Antimicrobial Activity of Dried Spearmint and Its Extracts for Use as White Cheese Preservatives

Mervat I. Foda[1], M. A. El-Sayed[1]., Marwa M. El-Moghazy[1], Amal- A. Hassan[2] & Nagwa M. Rasmy[2]

[1] Dairy Science Dept., National Researh Center, Dokki, Cairo, Egypt.

[2] Food Science and Technology Dept., Fac. of Agric. Ain Shams Univ., Shubra El-Khema, Egypt.

ABSTRACT

Spearmint which is common herb in the Mediterranean region was evaluated (dried or extracts) as inhibitor factor against the growth of twelve microbial strains belonging to bacteria, yeasts and moulds using filter paper disc agar diffusion technique. Water extract had no inhibitory effect on all strains under study. A moderate activity of ethanolic extract against Gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) was observed. Essential oil was the strongest antimicrobial inhibitor; therefore, its antifungal activity and minimum inhibitory concentration (MIC) were examined. To evaluate the antimicrobial activity of spearmint, white cheeses were prepared using different concentrations of dried spearmint and its essential oil. Such an oil showed significant reduction in the counts of total bacteria, proteolytic and psychrotrophic bacteria as compared with control samples. Moulds and yeasts were totally inhibited by using the essential oil.

Keywords: antimicrobial activity; spearmint (Mentha spicata); natural essential oil; herbs extracts; white cheese

INTRODUTION

Currently, there is a strong debate about the safety aspects of chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity. For these reasons, consumers tend to be suspicious of chemical additives and thus the demand for natural and socially more acceptable preservatives has been intensified (Skandamis *et al*., 2001). Many spices and herbs exert antimicrobial activity due to their essential oil fractions (Daferera *et al*., 2003). The most common herb in the Mediterranean region is spearmint (*Mentha spicata*), it is widely used as a source of essential oils for flavouring and recently has been used as a valuable source of the potent antioxidant for the nutraceuticals and cosmetic industries.

The aim of the present work was to evaluate the antimicrobial activity of dried spearmint and its extracts (essential oil, alcoholic and water extracts), and select the strongest microbial inhibitor to apply in dairy products such as white cheese.

MATERIALS AND METHODS

Materials

Dried spearmint (*Mentha spicata*) was obtained from Medicinal and Aromatic Plant Research Dept., Agriculture Research Center, Giza, Egypt. Buffalo's milk retentate was obtained from Dairy Industry Unit, Animal Production Research Institute, Ministry of Agriculture, Cairo, Egypt. The milk retentate contained 29.2% total solids; 15.5% fat; 10.5% total protein and 0.09%. titratable acidity. Microbial rennet (*Mucor mehiei*) was obtained from Novo, Denmark.

Microorganisms: Twelve microbial strains were used, these include two Gram-negative bacteria *Pseudomonas fluorescenes* NRRL 800 and *Escherichia coli* DSM 498, two Gram-positive bacteria: *Bacillus cereus* NRRL 3711 and *Staphylococcus aureus* ATCC 29213, and three yeasts:, *Candida lipolytica* NRRL 2034, *Pichia anomala* NCY 20 and *Saccharomyces cerevisiae* NRRL 1095, and five moulds, *Aspergillus niger* ATCC 102, *Aspergillus flavus* ATCC 247, Penicillium expansum ATCC 28877, *Geotricum candidum* NRRL 552, and *Fusarium moniliform* ATCC 206. These strains were obtained from the Microbiological Resources Center (MIRCEN), Fac. of Agric., Ain Shams Univ., Cairo, Egypt. Bacterial strains were cultured overnight at 37°C in Nutrient Broth, yeast strains were cultured overnight at 30°C in Malt Extract Broth; whereas moulds were cultured for 72 hrs at 27°C on Potatoes Dextrose Agar.

Methods

Extraction of the essential oil: Essential oil was extracted from dried spearmint by hydro distillation using a Clevengar-type apparatus according to the method described by Tepe *et al*., (2005). The GC-MS analysis of the essential oil was performed using a Varian gas chromatograph interfaced to a Finnigan SSQ 7000, England. Mass Selective Detector (MSD) with ICIS V2.0 data system for MS identification of the GC components. The separated components were identified by using National Institute of Standards and Technology (NIST) mass spectral library data n-alkans were used as reference points in the calculation of relative retention indices (RRI) for all volatile constituents. The quantitative determination was carried out based on peak area integration.

Preparation of alcoholic and water extracts: Ethanolic extract was prepared according to Puatanachokchai, *et al*., (2002). While, water extract was prepared according to Kansoh, (2001). Both extracts were lyophilized using a lyophilizer (Snijders type 2040, Netherlands).

Determination of antimicrobial activity: Antimicrobial activities of the various extracts of dried spearmint were determined by disc diffusion method as described by Sokmen, *et al*., (2004). Sterile paper discs (6 and 14 mm) were impregnated with 15µl of essential oil and 200 mg of the extracts respectively. The inoculated plates were incubated for 24 hrs at 37°C for bacteria, 48 hrs and 7 days at 30°C for yeasts and fungi respectively, then the inhibition zones of the microbial growth produced by different extracts were measured.

Determination of minimum inhibitory concentration (MIC): Spearmint essential oil at cncentrations of 0.4 to 5µl /ml were thoroughly mixed with sterilized nutrient agar (10 ml), then poured into Petri dishes and inoculated by streaking with suspension of each microorganism (Farag *et al*., 1989). The plates were incubated for 48 hrs at 37°C for bacteria and 30° C for yeast, and 7 days for moulds at 28°C and the lowest concentration of the extract required to inhibit the growth of the tested microorganism was designated as the MIC. Both microbial analyses were performed in triplicates.

Preparation of UF-white cheese with spearmint essential oil: White cheese was prepared according to Foda *et al*., (2006). Milk retentate was divided into 7 portions; each portion (1.0kg) was salted to a concentration of 3%, well mixed and pasteurized at 73°C for 15 sec. First portion was served as control and for the other six portions, different concentrations of spearmint essential oil (0.5, 0.75, 1.0, 1.5, 2.0 and 2.5 ml / kg retentate) were added at 40°C to prepare cheese treatments A, B, C, D, E and F, respectively. Curds were hold at 40°C for 30 min. in plastic containers after adding the rennet. All cheese samples were stored under refrigerator temperature (5°C±2) for 5 weeks. Samples were taken fresh and every week for analysis.

Preparation of UF-white cheese with dried spearmint: Milk retentate was divided into 4 portions; the first was served as control. Three different levels of dried spearmint, 0.25, 0.5 and 0.75 % were added before pasteurization to the three portions. Further steps were done as mentioned before with spearmint essential oil cheese.

Three replicates were prepared for each cheese treatment to determine their microbial contents.

Microbiological analysis of cheese: White cheese samples (11 g) were homogenized for 1 min. with 99 ml of sterilized tri-sodium citrate (2 % w/v). Decimal dilutions were prepared in 9 ml sterile Na Cl (0.85%). Microorganisms' content of cheese samples were determined as follow: Total viable counts were enumerated on Nutrient Agar following the pour plate method, incubated at 37°C for 48 hrs according to APHA (1992). For Coliforms, Violet Red Bile Agar was used according to Marshall (1992), the plates were incubated at 37°C for 24 hrs. Yeasts and moulds were counted on Potato Dextrose Agar after pH adjustment to 3.5, incubated at 25°C for 4-5 days. Aerobic psychotrophic bacteria were determined on Plate Count Agar (Oxoid Ltd) incubated at 7°C for 7-10 days according to APHA (1992). Proteolytic bacteria were counted on supplemented Plate Count Agar with reconstituted skim milk (10%), incubated at 32°C for 48 hrs as described by Frank, *et al*. (1992).

Statistical analysis: Statistical analysis of experimental data was performed by analysis of variance (ANOVA) producers using SAS PROC GLM/ STAT (SAS, 1998). Differences among means were identified using Duncan multiple range test.

RESULTS AND DISCUSSION

Chemical composition of spearmint-essential oil

The components of spearmint essential oil are shown in Table (1). It could be noticed that twenty

two components with different amounts were identified. The main components were carvone (68.58 %) and limonene (16.42 %), followed by β- Pinene (2.29%) and β-bourbonene (2.08%), the others ranged from 0.09 to 1.51%. These results are in agreement with those obtained by Dimandja *et al*., (2000) and Marongiu *et al*., (2001), who found that carvone and limonene are the main components of spearmint essential oil.

Antimicrobial activity of spearmint extracts

The *in vitro* preliminary screening of the antimicrobial activities of spearmint water, ethanolic extract and essential oil were tested against strains of bacteria, yeasts and mould using filter paper disc agar diffusion technique, the results are shown in Table (2). Data indicate that spearmint extracts (water or ethanolic) had no effect on all microorganisms under study except the moderate activity of ethanolic extract against Gram positive bacteria (*B cereus* and *Staph. aureus*). Also, essential oil had no effect on *P. flourescens*, while, yeasts and *P. expansum* were the most sensitive microorganisms. These results are in agreement with those obtained by Tepe *et al*., (2004) and Tepe *et al*., (2005) who reported that essential oils of some aromatic plants had strong antimicrobial activity, while the water and methanol soluble extracts remained almost inactive. Also, Chao *et al*., (2000) found that plant essential oils including spearmint had a broad spectrum activity against different microorganisms such as B. cereus, *Staph. aureus*, *E. coli* and *A. niger*.

Minimum Inhibitory Concentration (MIC)

The measurements of MIC of spearmint essential oil are shown in Table (3). It could be seen that

Table 1: Chemical composition of the spearmint essential oil

No	Compounds	RT (min)	(%)
1	α-Pinene	8.29	1.34
2	Camphene	8.78	0.14
3	**ß -Pinene**	**9.63**	**2.29**
4	3-Octanol	10.8	0.38
5	α -Myrcene	9.96	0.65
6	**Limonene**	**11.57**	**16.42**
7	γ-Terpinene	12.05	0.28
8	*trans*-Sabinene hydrate	12.8	0.18
9	α- Terpinolene	13.08	0.12
10	1-4-Terpineol	15.78	0.38
11	Terpene -4-ol	16.73	0.45
12	**Carvone**	**18.77**	**68.58**
13	Carvone oxide	19.28	0.09
14	**ß- Bourbonene**	**21.51**	**2.08**
15	Dihydro-α–ionone	21.91	0.14
16	trans-Caryophyllene	22.51	1.51
17	ß- Cubebene	22.58	0.14
18	α - Humulene	23.09	0.12
19	epi-Bicyclosesquiphellandrene	23.39	0.79
20	γ-Muurolene	23.50	0.24
21	Germacrene-D	23.84	0.54
22	γ-Cadinene	24.56	0.13
23	Calmenene	24.75	0.66
24	Spathulenol	26.19	0.80
	Total		**98.45**

spearmint essential oil had strong activity (1.7-2.0 µl/ml) on yeast strains, followed by fungi strains (2.5 µl/ml), while, Staph. aureus was the most resistant microorganism to the essential oil (4.0 µl/ml).On the other hand, spearmint essential oil had no effect on the growth of P. fluorescene. These results are in agreement with those obtained by Pintore, *et al*., (2002) and Wilkinson *et al*., (2003) who reported that Gram negative bacteria appeared to be less sensitive to the action of essential oils. It could be concluded that antimicrobial activity of spearmint essential oil could be related to its chemical composition which contains high amount of carvone (68.58 %) and limonene (16.42 %). According to Cimanga, *et al*., (2002) and Daferera *et al*., (2003) who reported that the compounds presented in greatest proportions are not necessarily responsible for the greatest share of the total activity; the in-

Table 2: Antimicrobial activity of extracts and essential oil of the spearmint

Microorganisms	Inhibition zone diameter (mm)*		
	Water extract	Ethanolic extract	Essential oil
Pseudomonas fluorescens	-	-	-
Escherichia coli	-	-	14.6 ± 0.57
Bacillus cereus	-	30.3 ± 0.57	13.0 ± 1.00
Staphylococcus aureus	-	23.0 ±2.00	14.6 ± 0.57
Candida lipolytica	-	-	90.0 ± 0.0
Saccharomyces cerevisiae	-	-	40.0 ± 0.0
Pichia anomala	-	-	60.0 ± 0.0
Aspergillus niger	-	-	26.3 ± 3.51
Aspergillus flavus	-	-	27.6 ± 3.05
Penicillium expansum	-	-	90.0 ± 0.0
Geotricum candidum	-	-	90.0 ± 0.0
Fusarium moniliform	-	-	66.6 ± 5.7

* The diameter of the filter paper discs (6 mm) for essential oil and (14 mm) for extracts was included.
(-) = not active (indicates that no inhibition zone could be determined)
Means ± Standard Deviation

Table 3: Minimum inhibitory concentration (MIC) of the spearmint essential oil against the twelve microorganisms

Microorganisms	Spearmint essential oil (µl/ml)
Pseudomonas fluorescene	-
Escherichia coli	2.2
Bacillus cereus	2.5
Staphylococcus aureus	4.1
Candida lipolytica	1.6
Saccharomyces cerevisiae	1.7
Pichia anomala	1.7
Aspergillus niger	2.5
Aspergillus flavus	2.5
Penicillium expansum	2.5
Geotricum candidum	2.0
Fusarium moniliform	2.5

(-) = not active (indicates that no inhibition zone could be determined)

volvement of the less abundant constituents should be considered.

Microbial changes of cheese by spearmint essential oil

Fig. (1) shows the microbial analysis of white cheese with different concentrations of spearmint essential oil (0.5 to 2.5 ml / kg retentate, which designated by A to F, respectively) during cold storage period. It could be noticed that fresh control cheese had higher total bacterial mean log count (Fig. 1a) decreased significantly (P<0.05) by adding spearmint essential oil with different concentrations. Also, these counts significantly (P< 0.05) decreased during the cold storage for 5 weeks. Proteolytic bacteria mean log count (Fig. 1b) decreased significantly (P<0.05) by elevating the concentrations of spearmint essential oil and during the cold storage compared with fresh control sample. The mean log counts of psychrotrophic bacteria are shown in Fig. (1c). It could be noticed that fresh and one week age white cheese were free of psychrotrophs, while, control cheese had higher mean log of this bacteria. It decreased significantly (P<0.05) by increasing the concentrations of spearmint essential oil. In general, essential oil of spearmint was effective as antimicrobial agent in soft cheese. These results are in agreement with those obtained by Tassou *et al*., (1995) who found that adding mint essential oil at concentrations of 0.5 to 2.0 % (v/w) to yoghurt and cucumber salad (tzatziki) stored at 4°C and 10°C, reduced the growth of *Salmonella enteritides*. Also, Mendoza-Yepes *et al*., (1997) reported that the mixture comprising of 50 % essential oils of rosemary, sage and citrus at concentration of 2500 ppm inhibited the growth of *Listeria monocytogenes* in Spanish soft cheese stored at 7°C. Hussein, (2004) reported that spearmint essential oil at concentrations of 500 and 1000 mg/kg extended the shelf life of *Tallaga* cheese from 10 to 15 days as compared with control cheese. Microbial analysis did not detect any colony of coliform groups or moulds and yeast in white cheese with different concentrations of spearmint essential oil during the cold storage for 5 weeks. While, after the first week of cold storage, some colonies of moulds and yeast were detected in control sample, increased significantly (P< 0.05) by prolonging the cold storage for 5 weeks.

Microbial changes of UF- white cheese with dried spearmint

The changes in the mean log count of total bacteria in control and UF- white cheeses with different concentrations of dried spearmint are shown in Fig. (2a), It could be noticed that mean log of total bacteria count in white cheese increased significantly (P<0.05) as a result of adding dried spearmint with different concentrations as compared with control sample. On the other hand, this count increased significantly (P<0.05) by prolonging the cold storage period for 5 weeks. These results could be attributed to the small concentration of active antimicrobial compounds in the dried herb and their ability to release from the herb tissue to the surrounding media. Leuschner & Ielsch, (2003) reported that food containing high protein and fat prevent the penetration of inhibitory substances of spices. Agboola & Tesic, (2002) found no significant differences in total bacterial counts (Lactocccus and Lactobacillus) between control and semi hard cheese samples containing 1g mint / kg cheese. Fig. (2b) shows that control cheese had lower proteolytic bacteria counts increased significantly (P< 0.05) by adding dried spearmint, while no significant effect was traced among the concentrations. Prolonging the cold storage period for 5 weeks increased mean log counts of proteolytic bacteria significantly (P< 0.05). These results are partially in agreement with those obtained by Foda *et al*., (2006) who mentioned that increasing the concentrations of thyme or celery in cheese samples increased log count of proteolytic bacteria significantly. Also, Abou-Zeid (1992) reported that cheese with parsley had higher total proteolytic bacteria count than regular cheese. These results indicated that some of crude herbs had stimulator effect on cheese microflora. Fig. (2c) shows that adding dried spearmint with different concentrations decreased mould and yeast log counts significantly (P<0.05), while no significant effect could be figured out among the spearmint concentrations. Fresh and one week age cheese were free of mould and yeast colonies, and then detected during the cold storage. These results are in agreement with those obtained by Foda *et al*., (2006) who found that increasing the thyme or celery concentrations (1or 2%) in UF-soft cheese led to decline the mould and yeast counts in cheese samples significantly. Sagdic *et al*., (2003) found that total mould and yeast were ≤ 4 log10 g^{-1} in (60%) of 15 commercial Turkish herby cheeses. Control cheese contained higher psy-

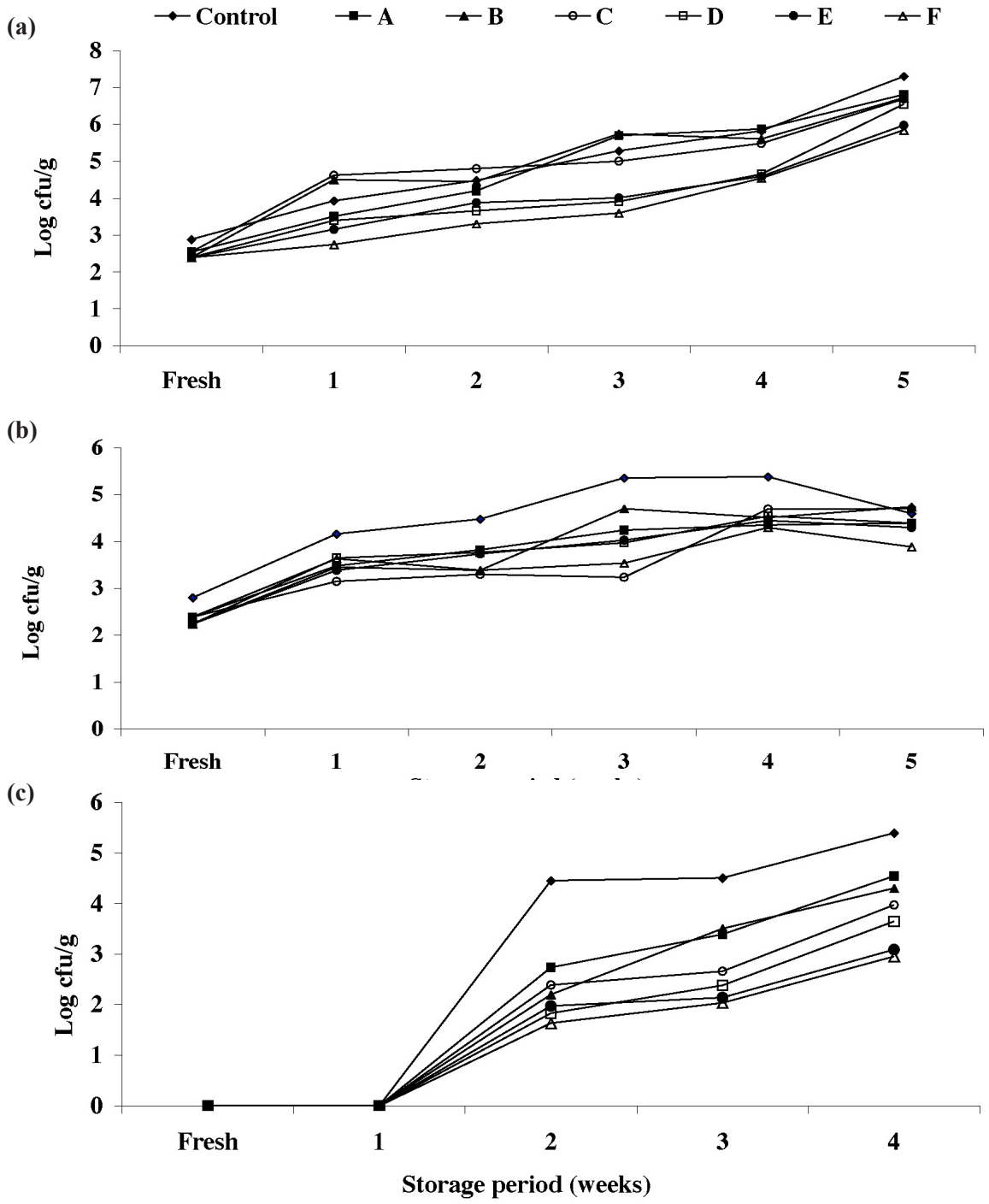

Fig. 1: Microbiological evaluation of white cheese with different concentrations of spearmint essential oil during cold storage for 5 weeks

a= total bacterial count, b= proteolytic bacteria, c= psychrotrophic bacteria. A= cheese with 0.5 (ml/kg retentate), B= cheese with 0.75 ml, C= cheese with 1.0 ml, D= cheese with 1.5 ml, E= cheese with 2.0 ml, F= cheese with 2.5 ml

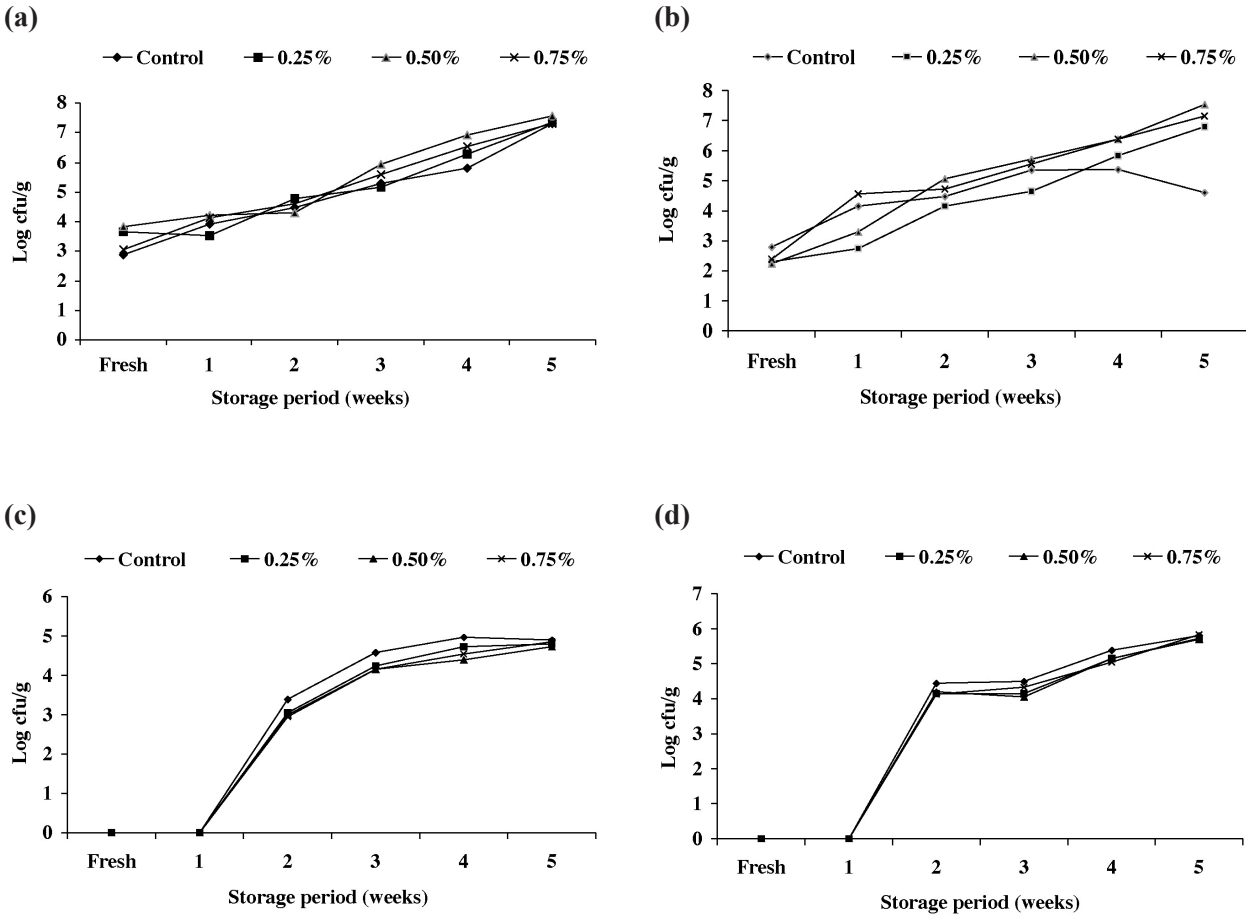

Fig. (2): Microbiological evaluation of white cheese with different concentrations of dried spearmint during cold storage for 5 weeks.

a= total bacterial count, b= proteolytic bacteria, c=yeast &mould, d= psychrotrophic bacteria

chrotrophic counts (Fig. 2-d), but decreased significantly (P<0.05) by adding dried spearmint, while increasing its concentrations had no significant effect. Fresh cheese and one week age cheese were free of psychrotrophic counts, but increased significantly (P< 0.05) by prolonging the storage period 5 weeks. Tarakci *et al*., (2004) stated that psychrotrophs and proteolytic bacteria counts decreased with increasing black cumin concentrations. Also, Foda *et al*., (2006) found that control cheese contained higher psychrotrophic mean log counts, and herbs concentration has no significant effect. While these counts increased up to three weeks then decreased significantly (P<0.05) at late storage period. Microbial analysis, of UF- white cheese with dried spearmint, did not detect any colony of coliform groups either in control sample or UF-white cheese with different dried spearmint concentrations during the cold storage for 5 weeks.

CONCLUSION

The present work examined water and ethanolic extracts of spearmint along with its essential oil against twelve microorganisms, Gram (+), Gram (-) bacteria, yeast and moulds. Essential oil showed best results against all strains under study with Pseudomonas fluorescenes, being an exception.

- Adding dried spearmint to white cheese increased the mean log count of total bacteria and proteolytic bacteria significantly (P<0.05), while moulds and yeast and psychrotrophic counts decreased significantly, (P<0.05).
- Adding spearmint essential oil with concentrations of (1.5 -2.5 ml /kg retentate) decreased the mean log count of total bacteria significantly (P<0.05). Moulds and yeast colonies were detected only in control samples. Proteolytic and psychrotrophic bacteria counts decreased significantly, (P<0.05) during the cold storage period.
- Spearmint essential oil prolonged the shelf life of white cheese.

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تقييم النعناع البلدي الجاف ومستخلصاته المختلفة كمادة حافظة للجبن الأبيض

ميرفت إبراهيم فوده [١]، مجدي محمد أحمد السيد[١] ، مروة محمد المغازي[١] ،

امال احمد حسن[٢] ، نجوي موسي رسمي[٢]

[١] قسم الألبان – المركز القومي للبحوث – الدقي – القاهرة

[٢] قسم علوم الأغذية – كلية الزراعة – جامعة عين شمس – شبرا الخيمة – القاهرة

يهدف هذا البحث الي تقييم النعناع البلدي الجاف والمستخلص المائي والكحولي له وكذلك الزيت العطري كمواد مضادة للنشاط الميكروبي، استخدمت طريقتا أقراص الانتشار والآجار المخفف مع ١٢ سلالة ميكروبية مختلفة من البكتريا و الخمائر والفطريات. أظهرت النتائج المتحصل عليها أن المستخلص المائي ليس له تأثير مثبط علي أي من السلالات الميكروبية تحت الاختبار، أما المستخلص الكحولي فقد أظهر تأثيرا بسيطا علي السلالتين التابعتين للبكتريا الموجبة لجرام فقط ، بينما أظهر الزيت العطري تأثيرا مثبطا قويا علي نمو كل السلالات المختبرة ، لذا تم دراسة النشاط المضاد للفطريات وتقدير أقل تركيز مثبط. كما تم تحضير نوعين من الجبن الأبيض باستخدام النعناع البلدي الجاف والزيت العطري بتركيزات مختلفة وذلك لتقييمه كمادة حافظة، أوضحت النتائج ان إضافة زيت النعناع العطري أدي الي خفض معنوي في اعداد البكتريا الكلية و البكتريا المحللة للبروتين و البكتريا المحبة للبرودة مقارنة بالجبن الأبيض العادي، كما أدي الي تثبيط كامل لنمو الخمائر و الفطريات.