	Ampicillin	0.7	1.5	1.8	0	1.2	1.5
	Gentamycin	1.5	1.6	1.6	2	2	1.5

Table 2 Growth inhibition percentage of Stevia rebaudiana (Bertoni) Bertoni extracts at concentration 3.125 mg/ml

	S. aureus	*E. coli*	*Y. pseudotuberculosis*	*B. lentus*	*B. cereus*	*A. hydrophila*
Acetone extract	64.62±3.30 a	60.88±1.33 a	66.75±2.65 a	62.45±3.88 a	70.00±1.73 a	58.95±2.08 a
Methanol extract	43.01±1.21 b	43.01±7.10 b	56.68±3.17 bc	41.86±4.33 b	60.46±2.63 a	47.48±0.93 bc
Ethanol extract	53.4±8.20 c	50.28±2.58 b	61.58±3.75 ac	53.11±2.70 a	59.51±1.80 a	54.19±2.00 ac

To determine the effect of concentration on antimicrobial feature of *S. rebaudiana*, nine concentrations were prepared for every extract to check against every pathogen using spot diffusion technique. After 24 h incubation, results were recorded by measuring the diameter zone of inhibition (DZI). Among six extracts, largest DZIs were seen for acetone extract except against *A. hydrophila*, so acetone extract revealed the best outcomes when compared to other extracts. Besides, acetone extract antimicrobial activity was greater against Gram-positive microorganisms than Gram-negative ones. On the opposite, water and hexane extracts exhibited no evidence of antimicrobial potential except against *B. cereus*. Although acetone and ethanol extracts had great potential for *S. aureus* growth inhibition, but as a conflict, Ghosh *et al*. (2008) indicated that these two extracts didn't have any effects on inhibition of this microorganism growth [19]. Besides that, mentioned research showed ethanol extract had no effect on *E. coli* growth, but DZI of ethanol extract against *E. coli* was 14mm in 100mg/ml concentration in present study. There's a controversy surrounding water extract; in contrast to current investigation, Tadhani *et al*. (2006) showed that some effects were observed by water extract against *S. aureus* [22], but Jayaraman *et al*. (2008) stated similar results with our investigation outcomes which no evidence of antimicrobial activity was observed by water extract against *S. aureus* [20]. As another disagreement about water extract, Ghosh *et al*. (2008) indicated this extract can prevent the growth of *E. coli* [19], but we observed consistent data with Tadhani *et al*. (2006) and Jayaraman *et al*. (2008) results which water extract has no effect on this microorganism growth [20, 22]. Beside water extract, Tadhani *et al*. (2006) also indicated that hexane extract has the antimicrobial potential against several pathogens which is not in line to our disquisition too[22], since current study showed this extract was effective just against *B. cereus*. Various factors are involved in these different results. All steps in investigation, like collecting and drying the leaves, extraction protocol, antimicrobial assessment process, and even result recording can cause these differences. Another probably reason of differences in results of this study with others can be result of different geographical area and the effects of weather and soil type. Based on current study results, Because the concentration can highly affect the antimicrobial potential of *S. rebaudiana* and to date no reports on side effects of this herb have been published [29-31], so high concentrations of this herb or its extracts can be used or added to numerous products for example in foods as baiter (due to its high sweetness) or to prevent of foods spoilage and also can be used in various drugs and mouth washes.

Conclusion

In our knowledge, this study is the first research about comparative and dose-dependent evaluation of antimicrobial activity of *S. rebaudiana* in Iran at this potential. Results of this study showed that *S. rebaudiana* is a great source of antimicrobial components. Antimicrobial activity which was studied in present work is one of numerous remedial features of *S. rebaudiana*; that is why this plant is named as herbal drug of 21th century. This plant or its extracts can be used for different medical goals in different products as a protective agent or treatment one. Although this *in vitro* study greatly proved *S. rebaudiana* has powerful antimicrobial potential, but as an idea, we suggest to determine the antimicrobial activity of this plant against microorganisms collected in some hospitals known as antibiotic-resistant microorganisms. We should pay attention to this point which if we want to use this material in clinical usages; *ex vivo* and *in vivo* studies are required.

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