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Antibacterial Effects of *Stevia rebaudiana Bertoni* Extract on Pathogenic Bacteria and Food Spoilage

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ABSTRACT: *Stevia rebaudiana* Bertoni, is an ancient perennial shrub of South America, with great potential as a crop for the production of high-power natural sweetener. Due to its chemical composition and content of the phytochemical compounds, it is suitable as a raw material for the extraction and production of functional foods. Diterpene glycosides component of low-calorie sweetener is approximately 300 times sweeter than sucrose. In this study, the antimicrobial properties of Stevia rebaudiana Bertoni extract on pathogenic and food spoiling bacteria were tested by the disk diffusion method. The effect of *Stevia rebaudiana* extract on gram-positive and gram-negative bacteria were examined and on *Bacillus cereus*, *Lactobacillus Plantarum*, *Leuconostoc mesenteroides*, *Salmonella typhimurium* and *Salmonella enterica* exhibited inhibitory and germicidal effect, but the effects on *Listeria monocytogenes* was lower than the rest of organisms. The Minimum Inhibitory Concentrations (MIC) and Minimum Bacterial Concentrations (MBC) of Stevia extract was 1250 and 10000-1250 mg /ml respectively. The results showed that Stevia extract can prevent the growth of food pathogens and spoilage bacteria. Therefor we can use it as a natural sweetener and preservative in food products.

Keywords: *Antimicrobial Properties, Preservatives, Stevia Rebaudiana Bertoni, Sweetener*.

Introduction

Antimicrobial compounds with diverse chemical structures and the continuing need to explore new mechanisms regarding the risk of new infections and antibiotic resistance have been the subject of research for some time now (Cowan, 1999). Search for new drugs for treatment of various diseases, particularly the herbal extracts have been attracting the attention of the researchers. The use of plant extracts and biological compounds with known antimicrobial properties can be a useful therapeutic approach (Jayaraman, 2008). The results of the research work conducted in the late 19th and 20th centuries and the advent of streptomycin and other antibiotics have provided an appropriate condition for testing antibiotic or antimicrobial activities of many useful plants (Doss & Dhanabalan, 2009).

The leaf of many plants contain components containing tannins, essences and other aromatic constituents. In addition, many biological functions and plant antibacterial effects are depended on tannins and flavonoids. Plants are able to produce unlimited amounts of aromatic components where most are based on phenolic compounds. These constituents protect the plants from microbial infection and deterioration. Some of these bio-compounds are notably able to reduce the risk of cancer due to the antioxidant properties and inflammatory effects they possess. Some clinical studies have illustrated that bio-

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compounds can prevent the colon cancer and other types of diseases (Jayaraman, 2008).

Stevia might prevent the growth of some bacterial species and other infective elements (Patil *et al*., 1996; Sivaram & Mukundam, 2003). Some believe stevia can prevent and protect the persons from cold and flu. The dry extract from the leave of stevia (*Stevia rebaudiana* Bertoni) contains sweet diterpene glycosides, flavonoids, alkaloids, chlorophylls, xanthophylls, hydroxycynamic acids (caffeic, chlorogenic, etc.), soluble oligosaccharides, free sugars, amino acids, lipids, essential oils and trace elements. The present study mainly aims to study the antibacterial activity of the methanolic extract of *Stevia rebaudiana* Bertoni leaves against the selected microorganisms.

Materials and Methods

- *Materials*

Dried stevia leaves were supplied by Golsaran Shomal Company. Leaves were packed in vacuum bag and stored at -18℃ until used.

The bacterial strains used were provided vials of lyophilized from the Persian Type Culture Collection. (Salmonella typhi PTCC 1609, Salmonella enterica subsp. Enterica PTCC 1709, Lactobacillus plantarum PTCC 1058, Leuconostoc mesenteroides subsp. Mesenteroides PTCC 1059, Listeria monocytogenes PTCC 1298, Bacillus cereus PTCC 1015*).*

- *Methods*
- *Preparation of extracts*

Dry powdered stevia leaves were subjected to a mixture of water:metanol (20:80) using a shaker for thirteen hours at room temperature. The extracts were filtered using whatman No.1 filter paper and the filtered solvents were concentrated using a rotary evaporator (Model Laborata4000, manufacturers Haydvolf). The concentrated extracts were dried in a freeze dryer (Operun-FDB550 South Korea).

- *Preparation of inoculum*

Stock cultures were maintained at 4°C on nutrient agar slants. Active cultures for experiments were prepared by transferring a loopful of culture to 10 mL of nutrient broth and incubated at 37°C for 24 h for bacterial proliferation.

- *Disc diffusion*

In order to study the anti-bacterial activity of the extracts of stevia, the disc diffusion methods were employed. In this experiment, microbial suspension equivalent to 0.5 McFarland suspension was prepared and 0.1 % inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 min. The sterile disc blank was placed on the media with the help of a sterile forcep and then different concentrations of plant extracts consisting of 25, 50, 100 and 200 mg/ml were applied on the disc.

- *MIC (Minimum Inhibitory Concentration)*

MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of microorganism after overnight incubation. In this part, different plant extracts and antibacterial activities of the extracts were studied at seven different concentrations of 625, 1250, 2500, 5000, 10000, 20000, 40000 mg/ml.

- *MBC (Minimum Bactericidal Concentration)*

In order to determination MBC, 5ml of wells with no turbidity were plated on Moller Hilton agar medium for 24 hours at 37 ℃ and minimum bactericidal concentration was determined as the lowest concentration that inhibits no bacterial growth.

Results and Discussion

Plant extracts have been used in food, medicine, natural remedies, and supplements

for thousands of years (Joshi *et al*., 2009). Plant extracts are considered as new sources of products to possess antibacterial characteristics against pathogenic bacteria. Laboratory experiments have confirmed the influence of plant extracts on the inhibition of bacterial growth. This influence can be categorized as strong, moderate and weak (Joshi *et al*., 2009). Hydrophobicity, a vital characteristic of plant extracts, enables the extracts to bond to the lipidic layer of the cellular membrane of the bacteria and their mitochondria cause this layer to rupture and the important molecules and ions exit from the cell, leading to the eventual death of the bacteria (Joshi *et al*., 2009). The result of the analysis of variance indicated that bacterial strains showed significant differences in resistance against the preventative effects of the Stevia extracts.

Table 1 and Figure 7 illustrate the inhibition zones formed around the methanolic extract of the Stevia leaf at 25-200µgml^{-1}. Statistical analysis of the screening of the antibacterial properties of the Stevia extract indicated that the greatest diameter of non-growth inhibition zones belonged to *Salmonella typhimurium* (Figure 1), *Bacillus cereus* (Figure 2), and *Salmonella enterica* (Figure 3) respectively, while the least diameter was related to *Listeria monocytogenes* (Figure 4). The diameter of non-growth inhibition zones was statistically significant in all dilutions except dilution 50 in *Lactobacillus planterum* (Figure 5) and *Leuconostoc mesenteroides* (Figure 6).

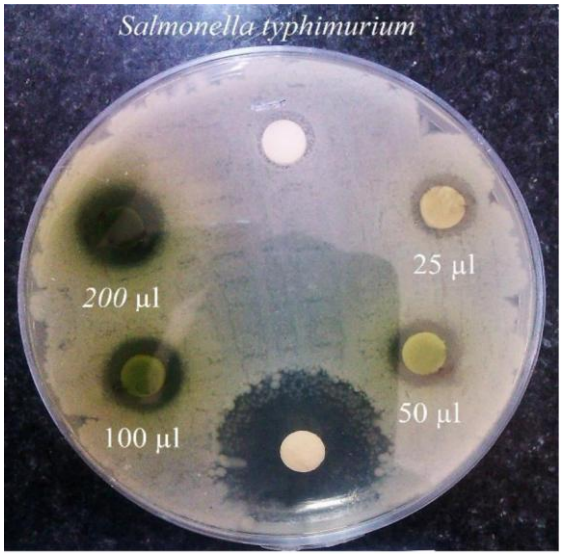

Fig. 1. Antibacterial activity of water: methanol extract of *Stevia rebaudiana* leaves against *Salmonella typhimurium.*

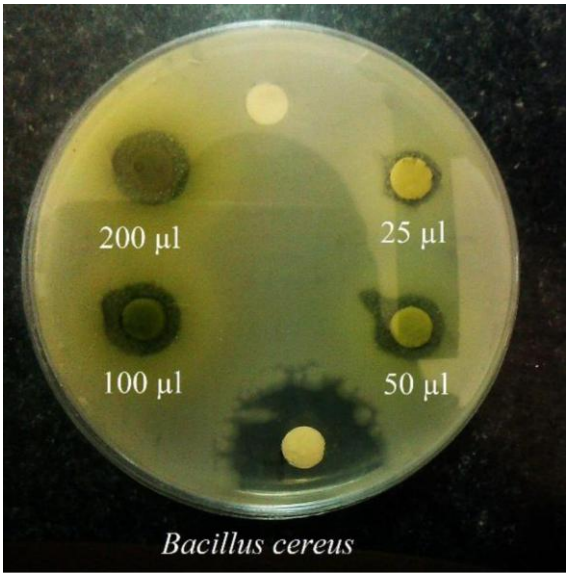

Fig. 2. Antibacterial activity of water: methanol extract of *Stevia rebaudiana* leaves against *Bacillus cereus.*

Table 1. Antimicrobial activity of water: methanol extract of *Stevia rebaudiana* leaves (zone of inhibition in mm).

Strain concentration	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
Salmonella enterica	7.2^{c}	8.9^{b}	10.22^{c}	10.53^{c}
Salmonella typhimurium	10^{a}	10.85^{a}	12.46^{a}	13.53^{a}
Lactobacillus plantarum	7.15^{c}	8.05^{bc}	9.69^{d}	10.2^{d}
Leuconostoc mesenteroides	7.67^{c}	8.67^{bc}	9.4^{d}	10.2^{d}
Listeria monocytogenes	6^{d}	7.67^{c}	8.55^{e}	8.9^{e}
Bacillus cereus	9.15^{b}	10.89^{a}	11.75^{b}	12.66^{b}

Dissimilar letters indicate significant differences at each concentration level of 5%.

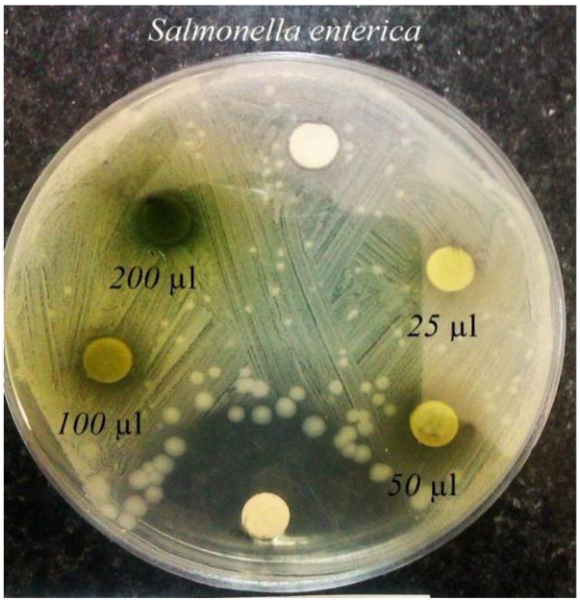

Fig. 3. Antibacterial activity of water: methanol extract of *Stevia rebaudiana* leaves against *Salmonella enterica*.

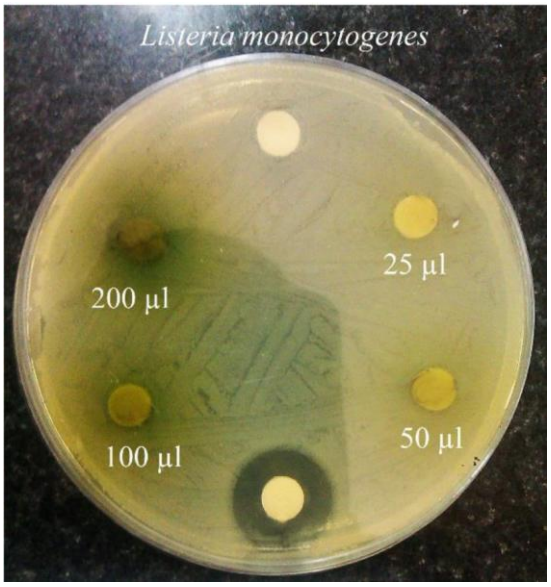

Fig. 4. Antibacterial activity of water: methanol extract of *Stevia rebaudiana* leaves against *Listeria monocytogenes.*

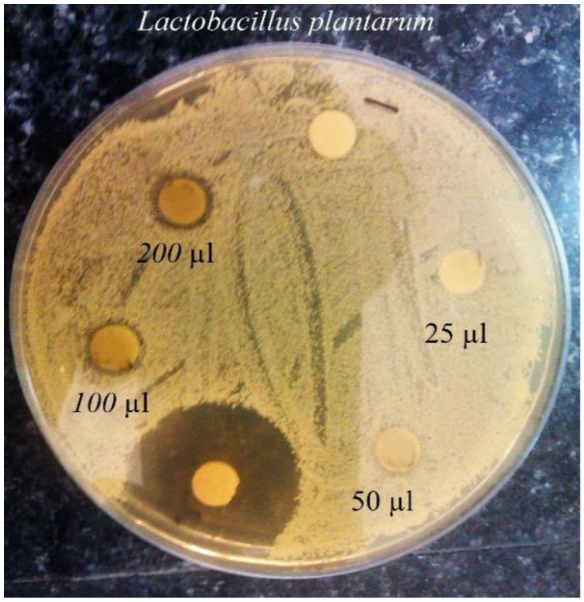

Fig. 5. Antibacterial activity of water: methanol extract of *Stevia rebaudiana* leaves against *Lactobacillus plantarum.*

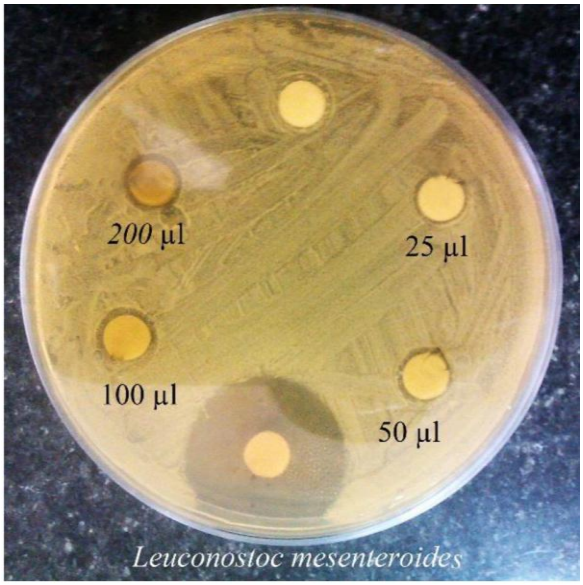

Fig. 6. Antibacterial activity of water: methanol extract of *Stevia rebaudiana* leaves against *Leuconostoc mesenteroides.*

- *The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)*

MIC is the least possible concentration in which the extent of survival of the inseminated bacteria can be compared with that of the initial insemination (Burt, 2004). The results of this analysis are presented in Table 2.

The MIC measured for all the tested organisms was 1250ppm. Previous investigations revealed that in some *Stevia Rebaudiana* extracts such as flower and leaf, the petroleum ether extract contained very small quantities of MIC. The petroleum

ether flower extract had the lowest MIC content (0.290 – 1.562 μgml^{-1}) in all the tested bacteria. The least value for MIC (0.390μgml^{-1}) was observed for *Bacillus subtilis, pseudomonas vulgaris, streptococcus pneumoniae*. The MIC content in the methanol plant extract was found to be in the range of 0.781 to 6.25μgml^{-1} while the in the leaf extract was limited to 3.125 to 12.5μgml^{-1} for all organisms. The flower extract contained the least amount of MIC as compared to the leaf extract for all the organisms tested (Preethi *et al.*, 2011).

MBC is the lowest concentration that indicated no significant growth following re-culturing in liquid media or where 99.9% of the initially inseminated bacteria were destroyed (Burt, 2004). The results of this analysis are presented in Table 2. The lowest amount of MBC was observed against *Salmonella typhimurium* and *Salmonella enterica* (Figure 8) which is an indicator of their sensitivity to Stevia extract. *Listeria monocytogenes* and *Bacillus cereus* (Figure 9) were the most resistant bacteria with highest MBC. *Lactobacillus Plantarum* and *Leuconostoc Mesenteroides* showed an intermediate resistace sensitivity (Figure 10).

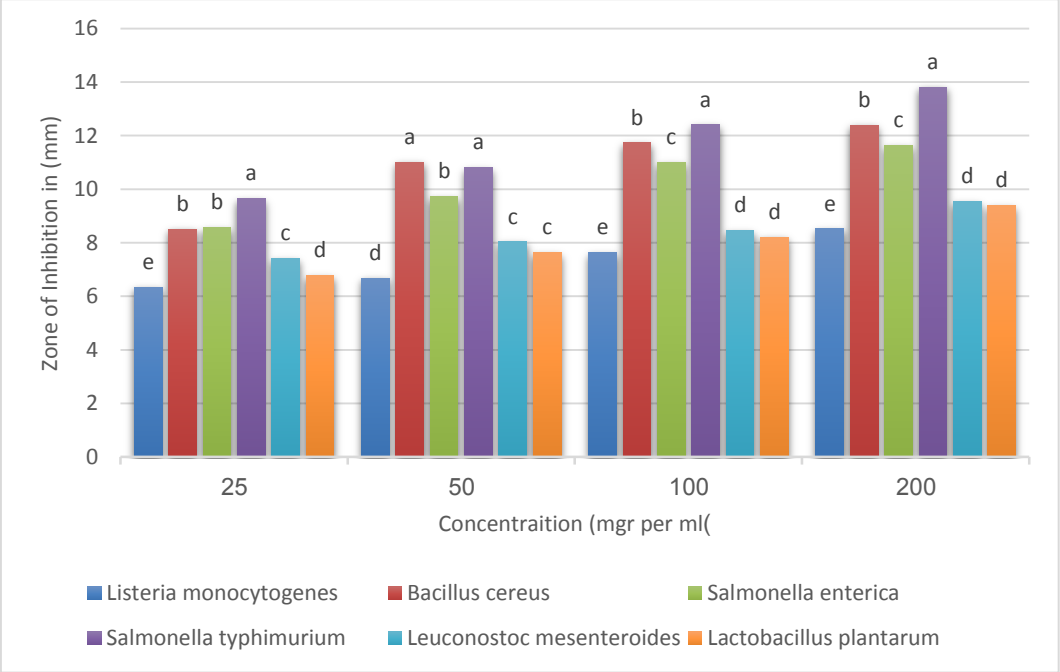

Fig. 7. Graphical presentation of antibacterial activity of water: methanol extract of *Stevia rebaudiana* leaves

Table 2. MIC and MBC concentrations of water:methanol extract of *Stevia rebaudiana* leaves (in ppm) against the tested bacteria.

strain	MIC	MBC
Salmonella enterica	1250	1250
Salmonella typhimurium	1250	1250
Lactobacillus plantarum	1250	5000
Leuconostoc mesenteroides	1250	5000
Listeria monocytogenes	1250	10000
Bacillus cereus	1250	10000

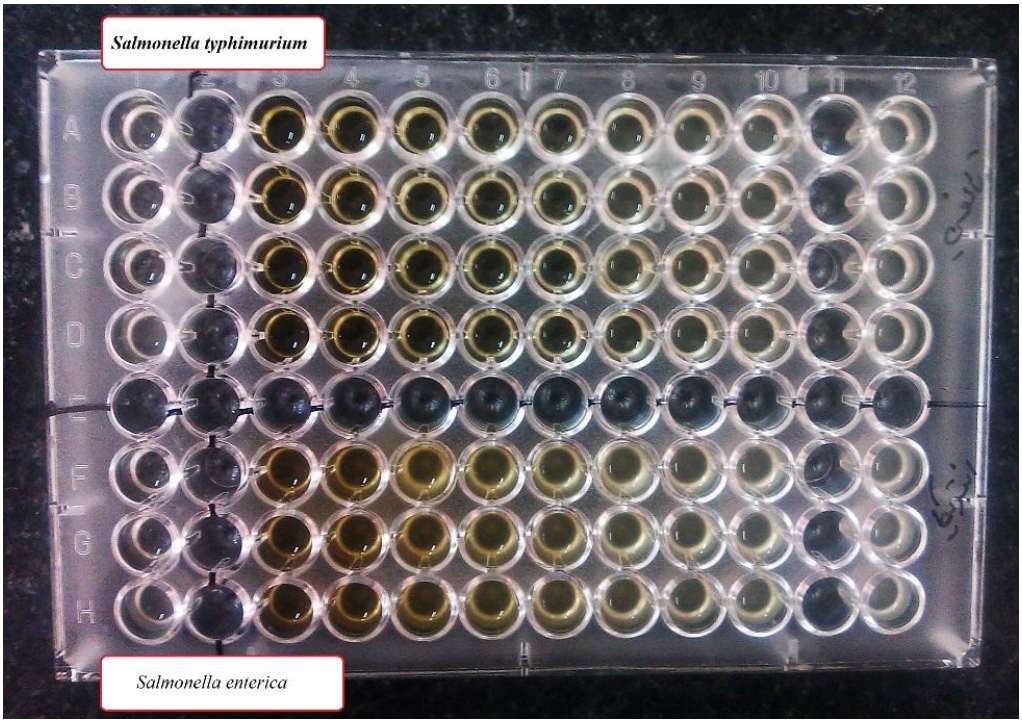

Fig. 8. *MIC and MBC concentrations of water:methanol leaves extract of Stevia rebaudiana (in ppm) against Salmonella enterica and Salmonella typhimurium.*

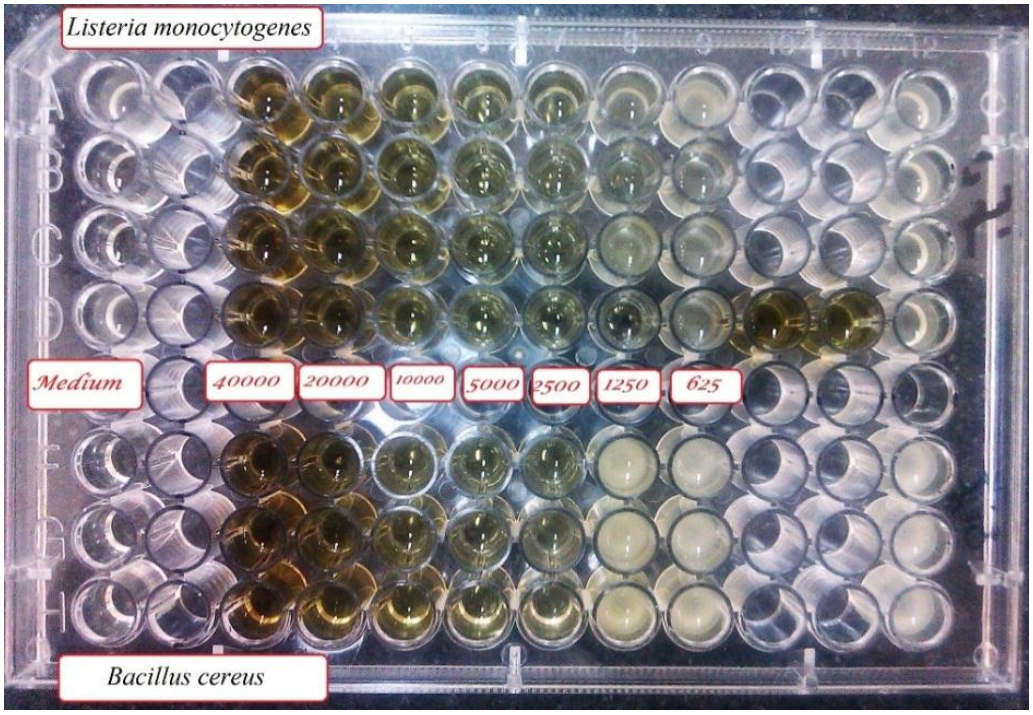

Fig. 9. MIC and MBC concentrations of water:methanol leaves extract of *Stevia rebaudiana* (in ppm) against *Listeria monocytogenes* and *Bacillus cereus.*

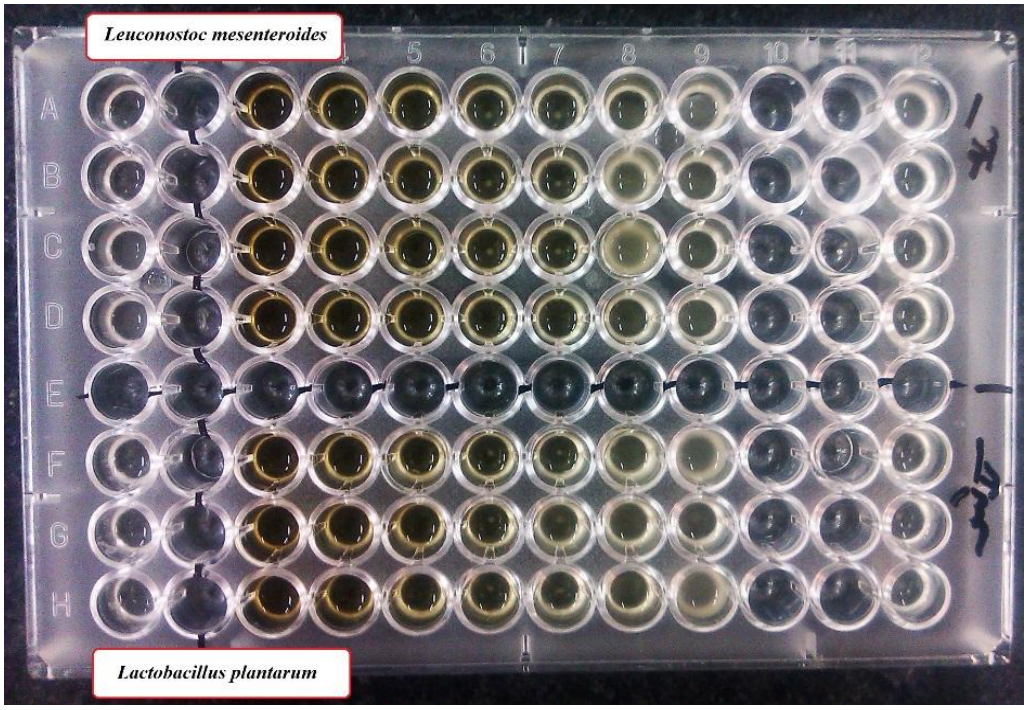

Fig. 10. MIC and MBC concentrations of water:methanol leaves extract of *Stevia rebaudiana* (in ppm) *against Lactobacillus plantarum* and *Leuconostoc mesenteroides.*

Conclusion

Stevia Rebaudiana, an ancient plant local to South America, has a very high potential as an agricultural produce for the manufacture of strong natural sweeteners. Because of its chemical composition and phytochemical contents, this produce is used as a suitable raw material for the extraction and manufacture of functional foods. The evaluation of the antibacterial activity of the Stevia extract showed that the highest growth inhibitory effect relative to the diameter of inhibition zones belonged to *salmonella typhimurium*, *Bacillus cereus*, and *salmonella enterica* respectively, while the least diameter was related to *listeria monocytogenes*. The lowest MBC was observed in *Salmonella typhimurium*, *Salmonella enterica* while the highest amount belonged to *Listeria monocytogenes* and *Bacillus cereus*.

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