Evaluation of Miswak antibacterial activity and its addition on chocolate milk sensory properties

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Abstract: The Antibacterial activity of ***Salvadora persica*** L (Miswak) aqueous extract (SPAE) against some pathogenic bacteria using agar diffusion method was evaluated. Also, the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) were determined. Chocolate milk treatments by adding the appropriate MLC of SPAE for each tested pathogenic bacteria were prepared and evaluated for their sensory properties. The results showed that the 400mg/ml of (SPAE) was the most effective against all tested pathogenic bacteria. The lowest MIC value and MLC value were noticed for *E. coli* (1.56 mg/ml and 3.12 mg/ml), respectively. The results of chocolate milk showed that the treatment 4 (Chocolate milk supplemented with 12.5 mg/ml of (SPAE)) recorded the lowest aroma and taste scores. No significant differences among the control, T2 (3.12 mg/ml of SPAE) and T3 (6.25 mg/ml of SPAE) as respect to tested sensory properties. Therefore, the SPAE can be used as a natural preservative in dairy industry.

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Key words: Miswak, agar diffusion method, MIC, MLC, Chocolate milk

1. Introduction:

The toothbrush tree, *Salvadora persica* L, also called Miswak, belonging to the Salvadoraceae family, is one of the most important among 182 species of plants being used as chewing sticks. It has been widely used in many Asian, African, and Middle Eastern countries. The roots, twigs, and stems of this plant have been used for oral hygiene and small miswak sticks have been used as toothpicks for maintaining oral hygiene (Sher *et al*., 2011).

Using Miswak as a chewing stick is highly recommended as a Sunnah practiced by the Prophet Mohammad (peace be upon him) and his companions to achieve daily dental care, and the prophet emphasised the importance of using Miswak for oral hygiene (Riggs *et al*., 2012).

Previous in vitro studies have reported the antibacterial and antifungal effects of miswak on cariogenic bacteria and periodontal pathogens including *Staph. aureus*, *Streptococcus mutans*, *St. faecalis*, *St. pyogenes*, *L. acidophilus*, *Ps. aeruginosa*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Haemophilus influenzae*, and *C. albicans* (Naseem *et al*., 2014). Al-Bayati and Sulaiman (2008), investigated the antimicrobial activities of the aqueous and methanol extracts of *Salvadora persica L*., against 7 isolated oral pathogens: *Staph. aureus*, *St. mutans*, *St. faecalis*, *St. pyogenis*, *Lactobacillus acidophilus*, *Ps. aeruginosa*, and *Candida albicans*. They found that the aqueous extract inhibited all isolated microorganisms, especially the *Streptococcus* species, and was more efficient than the methanol extract. Al-Lafi and Ababneh (1995), reported that extracts of miswak posses various biological properties, including significant antibacterial effect.

Abhary and Al-Hazmi (2016) indicated that Miswak contains more than one type of antimicrobial agent that inhibits the growth of both Gram positive and negative bacteria. The zone of inhibition for three different extracts was measured in *E. coli*, *Staph. aureus*, *L. acidophilus*, *St. mutans* and *Ps. aeruginosa*; the results showed a strong antimicrobial activity in the aqueous extract and less activity in alcoholic and nonpolar extracts.

No studies on the antibacterial activity of Miswak against non-oral pathogens were found. Therefore, this study was carried out to (1) evaluate the antibacterial activity of Miswak against some non-oral pathogens and (2) study the effect of adding Miswak on chocolate milk organoleptic properties.

2. Materials and methods:

1- Pathogenic bacteria:

Staph. saprophyticus ss saprophyticus and *E. coli* were isolated from Ras cheese and raw milk and identified using Biolog system in former study (Yassin,2018*). Ps. aeruginosa sh* (28) was obtained from Agricultural Microbiology Department, Faculty of Agriculture, Cairo University

2-Miswak Collection:

Salvadora persica chewing sticks were purchased from the local market of El-Fayoum City, Egypt, which imported from Pakistan by Al-Falah IMPEX for processors, Packers and Exporters Company. The sticks were washed with distilled water, cut into small pieces, and allowed to dry at room temperature for 2 weeks. Then, they were ground to powder using electrical blinder.

3-Extracts Preparation:

Preparation of aqueous extract was carried out by mixing 100 g of *Salvadora persica* powder with 1 L of distilled water for 24 h. The mixture was then filtered using Whatman No. 1 filter paper, and the filtrate was then evaporated in vacuum evaporator at 60◦C (Darmani *et al*., 2003). Before testing, the miswak extracts were freshly reconstituted in water at a final concentration of 400mg/ml which was used for preparing serial dilutions (400–50mg/ml).

4-Antibacterial Activity of Miswak

The antibacterial activity of Miswak extracts was examined using the agar diffusion, minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) methods.

4-A-Agar Diffusion Method

The antibacterial activity was performed on Nutrient agar plates using the agar diffusion method. Briefly, 1 ml of bacterial suspension was added to 100 ml of dissolved Nutrient agar flask, and then the medium was poured with the bacterial suspension in sterile plates. The required number of wells, each 5 mm in diameter, were cut out of the agar using a sterile glass capillary ensuring proper distribution of holes in the periphery and one in the center for each agar plate. Then, wells were filled with 50 μL of sterile aqueous extract made from *Salvadora persica* stock solution (50, 100, 200, and 400mg/mL). This was followed by 2 h pre-incubation at cooling temperature for proper diffusion of the plant extract into the media. Then, the plates were incubated at 37◦C (*Staph. saprophyticus ss saprophyticus* and *E. coli*) and 30◦C (*Ps. aeruginosa sh* (28)) for 24h (Karou, *et al*., 2005). The diameters of inhibition zones were measured after incubation time.

4-B- Determination of MIC and MLC of the aqueous extract of *Salvadora persica* on pathogenic bacteria

MIC and MLC values of the aqueous extract of *Salvadora persica* were determined by broth dilution method (Ellen *et al*., 1994), using *E. coli, Staph. saprophyticus ss saprophyticus* and *Ps. aerogenosa* as indicator microorganisms.

In this method, a standard known count of the tested organism used, which was visually determined in a liquid culture comparing the turbidity of the liquid medium to a standard that represents a known number of bacteria in suspension.

Turbidity standards can be prepared by mixing chemicals that precipitate to form a solution of reproducible turbidity. Such solutions, using barium sulphate for example, were developed by MacFerland to approximate number of bacteria in solution of equal turbidity as determined by colony count.

a- Preparation of standard barium sulphate suspension

A chemically induced precipitation reaction can be used to approximate the turbidity of bacterial suspension:

- Set up 10 new, cleaned and rinsed test tubes of equal size and of good quality.
- Prepare 1% chemically pure sulphuric acid and 1.175% aqueous of barium chloride ($BaCl_2.2H_2O$).
- Slowly and with constant agitation, add the designated amounts of the two solutions to the tubes as shown in the test to make a total of 10 ml per tube.
- Seal the tubes. The suspended barium sulphate precipitate corresponds approximately to homogenous bacteria cell densities per milliliter throughout the range of standards.
- Store the tube in the dark at room temperature. They should be stable for 6 months.

Cell density proportional to $BaCl_2.2H_2O$ standard (W.L610nm)

Tube number	0.5	1	2	3	4	5	6	7	8	9	10
$BaCl_2.2H_2O$ (ml)	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
H_2So_4(ml)	9.95	9.90	9.80	9.70	9.60	9.50	9.40	9.30	9.20	9.10	9.00
Approximate cell density ($x10^8$/ml)	1.5	3	6	9	12	15	18	21	24	27	30

b- Broth dilution method for determination of the MIC and MLC of the aqueous extract of *Salvadora persica*

Inoculum Preparation:

All bacterial isolates were grown to the exponential phase in Nutrient broth medium at 37◦C for 18h. The bacterial growth was estimated as turbidity using spectrophotometer to measure the light absorption of the microbial mass as determined by the optical density readings at 620 nm (OD_{620}). Growth was checked every 30 minutes, and the exponential phase of bacterial growth was identified by the increased OD_{620} reading. Then, the inoculum density of each bacterial suspension was adjusted to a final

density equivalent to 0.5 McFarland Standard (1x 10^6 CFU/mL) in sterile Nutrient broth medium.

For broth dilution method:

Different concentrations of the aqueous extract of *Salvadora persica* prepared in serial two fold concentration. Then, placed in tubes of Nutrient broth medium. The aqueous extract was preceding by prepared in concentrated solutions and then diluted to the appropriate concentrations in broth.

To perform the classic broth dilution test, standard inocula of the organism are added to an equal volume (often 1 ml) of each concentration of aqueous extract of *Salvadora persica* and to a tube of the growth medium without aqueous extract of *Salvadora persica* which serves as a control. Notice, that adding a bacterial suspension will dilute both the suspension and the concentration of aqueous extract of *Salvadora persica* in the tube; this must be taken into account during preparation of inocula and the dilutions of aqueous extract of *Salvadora persica*. An inoculated tube of medium is incubated to serve as a negative growth control. In the present experiment one ml inocula of bacterial suspension was placed in one ml of double strength medium contains aqueous extract of *Salvadora persica* in serial two-fold dilutions (0.049, 0.098, 0.195, 0.390, 0.781, 1.5625, 3.125, 6.25, 12.5, 25 mg/ml broth medium).

After 24 hours incubation, the tubes were examined for turbidity, indicating growth of microorganism. The organism grown in control tube and in any other tube that does not contain enough aqueous extract of *Salvadora persica* (antimicrobial agent) to inhibit growth. The lowest concentration of the aqueous extract of *Salvadora persica* that inhibits growth of the organism, as detected by lack of visual turbidity (matching the negative growth control), is designated the minimum inhibitory concentration (MIC).

The (MIC) measures the ability of the aqueous extract of *Salvadora persica* to inhibit multiplication of the organism. Thus, the organisms in the inocula may be merely inhibited by the aqueous extract of *Salvadora persica* and will be able to recommence growing if the aqueous extract of *Salvadora persica* influence is removed. In this case the aqueous extract of *Salvadora persica* is inhibitor. For certain cases it is important to determine the ability of an agent to actual killing the microorganism. To measure the ability of the aqueous extract of *Salvadora persica* to kill the microorganism, the test was performed using a modification of the broth dilution test. When the initial microorganism suspension is being inoculated into the tubes of broth, a known portion is removed for the growth control tube immediately after it was inoculated and this aliquot is plated to Nutrient agar for determination of actual CFU in the inocula, 0.001 ml of the growth control tube can be subcultured in plate for CFU. If the organism concentration is 5x105/ml in the tested tube, there should be around 250 CFU on the sub culture plate made from a 1:2 dilution of the growth control tube (0.001 of suspension of $2.5x10^5$ organisms contains $2.5x10^2$ organisms). The 1:2 dilutions are necessary in order to be able to count the colonies on the plate (30-300 CFU).

A known quantity (0.1 ml) of inocula from each of the tubes of broth that showed no visible turbidity after 24 hours incubation is subcultured to Nutrient agar plates. The small amount of the antimicrobial agent (aqueous extract of *Salvadora persica*) that is carried over with these inocula is easily removed by diffusion into the agar and the effect is negated by spreading the inocula over a large area. The number of colonies that grow on this subculture after overnight incubation is then counted and compared to the number of CFU/ml in the original inocula. Within those tubes that showed no turbidity, microorganisms were either still viable or they were killed by the aqueous extract of *Salvadora persica* (antimicrobial agent). The lowest concentration of aqueous extract of *Salvadora persica* that allowed less than 0.1% of original inocula to survive is said to be minimum lethal concentration (MLC).

5- Examination the influence of *Salvadora persica* aqueous extract in killing the pathogenic bacteria in milk:

1- Three sterile conical flasks, each containing 100 ml of sterile milk were inoculated with one of the tested pathogenic bacteria (*Staph. saprophyticus ss saprophyticus*, *E. coli* and *Ps. aeruginosa sh (28)* with count (~1 x 10^6/ml).

2- Aqueous *Salvadora persica* extract was added with appropriate MLC of each tested pathogenic bacteria.

3- One ml from each conical flask, containng *Salvadora persica* aqueous extract and the pathogenic microbe, was inoculated in Nutrient agar plates.

4- Inoculated plates were incubated at 37^{o}C for 24 hours.

6-Manufacture of Chocolate Milk (El- Hagarawy, 1963):

(6% w/v) sucrose (Al- Zhor factory, Fayoum governorate, Egypt), (1.5% w/v) Coccoa and (0.4% w/v) gelatin (Queen company, 6 th October, Egypt) were added to cow milk (Faculty of Agriculture, Fayoum Univ., Egypt., moisture86.50%, fat 4.39%, protein 3.30%, lactose 4.44%, solids not fat 9.11% and ash 0.73%). All these ingredients were mixed, pasteurized at 72 ^{o}C /15 s and cooled to 5^{o}C.

Four treatments of chocolate milk were prepared after cooling. Chocolate milk without *Salvadora persica* aqueous extract (control, T1) and the other

three chocolate milk treatments were prepared by adding the appropriate Miswak MLC for each tested pathogenic bacteria.

7-Organoleptic properties:

The organoleptic properties of chocolate milk treatments were evaluated by 13 panels of members of Microbiology Department and Dairy Science Department, Faculty of Agriculture, Fayoum University.

Chocolate milk treatments were evaluated according to the score card sheet of **Morais *et al.* (2014).** The total score (50 points) was divide into 10 points for Appearance, 10 points for Aroma, 10 points for Taste, 10 points for Texture and 10 points for Overall impression.

8-Statistical analysis:

Data were statistically analyzed using General Linear Models procedure of Statistical Package for Social Sciences (SPSS) Version 17.0.0 software. Duncan's (1955) multiple range tests were used to compare between the means (SPSS, 2008).

Results and discussion:

Antibacterial activity of *Salvadora persica* aqueous extract (SPAE) (L. Miswak) as a natural preservative against some pathogenic bacteria

The antibacterial activities of (SPAE) against the three pathogenic bacteria *(Staph. saprophyticus, Ps. aeruginosa* and *E. coli)* were studied using the well diffusion agar method. As shown in Table (1) 50, 100, 200 and 400 mg/ml of (SPAE) were used against the three pathogenic bacteria. The 400mg/ml of (SPAE) was the most effective against all strains (Photo. 1). The highest growth inhibition was recorded against *Ps. aeruginosa* followed by *Staph. saprophyticus* and *E. coli.*

Table (1) The diameter of inhibition zones (mm).

bacterial isolates	Aqueous extract (mg/mL)			
	50	100	200	400
Staph. saprophyticus	N.D	N.D	5	7
E. coli	N.D	N.D	3	6
Ps. aeruginosa	N.D	N.D	6	11

N.D= Not Detected

Antibacterial resistance has always been a global health concern challenging treatment of human infections caused by bacterial pathogens (Magiorakos *et al*., 2012). This problem has opened a wide range of research studies investigating the possible use of natural plant extracts in traditional medicine. In the present study, three pathogenic bacteria were used in screening antimicrobial activity of aqueous and extract of *Salvadora persica*. In this study, the 400mg/mL of Miswak extracts (SPAE) represent the most effective one on all the three pathogenic bacteria, this result is in agreement with previous findings from other studies as Al-Bayati and Sulaiman (2008), Al-Sieni (2014), Naseem *et al*. (2014) and Al-Ayed *et al*. (2016), they investigated that the aqueous extract inhibited microorganisms and was more efficient than the methanol extract. It is well known that the antimicrobial property of *Salvadora persica* extracts is attributed to the different phytochemical constituents.

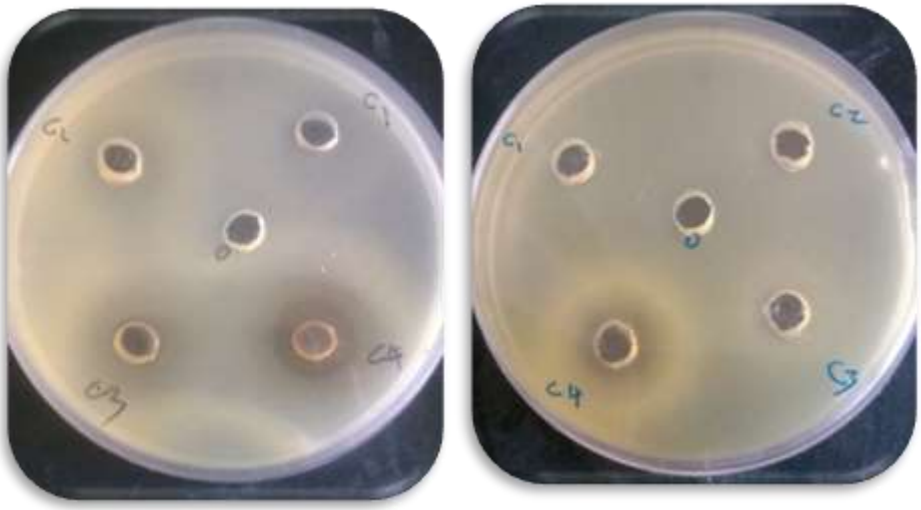
Mohammed (2013) investigated the phytochemical constituents of *Salvadora persica* extracts and revealed the presence of flavonoids, sterols, saponins, tannins, basic alkaloids.

Sofrata *et al*. (2011) identified a volatile compound: benzyl isothiocyanate (BITC) in *Salvadora persica* extracts, that BITC exhibited rapid and strong bactericidal effect against Gram-negative bacteria but low effect on Gram-positive bacteria. They speculated that BITC might penetrate through the outer bacterial membrane and possibly interfere with the bacterial redox systems and thus hamper the ability of the bacterium to maintain its membrane potential.

The MIC values of aqueous extract are presented in Table (2). The lowest MIC value and MLC value was seen for *E. coli* (1.56 mg/ml and 3.12 mg/ml) respectively, followed by *Staph. saprophyticus* (3.12 mg/ml and 6.25 mg/ml) respectively. The highest MIC and MLC value was recorded for *Ps. aeruginosa* (6.25 mg/ml and 12.5 mg/ml) respectively.

Table (2) The MIC and MLC values of (SPAE) against the undesirable microorganisms.

bacterial isolates	MIC values (mg/mL)	MLC values (mg/mL)
Staph. saprophyticus	3.12	6.25
E. coli	1.56	3.12
Ps. aeruginosa	6.25	12.5



(**SPAE) against *St. saprophyticus*** **(SPAE) against *E.coli***

(**SPAE) against *Ps. aeruginosa***
Photo (1): Antimicrobial effect of (SPAE)

In this study, the aqueous extract of *Salvadora persica* had promising MIC values against *E. coli* and *Ps. aeruginosa* in agreement with Al-Ayed *et al*. (2016), but the MIC value of *Staph. saprophyticus* is lower than methicillin-resistant *Staphylococcus aureus* (MRSA) value as obtained by Al-Ayed *et al*. (2016) study. Previous studies have reported that *Salvadora persica* extracts were effective against *Staph. aureus* and *Ps. aeruginosa* (Mohammed, 2013 and Alireza *et al*., 2014).

This finding is of considerable concern. These emerging pathogens become a significant health problem because of their remarkable ability to innate and acquire resistance to multiple antimicrobial classes and to survive in nosocomial environments (Elabd *et al*. 2015).

Antibacterial effect of *Salvadora persica* aqueous extract (SPAE) on pathogenic bacteria in milk:

Table (3) illustrates the antibacterial effect of *Salvadora persica* aqueous extract (SPAE) on pathogenic bacteria in milk.

Table (3): Antibacterial effect of *Salvadora persica* aqueous extract (SPAE) on pathogenic bacteria * in milk:

Treatments	pathogenic bacteria count CFU/ml
A	N.D
B	N.D
C	N.D

*****Initial count of pathogenic bacteria: 10^6 CFU/ml
A: *E.coli* + 3.12 mg SPAE/ml
B: *Staph. saprophyticus*+ 6.25mg (SPAE)/ml
C: *Ps. aeruginosa sh* (28)+ 12.5mg SPAE/ml
N.D= Not detected

Organoleptic properties of chocolate milk with *Salvadora persica* aqueous extract (SPAE) (L. Miswak)

Table (4) and Fig (1) illustrates the organoleptic properties of chocolate milk with *Salvadora persica* aqueous extract (SPAE) (L. Miswak)

Addition of (SPAE) to chocolate milk had effect on aroma, taste and overall impression, while no significant effect as respect of appearance and texture.

Table (4): Organoleptic properties of chocolate milk with (SPAE)

Treatments*	Appearance	Aroma	Taste	Texture	Overall impression
T_1	9.23[a]	9.00[a]	9.23[a]	9.23[a]	8.85[a]
T_2	9.23[a]	9.23[a]	9.38[a]	9.15[a]	9.00[a]
T_3	9.31[a]	8.54[a]	8.54[a]	9.23[a]	8.46[a]
T_4	9.15[a]	8.00[b]	7.31[b]	9.00[a]	6.92[b]
SE±	0.14	0.13	0.29	0.12	0.24
Sig.	NS	*	***	NS	***

***T1=** Chocolate milk control without (SPAE)
***T2 =** Chocolate milk with adding 3.12 mg/ml of (SPAE)
***T3=** Chocolate milk with adding 6.25 mg/ml of (SPAE)
***T4=** Chocolate milk with adding 12.5 mg/ml of (SPAE)
***SE=** Standard Error
***Sig**= Significant
***NS=** Non- significant

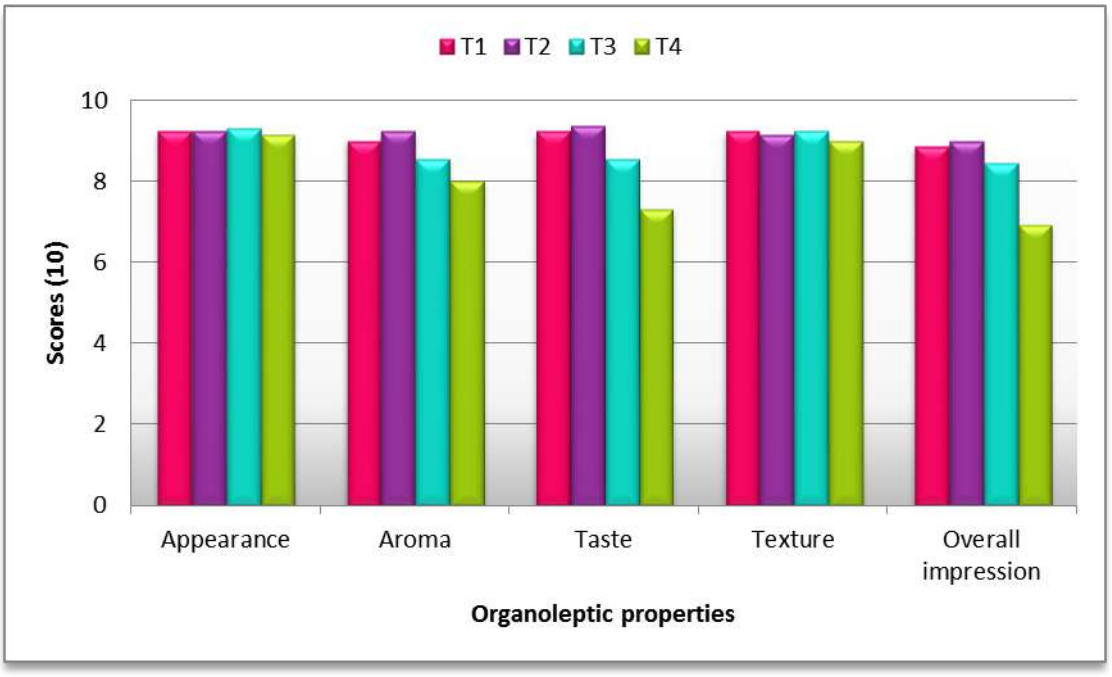


Figure (1): Organoleptic properties of chocolate milk with (SPAE)

The highest aroma score was recorded by chocolate milk supplemented with 3.12 mg/ml of (SPAE) (T2) of 9.23 points, whereas T4 (chocolate milk supplemented with 12.5 mg/ml of SPAE) recorded the lowest score of 8.00 points. There was insignificantly effect on taste between T1 (chocolate milk without additive SPAE), T2 (chocolate milk supplemented with 3.12 mg/ml of SPAE) and T3 (chocolate milk supplemented with6.25 mg/ml of SPAE). T1, T2 and T3 recorded the highest score points of taste (9.23, 9.38 and 8.54), while T4 (chocolate milk supplemented with12.5 mg/ml of SPAE) significantly recorded the lowest point 7.31 on taste.

There was insignificant effect on overall impression between T1, T2 and T3 (T1 chocolate milk without addition SPAE), T2 (chocolate milk supplemented with 3.12 mg/ml of SPAE) and T3 (chocolate milk supplemented with6.25 mg/ml of SPAE), T1, T2 and T3 both of them recorded the highest score points of the overall impression (8.85, 9.00 and 8.46), whereas T4 (chocolate milk supplemented with 12.5 mg/ml of SPAE) recorded the lowest score of 6.92 points.

So, there was an insignificant effect for addition of SPAE to chocolate milk either on appearance or texture.

Conclusion:

Salvadora persica L (Miswak) aqueous extract (SPAE) showed antibacterial effect at high tested concentrations and the 400mg/ml of (SPAE) was the most effective against all tested pathogenic bacteria. The lowest MIC value and MLC value was seen for E. coli (1.56 mg/ml and 3.12 mg/ml), respectively. No significant differences among the control, T2 (3.12 mg/ml of SPAE) and T3 (6.25 mg/ml of SPAE) as respect of tested sensory properties. Therefore, the SPAE can be used as a natural preservative in dairy industry.

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