Egypt. J. Chem. **Vol. 67**, No. 7 pp. 413 - 420 (2024)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Enhancement of Eco-Friendly Ultrasound Process on the Microbiological Quality of Stevia, Mint and Lemon Extracts Stored At Refrigerator Temperature for Four Weeks

Shaaban M. Abdel-Fatah[a] , Wafaa A. Ibrahim[b] , Atef A. Abou-Zaid[b] , Nadir A. S.[b] , Mostafa T. Ramadan[b] , yahia H. Abu Sree[a] , and Hesham A. Eissa[b]*

[a]*Food Toxicology and Contaminants Department, Food Industries and Nutrition Research Institute, National Research Centre, 12622, Egypt*

[b]*Food Technology Department; Food Industries and Nutrition Research Institute, National Research Centre, 12622, Egypt*

Abstract

Now, Ultrasound technology is one of the developed arising technologies to minimize processing, maximize quality and ensure the safety of food products. In recent times, it has been used as a volition processing option to conventional thermal approaches. The aim of this study was to evaluate the ultrasonic process on the microbiological quality of Stevia, Mint and Lemon extracts. Microbiological tests for the enumeration of yeasts and molds, total aerobic mesophilic bacteria and coliforms, were carried out in samples obtained from stevia leaf powder. The load of aerobic mesophilic bacteria, molds, and yeast; were in the allowable limits according to the standard specifications for the ultrasonicated stevia syrup. Total coli forms were found to be absent in all samples during storage period for 4 weeks under cooling conditions. In conclusion the use of ultrasound technology in this study had an effective effect in reducing the microbial load of all extracts under study, and this encourage its use as one of the important means of food preservation - in addition to that it is non-toxic, environmentally friendly and an emerging green technology because it saves a lot of energy and increases production.

Keywords: Eco-friendly; Ultrasound process; Microbiological quality; Stevia; Mint; Lemon.

1. Introduction

Instead of traditional food spoilage controls, the trend is now toward using ultrasound, ultraviolet light, and natural antimicrobial systems to extend the shelf life of fresh -like foods [1]. Ultrasound inactivates many enzymes and microorganisms pasteurize under moderate temperature conditions, which may improve food quality as well as ensure food stability and safety. Therefore, it can be used in pasteurization and food preservation. There are three different ways to apply ultrasound to products: By application directly to the product, b) coupling to the device and c) immersion in an ultrasonic bath [2] . Temperature and time are the most important factors on which the effectiveness of heat treatment depends. There is a proportional relationship between processing volume, time and process temperature with the amount of nutrient loss, the development of undesirable flavours, and the deterioration of the functional properties of food products. Ultrasound power of about 100 W was found to be optimal for maximum microbial inactivation. Microorganisms with "softer" and thicker capsules have been shown to be highly resistant to ultrasonic treatment [3]. In industrialized countries around the world, metabolic syndrome has become associated with abdominal obesity, increased risk of cardiovascular disease, and type 2 diabetes. This syndrome can be traced back to our human ancestors, who relied on carbohydrate -rich grains and ripe fruits to meet their energy needs. The innate

***Corresponding Author:** email: heshamin62@gmail.com.;(Hesham A. Eissa).
EJCHEM use only: Received date 19 October 2023; revised date 30 December 2023; accepted date 05 January 2024
DOI: 10.21608/EJCHEM.2024.236080.8751

attraction to sweetness has become a serious health problem, especially when food is plentiful and calories excessive [4, 5] . Processed low -calorie and no -calorie sweeteners such as saccharin and aspartame have been introduced, particularly within the industrialized part of the world, to reduce the contribution of sucrose and alternative high -energy sweeteners to excessive human caloric intake [6] . Compared to sucrose and fructose syrups, chemically manufactured artificial sweeteners, such as saccharin and aspartame, have sensory disadvantages, and consumer demand for zero -calorie natural sweeteners rather than artificial sweeteners has stimulated the search for natural and generally nonnutritive sweeteners. Steviol glucosides from Stevia rebaudiana leaves are a natural alternative [7] . For centuries in South America the leaves of the sweet herb "Stevia rebaudiana" have been used as natural sweeteners. This herb contains a mix of steviol glycosides. The nutritional safety of these compounds “steviol glycosides" has been confirmed through studies of their metabolism and use as a food additive, which has led to their general use in the United States and the European Union [7,8,9, 10] . Recently, there is a great effort to produce natural sweeteners through a strategic plan to grow new crops that could be suitable for cultivation in the new reclaimed areas and that might reduce the food gap, especially in the field of sugar and sweeteners. In Egypt some trials are in the beginning for Stevia cultivation. The present study was conducted to evaluate the ultrasound process on the microbiological quality of Stevia, Mint and Lemon extracts as one of the important means of food preservation - in addition to that it is non -toxic, environmentally friendly and an emerging green technology.

2. Materials and Methods

The aim of this study was to evaluate the possible effect of ultrasonication on the microbiological quality of stevia, mint and lemon extracts stored under refrigerator temperature for four weeks. Samples were taken from the stored extracts at the time periods of zero, two, and four weeks in order to determine the aerobic mesophilic bacteria, total coliforms count, and mold & yeast counts in the samples taken. Then determine the extent to which this treatment can be used to extend the shelf life of some natural extracts.

2.1. Stevia leaves, mint and whole lemon preparation

The fresh green leaves of stevia rebaudiana Bertoni were obtained from Sakha Agric. Res. Station Farm in Kafr El -Sheikh Governorate, Egypt. The leaves were allowed to dry using an oven at 60 °C for 16 h. The dried leaves were then blended to powder using a high -speed blender (10000/min). The powder samples were stored in polyethylene bags at 4oC until used. The fully matured, freshly harvested mint leaves or whole lemon pieces (Superstar cultivar) provided by the local market, Cairo, Egypt. Mint leaves or whole lemon pieces obtained from field experimental in institute of agronomy crops at Agricultural Research Institute, Giza, Egypt. Mint leaves or whole lemon pieces’ samples were used for all processing trials and was stored at 4 °C after receipt and processed within 24h.

2.2. Preparation and extraction of stevia, mint or lemon extracts:

Preparation and Extraction of Stevia leaves, mint leaves or whole lemon pieces were washed, weighed and immersed into 1% sodium meta - bisulfite solution (SO_2). They were rinsed with water and homogenized using electric pulpier (blender). Stevia, mint or lemon was extract. The stevia, mint or lemon extraction was then strained with double -layer cheesecloth. It filled the Stevia, mint and lemon extracts into clean, dry sterilized bottles hot. The bottled Stevia, mint and lemon extracts was cooled under running cold water and stored at room temperature for analysis (Figure -1). The stevia extract was mixed with mint extract and lemon extract by the ratio 1:1.

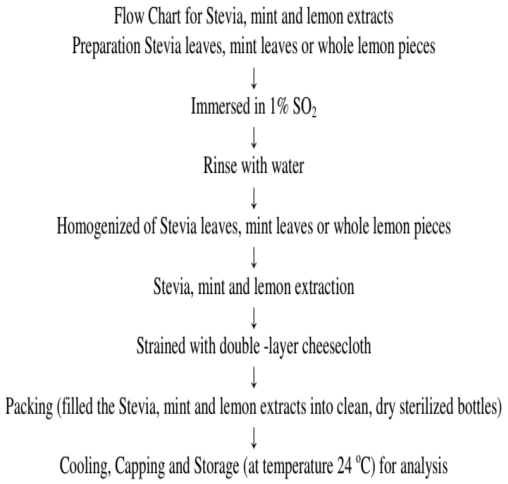

Figure 1: Flow char or steps in the preparation and extraction of Stevia, mint and lemon extracts

2.3. Ultrasonic process

To study the effect of ultrasonic process on the microbial load of stevia syrup, Ultrasonic -assisted stevia syrup had done using ultrasound generator probe (JY98 -III DN, Nanjing Fei qi industry &Trade Co., Ltd. Nanjing -China). The actual power delivered into the extraction system was 480 W (at 40% amplitude). An ultrasonic probe with a tip diameter of 20 mm was fitted into a glass beaker and the tip was inserted at the 15 mm height of the stevia syrup. After ultrasonication, the syrup was kept under 5 °C until used for the microbial test.

2.4. Microbiological Assay

2.4.1. Aerobic plate count (APC)

For determination of aerobic mesophilic bacteria, one ml of each stevia syrup was homogenized in 9 ml of 0.1% peptone water in an Erlenmeyer flask. Subsequent decimal dilutions were prepared in sterile peptone water. One mL of each decimal dilution was added to 12 -15 mL plate count agar (PCA) in duplicate, the plates were incubated for 24 -48 h in 30 °C, and then the bacterial colonies were counted [11]

2.4.2. Total coliforms count:

One ml of subsequent decimal dilutions up to 1:1000 (previously prepared for the APC) was transferred to Petri dishes in duplicate. Ten ml of Violet Red Bile Agar (VRBA) was poured, swirled to mix and let to solidify. Then, it was overlaid with 10 ml of melted medium. The plates were incubated at 35 ◦C for 24 h. Purple -red colonies (0.5 mm or larger in diameter) surrounded by a zone of precipitated bile acids were counted. To confirm that the colonies were coliforms, 10 representative colonies were picked and each of them was transferred to a tube of Brilliant Green Lactose Bile Broth. The tubes were incubated at 35 ◦C and were examined at 24 and 48 h for gas production [11].

2.4.3. Mold and yeast counts

Mold and yeast counts were performed on Sabouraud dextrose agar (SDA) according to the International Organization for Standardization [12] . One mL of each decimal dilution was placed on plate surface that contained Sabouraud dextrose agar (SDA) and distributed by a sterilized swab. Plates were incubated for 5 days at 25°C. Colonies were counted and expressed as CFU/ml.

3. Results and Discussions

Microbiological analysis

The dry *S. rebaudiana* leaf powder and its extract samples with or without lemon or mint extracts (Stevia plus Lemon & Stevia plus mint) were used and microbiologically analyzed in this study. The growth of moulds and yeasts, total mesophilic bacteria and coliforms (expressed as log CFU mL^{-1}) of the tested samples after different storage periods for 4 weeks under refrigerator conditions, are shown in Tables 1 -4 and Figures (2 - 5). Except for total coliforms, TPC, yeast and mold count increased during the storage period (Figures 2 -4). At zero time of storage, there were no changes in the microbial growth in all the tested extracts, except for the MB values, where these values of the unsonicated extracts recorded $0.64x10^{2}$, $0.51x10^{2}$, and $0.38x10^{2}$ CFU/mL for the extracts of stevia, stevia plus lemon, and stevia plus mint; respectively. For the ultrasonicated extracts there were no growth recorded for the MB values at zero time period (Table 1 & Figure 2).

Table (1): Changes in microbial quality at 0 day storage period at 5 ^{0}C of the tested extracts as affected by of ultrasonication process

Microbial Type	Stevia extract		Stevia extract plus lemon extract		Stevia extract plus mint extract	
	Unsonicated	Ultrasonicated	Unsonicated	Ultrasonicated	Unsonicated	Ultrasonicated
MB (CFU/ml)	$0.64x10^{2}$	ND	$0.51x10^{2}$	ND	$0.38x10^{2}$	ND
Yeast (CFU/ml)	ND	ND	ND	ND	ND	ND
Mold (CFU/ml)	ND	ND	ND	ND	ND	ND
Total Coli forms (CFU/ml)	ND	ND	ND	ND	ND	ND

ND: Not detected

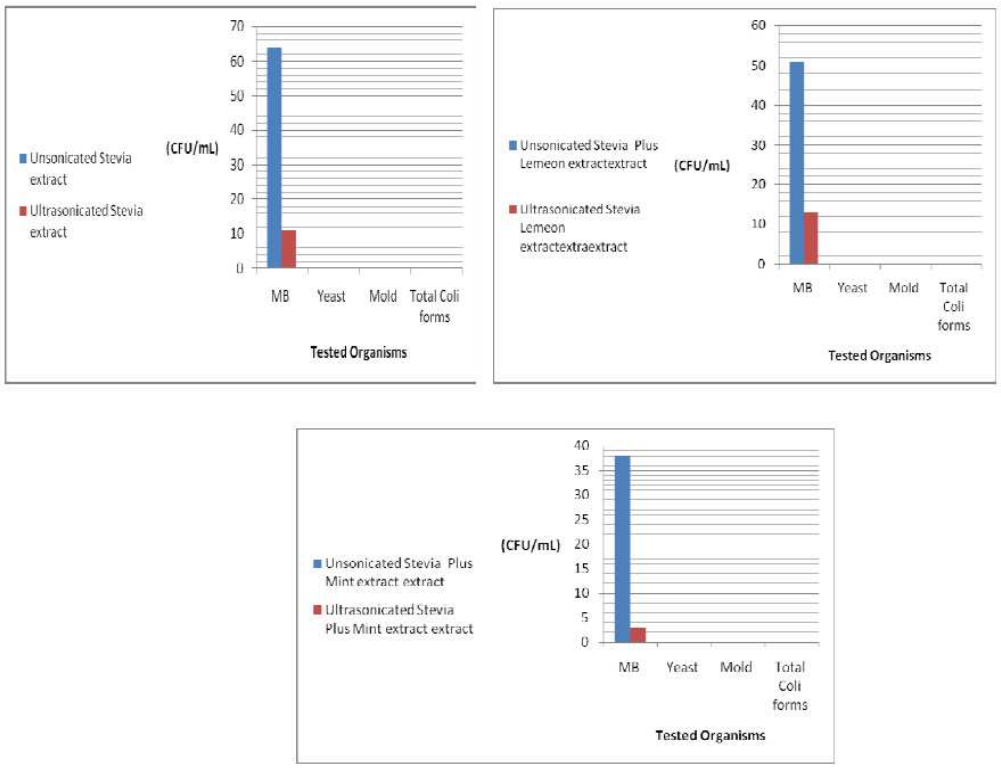

Figure 2: The effe

Figure 2: The effect of ultrasonication on microbial content at 0 day storage period.

After one week of storage, there were no changes in the microbial growth in all the tested extracts, except for fungal growth or the MB values with the unsonicated extracts, where the fungal growth recorded 4 CFU/mL in the extracts of stevia or stevia plus mint, while stevia plus lemon extract recorded 6 CFU/mL (Table 2 & Figure 3).

Table (2): Changes in microbial quality after 1 week storage period at 5 ^{0}C of the tested extracts as affected by of ultrasonication process

Microbial Type	Stevia extract		Stevia extract plus lemon extract		Stevia extract plus mint extract	
	Unsonicated	Ultrasonicated	Unsonicated	Ultrasonicated	Unsonicated	Ultrasonicated
MB (CFU/ml)	$2.12x10^2$	$0.56x10^2$	$2.68x10^2$	$0.31x10^2$	$1.75x10^2$	$0.76x10^2$
Yeast (CFU/ml)	ND	ND	ND	ND	ND	ND
Mold (CFU/ml)	4	ND	6	ND	4	ND
Total Coli forms (CFU/ml)	ND	ND	ND	ND	ND	ND

ND: Not detected

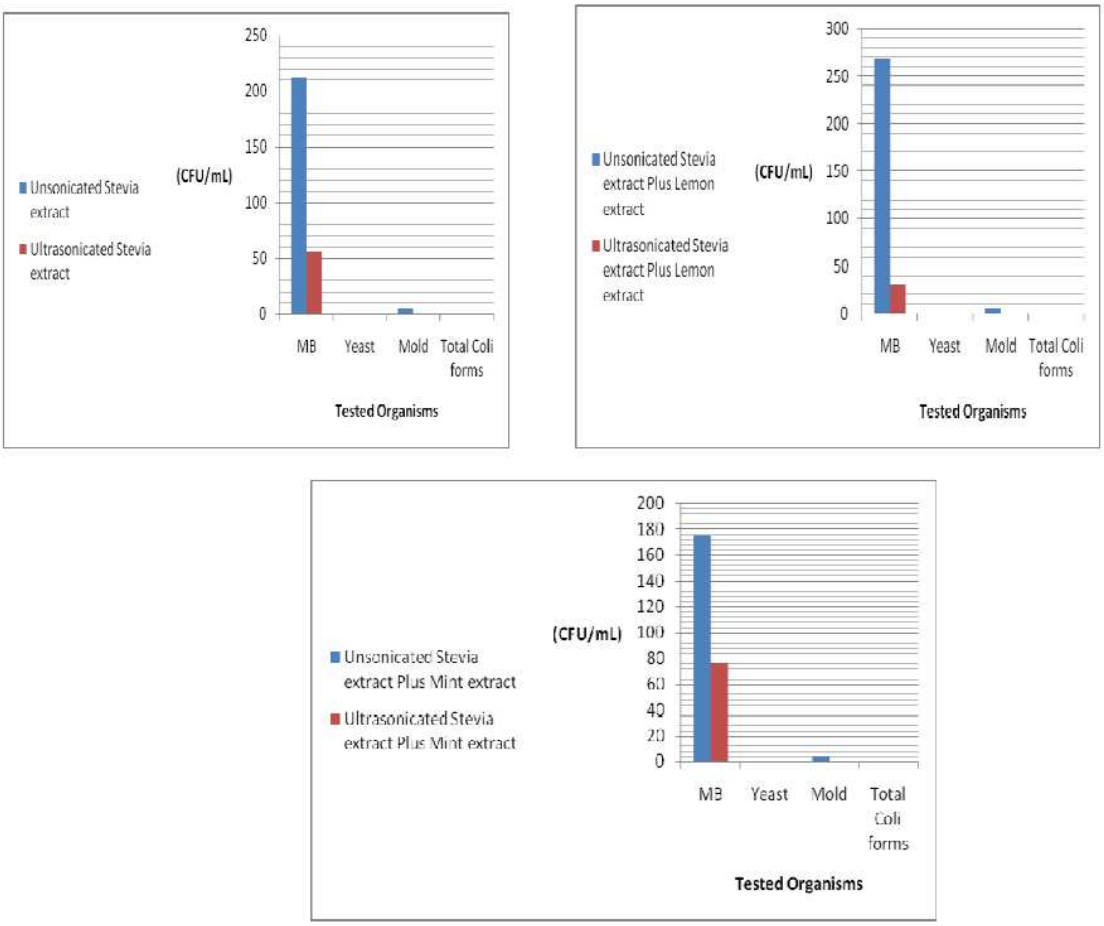

Figure 3: The effect of ultrasonication on microbial content at one week storage period.

The MB values (CFU/ml) of the ultrasonicated extracts recorded $0.56x10^{2}$, $0.31x10^{2}$, and $0.76x10^{2}$; for the extracts of stevia, stevia plus lemon, stevia plus mint, respectively. For the unsonicated extracts, The MB values changed to $2.12x10^{2}$, $2.68x10^{2}$, $1.75x10^{2}$; respectively. After two weeks of storage, no recorded values in total Coli forms (CFU/ml) four all tested extracts (Table 3 & Figure 4). But, there were slow changes in the microbial growth in fungal growth and MB values for all the tested extracts, where these growths recorded 5 CFU/mL in the extracts of stevia and 7 (CFU/ml) of stevia plus mint, while stevia plus lemon extract recorded 11 CFU/mL.

Table (3): Changes in microbial quality after 2 weeks storage period at 5 ^{0}C of the tested extracts as affected by of ultrasonication process

Microbial Type	Stevia extract		Stevia extract plus lemon extract		Stevia extract plus mint extract	
	Unsonicated	Ultrasonicated	Unsonicated	Ultrasonicated	Unsonicated	Ultrasonicated
MB (CFU/ml)	$3.34x10^{2}$	$0.75x10^{2}$	$3.81x10^{2}$	$1.38x10^{2}$	$3.7x10^{2}$	$1.43x10^{2}$
Yeast (CFU/ml)	ND	ND	ND	ND	ND	ND
Mold (CFU/ml)	5	ND	11	ND	7	ND
Total Coli forms (CFU/ml)	ND	ND	ND	ND	ND	ND

ND: Not detected

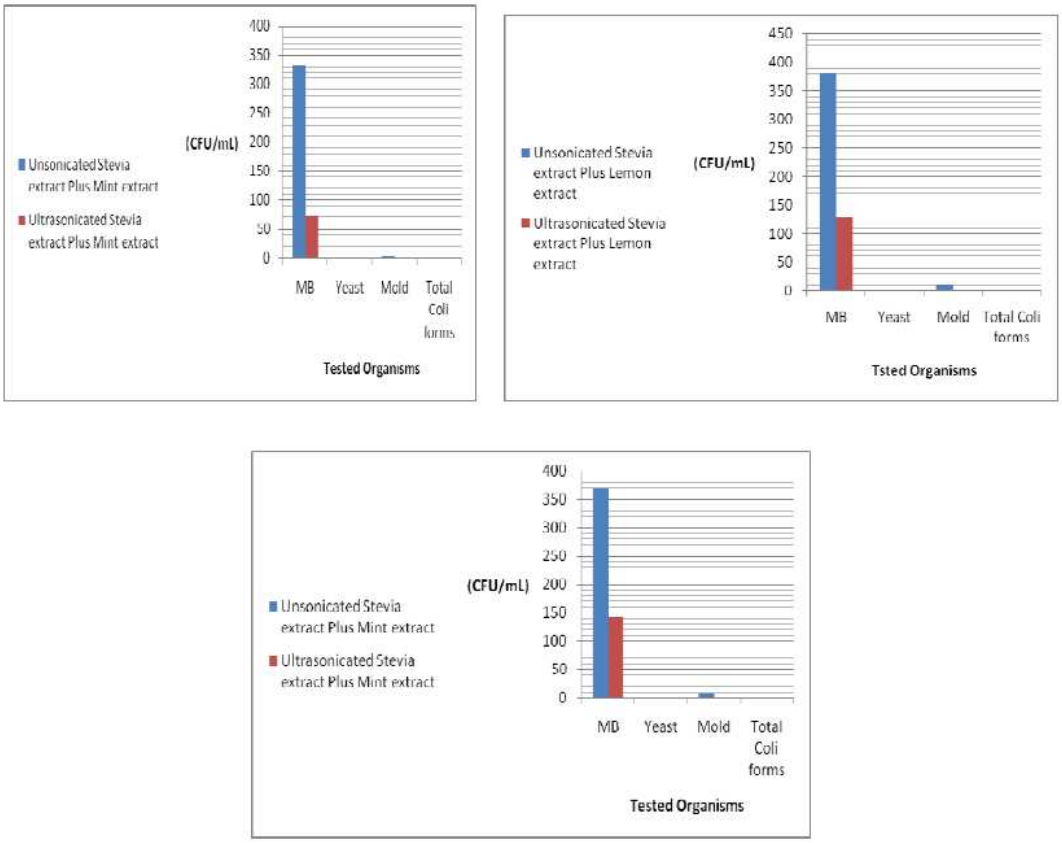

Figure 4: The effect of ultrasonication on microbial content at Two weeks storage period.

The MB values (CFU/ml) of the ultrasonicated extracts recorded $0.75x10^{2}$, $1.38x10^{2}$ and $1.43x10^{2}$; for the extracts of stevia, stevia plus lemon, stevia plus mint, respectively. For the unsonicated extracts, these values changed to 3.34x102, $3.81x10^{2}$, and $3.7x10^{2}$; respectively. After four weeks of storage, there were a slight increase in fungal growth and MB values for all the tested extracts of ultrasonicated extracts, but for the ultrasonicated extracts this increase was significantly higher (Table 4 & Figure 5).

Table (4): Changes in microbial quality after 4 weeks storage period at 5 ^{0}C of the tested extracts as affected by of ultrasonication process

Microbial Type	Stevia extract		Stevia extract plus lemon extract		Stevia extract plus mint extract	
	Unsonicated	Ultrasonicated	Unsonicated	Ultrasonicated	Unsonicated	Ultrasonicated
MB (CFU/ml)	$3.39x10^{3}$	$1.05x10^{2}$	$4.45x10^{3}$	$1.75x10^{2}$	$3.74x10^{3}$	$1.02x10^{2}$
Yeast (CFU/ml)	7	1	16	3	16	3
Mold (CFU/ml)	13	2	16	11	19	5
Total Coli forms (CFU/ml)	ND	ND	ND	ND	ND	ND

ND: Not detected

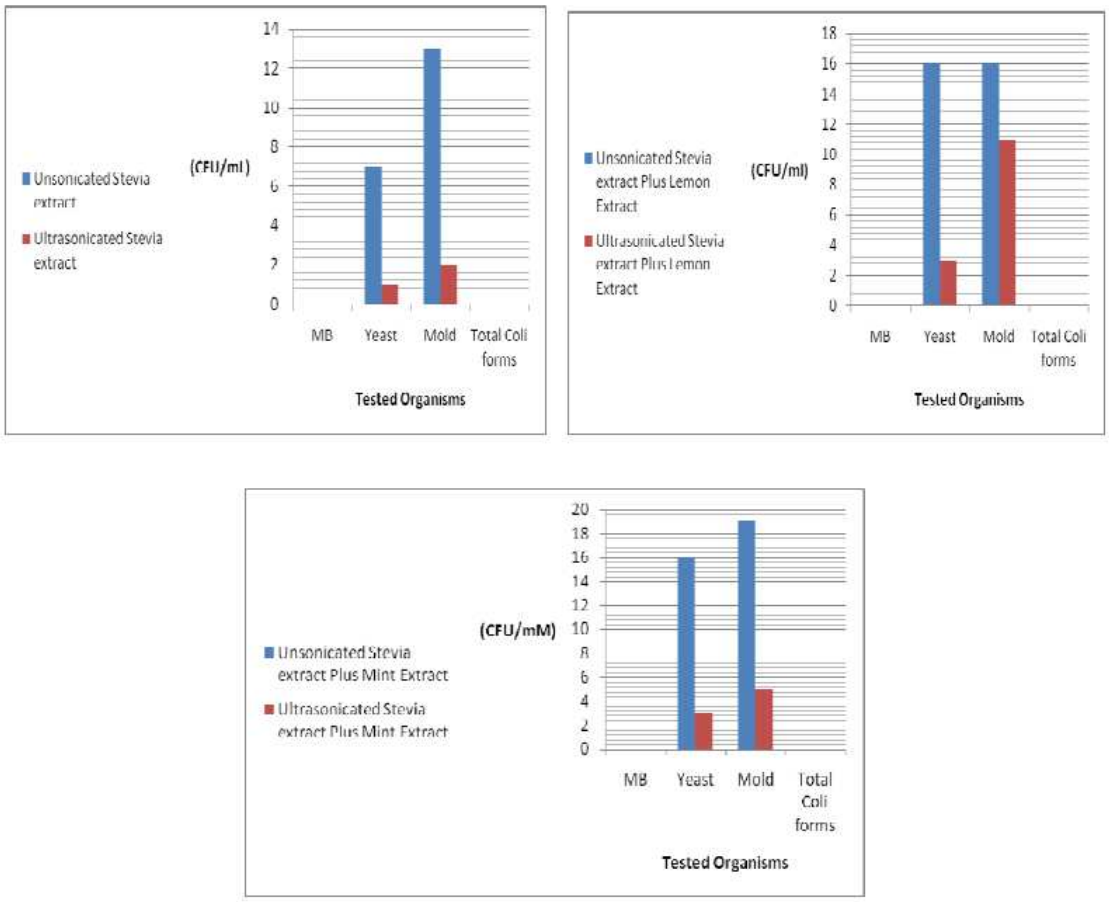

Figure 5: The effect of ultrasonication on microbial content at Four weeks storage period

The results of Yeast values (CFU/ml) were recorded in the unsonicated extracts of stevia plus lemon or stevia plus mint as 16 (CFU/ml), while stevia extract recorded 7 CFU/mL. These values of Yeast were changes as a result of ultrasonicated process to reach 3 CFU/mL for stevia plus lemon or stevia plus mint; and 1 CFU/mL, for the extracts of stevia. Data presented in Table 1 and shown in Figures (2 - 5) clearly indicate that the Ultrasonication and the adding materials (Mint & Lemonade) had a significant effect on the microbial populations occurred in the tested samples during the storage periods. Our results are in same trend with those found by Srikanth et al. [13] where the TPC, yeast and mold count were significantly decreased at ($p \leq 0.01$) level as the Aloevera extract percentage increased. For all the tested samples, Yeast and mould count, total plate count, and Coli forms, showed a respectable range of microbial growth till 30th day in reference to the Egyptian Standard Specifications. This study indicates the possible antimicrobial eventuality of the *S. rebaudiana* leaf extracts. Generally, decrease in total microorganism count of mould or yeast found during this study was because of the ultrasound method that creates cavitations as a result of changes in pressure and thus leading to destruction of microorganism. In this respect, Herceg et al. [17] mentioned that the high - power ultrasound (20 kHz, 42 W, 120 µm amplitude, for 9 min at 20°C) on Aspergillus spp. and Penicillium spp. in pure culture medium could reduce the count of P. expansum by 1.26 log, which is in agreement with our results. The mechanism of microbial destruction is mainly due to thinning of cell membranes, localized heating and production of free radicals [14, 15]. The study carried out by Dolatowski et al [16] proved that ultrasound processing has significant influence on microbiological contamination. Considering the previous studies that support our findings about the potential effect of ultrasound process in reducing the rate of microbial load, Cameron et al. [18] in milk showed that ultrasound process (20 kHz, 750 W) for

10 min could decrease an initial microbial load of 1×10^4 cfu/mL containing 4% fat and in normal saline to zero. The greatest reduction by sonication, on the growth of E. coli O157:H7, S. aureus, P. chrysogenum, and Cl. sporogenes at 20, 40 and 60 kHz was observed at 60 kHz. [19] . The rate of inhibition on microorganisms by ultrasound depends on some factors, including the initial microbial load of the microorganisms [20; 21]. Also, the viscosity of the medium, as indicated by the studies of Arroyo and et al [22].

4. Conclusion

In conclusion, it can be said that, S. rebaudiana Bertoni leaves have a profile as a healthy sweetener and are an important part of a healthy and nutritious diet. Also, treating the extracts under study using ultrasound technology had a positive effect in reducing their microbial load during different storage periods, which heralds the possibility of using this technology in food preservation and this encourage its use as one of the important means of food preservation in the future . In addition to that it is non -toxic, environmentally friendly and an emerging green technology because it increases production and saves a lot of energy.

5. References

[1] Zeuthen P, Ohlsson T, Bengtsson N. Safety criteria for minimally processed foods. Minimal processing technologies in the food industry. 2002; 196 -218.

[2] Bogala Madhu, M. Sai Srinivas, G. Srinivas and S. K. Jain. Ultrasonic Technology and Its Applications in Quality Control, Processing and Preservation of Food: A Review. Current Journal of Applied Science and Technology 32(5): 1-11, 2019.

[3] Yusaf T, Al -Juboori RA. Alternative methods of microorganism disruption for agricultural applications. Applied Energy. 2014; 114: 909 –923.

[4] Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of childhood and adult obesity in the United States, 2011 –2012. J Am Med Assoc. 2014; 311:806 –14.

[5] Diabetes data and statistics. http://www.cdc.gov/diabetes/data/. Accessed 27 Mar 2016.

[6] Nabors LO. Consumer perceptions of products containing sweeteners in USA. World Rev Nutr Diet. 1999; 85: 164 –70.

[7] Soejarto DD, Compadre CM, Medon PJ, Kamath SK. Potential sweetening agents of plant origin. II. Field search for sweet -tasting stevia species. Econ Bot. 1983; 37: 71 –9.

[8] Espinoza MI, Vincken J, Sanders M, Castro C, Stieger M, Agosin E. Identification, quantification, and sensory characterization of steviol glycosides from differently processed stevia rebaudiana commercial extracts. J Agric Food Chem. 2014; 62:11797 –804.

[9] Misra H, Soni M, Silawat N, Mehta D, Mehta BK, Jain DC. Antidiabetic activity of medium -polar extract from the leaves of Stevia rebaudiana Bert. (Bertoni) on alloxan -induced diabetic rats. J Pharm Bioallied Sci. 2011; 3:242 –8.

[10] Prakash I, Markosyan A, Bunders C. Development of next generation stevia sweetener: rebaudioside M. Foods. 2014; 3: 162 –75.

[11] American Public Health Association (APHA). Compendium of methods for the Microbiological Examination of Foods, fourth ed Washington, DC: American Public Health Association; 2001.

[12] International Organization for Standardization. Part 1:colony count technique in products with water activity greater than 0.95. Geneva: International Organization for Standardization; 2008. Microbiology of food and animal feeding stuffs —horizontal method for the enumeration of yeasts and moulds; p. 8. (ISO 21527 -1:2008). [Google Scholar]

[13] Srikanth, K., Kartikeyan, S and Adarsha, M. K. 2017a. Storage Studies of Aloe vera Extract Incorporated Peda. Intl. J. Food. Ferment. Technol. 7(2): 231 -239.

[14] Fellows, P.J. 2000. Food processing technology: Principles and practice, CRC Press, North America.

[15] Butz, P. and Tauscher, B. (2002) Emerging Technologies: Chemical Aspects. Food Research International, 35, 279 -284.

[16] Dolatowski, Z.J., Stadnik, J. and Stasiak, D. 2007. Applications of ultrasound in food technology. Acta Sci. Pol. Technol. Aliment. 6(3): 89 -99.

[17] Herceg, Z., A. R. Jambrak, V. Lelas, and S. M. Thagard. 2012. The effect of high intensity ultrasound treatment on the amount of Staphylococcus aureus and Escherichia coli in milk. Food Technol. Biotechnol. 50:46 –52.

[18] Cameron, M., L. D. McMaster, and T. J. Britz. 2009. Impact of ultrasound on dairy spoilage microbes and milk components. Dairy Sci. Technol. 89:83 –98.

[19] Jalilzadeh A., J. Hesari, S. H. Peighambardoust, and I. Javidipour. The effect of ultrasound treatment on microbial and p.J. Dairy Sci. 101:5809 –5820 https://doi.org/10.3168/jds.2017 -14352 .

[20] Tsukamoto, I. 2004. Inactivation of Saccharomyces cerevisiae by ultrasonic irradiation. Ultrason. Sonochem. 11:61 –65

[21] Hunter, G., M. Lucas, I. Watson, and R. Parton. 2008. A radial mode ultrasonic horn for the inactivation of Escherichia coli K12. Ultrason. Sonochem. 15:101 –109.

[22] Arroyo, C., G. Cebrian, R. Pagan, and S. Condon. 2011. Inactivation of Cronobacter sakazakii by manothermosonication in buffer and milk. Int. J. Food Microbiol. 151:21 –28.