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Effects of Carnation Essential Oil Extracted from Carnation Calli on Extending Shelf Life of Yoghurt

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Abstract

Carnation's essential oil with high content of eugenol (monoterpenes) was extracted from carnation plant calli. Eugenol had antimicrobial effect and could be used to prolong the shelf life of fermented products as yoghurt. Eugenol in carnation essential oil was added to milk, at the percentages of (0.2, 0.4, 0.6, and 0.8 µl/ml milk, respectively before using the milk in the yoghurt manufacture. Results indicated that eugenol in carnation essential oil possessed good inhibitory effect against selected pathogenic bacterial strains at different concentrations, but had no inhibitory effect on *Saccharomyces cerevisiae*. Addition of carnation essential oil affected the pH and titratable acidity of the prepared yoghurt. Moreover, total solids, total protein and fat-to-dry matter records were slightly affected. The total viable counts, also, counts of yoghurt starter cultures and *Lactobacillus acidophilus* in yoghurt samples enhanced to become a maximum after 10 days of storage and reduced thereafter. Yeasts and molds, and coliform bacteria were not detected in the treated yoghurt. In different samples of yoghurt, yoghurt containing 0.6 µl/ml eugenol was the most acceptable organoleptically. It can be concluded that 0.6 µl/ml eugenol, can be applied to prolong yoghurt storage time for more than 15 days.

Introduction

Carnations and pinks are generally names for several species of the genus *Dianthus*, which belongs to the Caryophyllaceae. The family contains 80 genera and 2,000 species which are either annual or perennial and majority of these herbs occur in the northern

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hemisphere. Over 300 *Dianthus* species have been known (Galbally and Galbally 1997, Jurgens et al. 2003). The carnation flower fragrance is predominantly due to eugenol, β-caryophllene and benzoic acid derivatives. The cultivar 'Eliat', indicated that the level of these compounds rises during flower development and coincides with a rise in flower fragrance (Zuker et al. 2002).

Yoghurt is one of the most common dairy products consumed all over the world, mainly in Europe, North America and the Middle East. High intake and popularity of yoghurt especially between women, children and teenagers are due to its beneficial and health effect (Güler and Park 2013). Traditional yoghurt is the coagulated milk product obtained by lactic acid fermentation through the action of *Lactobacillus delbreukii* sub sp. *bulgaricus* and *Streptococcus thermophillus*. The main role of these two starter bacteria in yoghurt manufacture is to develop lactic acid and synthesis of aromatic compounds. Yoghurt can be found in different textures (e.g. liquid, stirred and set) and fat contents. It is a perfect food matrix for delivering probiotics, due to their high consumer acceptability and improved viability of these organisms. Although quantitative microbiological standards differ all over the world, it is accepted that yoghurt contains 10^7 CFU/g of *Lactobacillus delbreukii* sub sp. *bulgaricus* and *Streptococcus thermophillus* (Assem et al. 2013). Therefore, the survival of yoghurt microorganisms during storage is an important criterion for the quality and health characteristic of the product. The number of viable microorganisms decreased during storage whereas the survival factors determine the rate of their decrease. Volatile oils are aromatic, oily liquids extracted from plant parts. Steam distillation is the most applicable method for commercial production of volatile oils. It has long been known that various essential oils have antimicrobial properties (Burt 2004) and those are able to be used as food flavor agents or preservers, and for pharmaceutical purposes (El-Nawawy et al. 1998). The essential oil mostly contained phenols and polypeptides that have been attributed in the antimicrobial properties of essential oils (Terzaghi and Sandine 1975). Many researches have indicated the efficiency of phytophenols as antimicrobial agents, mainly carvacrol from oregano and thyme and eugenol from cloves (Gilliland and Walker 1975). Most volatile oils have been recorded as general recognize as safe (GRAS) and the antimicrobial activity of essential oils and spices are attributed to prolong the storage time of diverse foods (Burt et al. 2004). Some studies suggested that lactic acid bacteria (LAB) are resistant to the inhibitory effect of spices and essential oils. Therefore, the vitality of LAB enhanced at attuned concentrations of volatile oils (Ismail et al. 2006). Smith-Palmer et al. (2001), studied the ability of plant essential oils; bay leaf, clove, cinnamon and thyme as food preservatives.

Eugenol has been known in numerous aromatic plants such as nutmeg, true cinnamon, Saigon cinnamon, basil and sweet basil. Nevertheless, *Eugenia caryophyllata* (= *Syzygium aromaticum*) can be considered the main natural source of this compound as it constitutes between 45 and 90% of the total oil. Commercial eugenol is obtained from

clove bud/leaf oil, cinnamon leaf oil or basil through distillation which is then further refined (Guy et al. 2012).

There is a rising tendency to change synthetic antioxidants with natural compounds. Many phenolic essential oil constituents (e.g., carvacrol, thymol) have established their ability as antioxidant molecules (Guy et al. 2012). The inhibitory effect of eugenol on lipid peroxidation was less (IC_{50} value of ≈ 80 μM) than isoeugenol (Guy et al. 2012). The mechanism of action of the two compounds was examined and it was recommended that the antioxidant ability of eugenol could be described by the creation of complexes with reduced metals.

The objective of this study is to evaluate the effect of carnation's essential oil with high content of eugenol (monoterpenes), at different concentrations, on prolonged the yoghurt storage time and the survival of lactic acid bacteria. The effect of these additives on the chemical, microbiological and organoleptic properties of the resulting yoghurt was also studied.

Materials and Methods

Seeds of Egyptian carnation were obtained from Horticulture Research Institute, Agricultural Research Centre, Giza, Egypt.

Fresh buffalo's milk, used in the manufacture of yoghurt, was obtained from the Faculty of Agriculture, Cairo University. Imported skim milk powder was purchased from the local market.

Lactobacillus acidophilus and *Streptococcus thermophilus* CH-1 were obtained from Chr. Hansen's Lab., Denmark, *Lactobacillus dulbueckii* subsp. *bulgaricus* Lb-12 DRI-VAC, Provided by northern Regional Research Laboratory. Illinois, USA. Cultures were propagated in sterilized reconstituted skim milk (10% total solids). Mixture (1 : 1 : 1) of the three strains was used in the manufacture of yoghurt.

Saccharomyces cerevisiae Y-2223 was provided by the Northern Regional Research Laboratory Illinois, USA (NRRL). *Escherichia coli* 0157: H7 and *Staphylococcus aureus* were isolated and serologically identified by Dairy Microbiological Lab., National Research Center. *Listeria monocytogenes* 598 was provided by the Department of Food Science, University of Massachusetts, Ambert MA, USA. *Salmonella typhimirum* was obtained from Hungarian National Collection of Medical Bacteria, OKI, Gyaliut 2-6, H-1966 Budapest, Hungary.

Seeds of carnation (*Dianthus caryophyllus*) were germinated on MS. Calli cultures were established on MS supplemented with 2 mg/l 2,4-D and 0.5 mg/l Kn from leaf explants. Methyl jasmonate was used at different concentrations to study its effect on both growth of calli cultures and eugenol content. Osmotic development was induced by adding mannitol at different concentration to investigate its effect on the growth of calli

and eugenol content in calli cultures. Essential oils were extracted by steam distillation from the obtained calli cultures and used as additives in yoghurt (Matter et al. 2017).

Screening of essential oils for antibacterial activity was done by the disc diffusion method (Prabuseenivasan et al. 2006) using pathogenic strains incubated at 37°C for 18 hrs in 10 ml of trypton soya broth. The strains were adjusted to 10^5 CFU/ml with sterile saline solution. Aliquots of 0.1 ml of the strain suspensions were spread evenly over the plates containing nutrient agar medium using a sterile swab. The different concentrations of eugenol incarnation essential oil, eugenol ranged from 10 to 25 µl were dissolved in dimethyl sulfoxide (DMSO) in concentration 1 : 1 for easy diffusion in agar medium. Under aseptic conditions, empty sterilized discs (Whatman 6 mm diameter) were impregnated with 20 µl of different concentrations (10, 15, 20, 25 µl) of the eugenol and placed on the agar surface. The plates were left for 30 min at room temperature to allow the diffusion of oil, and then they were incubated at 37°C for 24 hrs. After the incubation period, the zone of inhibition was measured in mm.

Different concentrations (0.2, 0.4, 0.6, and 0.8 µl/ml milk) of eugenol in carnation essential oil were added to different test tubes containing 10 ml sterilized skim milk (10% total solids (TS). The milk was inoculated with pathogenic strains (*E. coli, S. aureus)* and incubated at 37°C for 24 hrs. The count of *S. aureus* was enumerated using Braid Parker agar medium and the plates incubated at 37°C for 48 hrs. The count of *E. coli* was enumerated using violet red bile agar medium and the plates incubated at 37°C for 24 hrs.

Different concentrations (10, 15, 20, 25 µl/ml milk) of eugenol were added to different test tubes containing 10 ml sterilized skim milk. The milk inoculated with lactic acid bacteria strains (*L. bulgaricus, L. acidophilus* and *S. thermophilus*) and after that these milk incubated at 37°C for 24 hrs. The count of lactobacilli was enumerated using MRS agar medium and the plates incubated at 37°C for 48 hrs under anaerobic condition. On the other hand the count of streptococci was enumerated using M17 agar medium and the plates incubated at 37°C for 48 hrs under aerobic condition.

Fresh buffalo's milk was standardized (3% fat w/v) with skim milk powder (2% w/w), homogenized and heat treatment at 85°C for 5 min, then cooled to 42°C. The milk was divided into four equal portions. The first portion was kept as control. The eugenol in essential oil of carnation was added to the other three portions of milk in different concentrations (0.3, 0.6, 1.0 µl/ml). Milk samples from all treatments were inoculated with 3% (v/v) active yoghurt starter and probiotic strain and transferred to 100 ml cups then incubated at 42°C for 4 hrs. Afterward they were refrigerated at 4 ± 1°C and stored for 30 days.

Fresh and stored samples of yoghurt were analyzed for TS, fat content and titratable acidity (AOAC 2012). Total proteins (TP) were determined by the micro Kjeldhl method

(Ling 1963). The pH values of samples were measured using a digital pH meter (Hanna, Germany).

Antioxidant activity was determined by measuring the free radical scavenging ability of yoghurt using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) inhibition assay (Brand-Williams et al. 1995). The DPPH was used at a concentration of 60 μmol/l, dissolved in methyl alcohol. The solution was homogenized and transferred to a dark glass bottle. The solution was prepared and used only on the day of analysis. In the dark, aliquots of 0.1 ml sample (250 mg/ml) were transferred to test tubes containing 3.9 ml DPPH solution and homogenized by shaking. After 45 min, the scavenging activity was measured spectrophotometrically by the decrease in absorbance at 517 nm. Inhibition of free radical DPPH in per cent (I%) was calculated as follows:

$$I\% = 100 \times (A\ Control - A\ Sample) / A\ Control$$

where, A Control is the absorbance of the control reaction (containing all reagents without the test sample), and A Sample is the absorbance of the tested sample.

L. bulgaricus counts were determined using MRS agar. The plates were incubated at 37°C for 48 hrs under anaerobic condition. *S. thermophilus* counts were determined using M17 agar. The plates were incubated at 35°C for 48 hrs. *L. acidophilus* counts were determined using MRS agar supplemented with 0.05% bile salts as Gilliland and Walker (1990). The plates were incubated at 37°C for 48 hrs under anaerobic condition. Yeasts and molds counts were enumerated using potato dextrose agar acidified to pH 3.5 with sterile lactic acid solution (APHA 1994). The plates were aerobically incubated at 25°C for 4 days. Coliform bacterial counts were enumerated using violet red bile agar medium (Mossel et al. 1985). The plates were incubated at 37°C for 18 hrs. The results were recorded as log number of colony forming units per ml (log 10 CFU/ml).

The Cytotoxic effect of lyophilized yoghurt sample containing 0.6 µl/ml of carnation on human cell line (RPE1: normal retina cell line) and (HCT116: Colon cell line) was also determined. Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan as (Mosmann 1983). All procedures were done in a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). Cells were suspended in RPMI 1640 medium for RPE1 and HCT116 for A549. The absorbance was measured using a microplate multiwall reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. Statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program.

Samples of yoghurt were sensory evaluated for flavour (out of 60 points), body and texture (out of 30 points), and color appearance (out of 10 points) according to score card (Keating and White 1990).

All experiments were replicated and sub-sampled at least once. Results were analyzed using the general linear model (GLM) procedure of the SAS System 1996.

A protocol for mass production of calli cultures derived from carnation leaves was achieved. The calli cultures were exposed to an elicitor (methyl jasmonate) to study its effect on eugenol content in essential oils. Essential oils were tested in preservation of yoghurt (Matter et al. 2017).

Results and Discussion

Different concentrations of eugenol in carnation essential oil showed various degrees of inhibition against different pathogenic strains (Table 1). The results showed that *S. aureus* was found to be highly sensitive to the eugenol in carnation essential oil action with different concentration which the diameter of inhibition zone ranged between 7.00 and 12.00 mm, followed by *E. coli*, *L. monocytogenes* and *S. typhamurium.* There was no effect on *S. cerevisiae*. The antibacterial activity of carnation essential oil may be attributed to its major individual phenolic constituent eugenol. Oyedemi et al. (2009) found that the oil component eugenol was capable of making cell lysis by leak the protein and lipid contents. A vital characteristic of essential oils and their components is their hydrophobicity, which allows them to partition the lipids of the bacterial cell membrane and mitochondria, worrying the cell structures and rendering them leakier (Knobloch et al. 1986, Sikkema et al. 1994). Carnation oil has biological activities, such as antibacterial, antifungal, insecticidal and antioxidant properties, and is used usually as a preserving agent and antimicrobial material in food (Pérez-Rosés et al. 2016, Lee and Shibamoto 2001 and Nuñez et al. 2001).

Table 1. Antibacterial activity of eugenol (µl) in carnation essential oil on pathogenic strains.

Pathogenic strains	10 µl	15 µl	20 µl	25 µl
	Diameter of inhibition zone (mm)			
S. cerevisiae	ND	ND	ND	ND
S. typhimurium	6.50^{Bc}	6.00^{Bc}	9.00^{Ab}	11.00^{Ba}
S. aureus	7.00^{Bd}	8.00^{Ac}	10.00^{Ab}	12.00^{Aa}
L. monocytogenes	6.00^{Cc}	8.00^{Ab}	10.00^{Aa}	11.00^{Aa}
E. coli	8.00^{Ac}	8.50^{Ac}	9.00^{Ab}	10.00^{Ba}

Data expressed as mean of three replicates. Means in the same row showing the same superscripts are not significantly different ($p \leq 0.05$). Means in the same column showing the same capital letters are not significantly different ($p \leq 0.05$).

The antimicrobial effect of eugenol adding to milk was presented in Table 2. It is clear that essential oil have antimicrobial effect against pathogenic strains when used in concentration more than 0.4 µl/ml milk, because of the essential oil may be able to bind with milk protein as mentioned by Gaysinsky et al. (2007). Moreover, the concentration

above 0.6 µl/ml milk has antimicrobial effect, so, from this study it is suggested that the concentration of eugenol that used as preservative in dairy products should be not less than 0.6 µl/ml milk.

Table 2. Evaluation the antimicrobial effect of eugenol (µl/ml) in carnation essential oil in milk.

Pathogenic strains	Control	0.2	0.4	0.6	0.8
E. coli	+++	+++	++	+	+
S. aureus	+++	+++	++	+	+

+++: Found growth in 10^5 CFU/ml milk. +: Found week growth in 10^5 CFU/ml milk.

From the Table 3 it is clear that the eugenol has no effect on lactic acid bacterial count So, we can use these different concentrations in different dairy products as a bio-preservative with no effect on lactic acid bacteria.

Table 3. Effect of different concentrations of eugenol (µl) in carnation essential oil on lactic acid bacteria.

Lactic acid bacteria	Control	10	15	20	25
S. thermophilus	8.96^{d}	8.97^{cd}	9.00^{c}	9.15^{b}	9.30^{a}
L. bulgaricus	9.37^{ab}	9.38^{ab}	9.38^{ab}	9.40^{a}	9.35^{b}
L. acidophilus	9.39^{b}	9.42^{b}	9.43^{ab}	9.45^{ab}	9.50^{a}

Data expressed as mean of three replicates. Means in the same row showing the same small letters are not significantly different ($p \leq 0.05$).

Table 4 shows that the main gross chemical composition of fresh and stored yoghurt samples which fortified with different concentrations of eugenol. No significant differences were detected in the TS and fat-to-dry matter (F/DM) content of the various yoghurt either when fresh or in the storage period. During storage, both TS and F/DM increased slightly and could be attributed to moisture loss. Likewise, Thabet et al. (2014) also stated that there were no clear differences in TS and F/DM of yoghurt by addition of three diverse essential oils. The alteration in titratable acidity (TA) is a very significant factor, since it affects the storage time and the adequacy of yoghurt. It was observed that acidity records of the treated yoghurt enhanced with an increase in the storage period (Table 4). Significant differences were detected in the results of TA and pH content between yoghurt samples containing eugenol. TA in all samples of yoghurt which treated with eugenol increased slightly compared to untreated control yoghurt, indicated that the eugenol had an encouraging effect on the starter culture and total count (Al Otaibi and El. Demerdash 2008). It was detected that TA% increased regularly in storage period. The development of the changes in pH values of all treatments was reverse to that of acidity. Table 4 presented that the content of protein in all samples gradually increased till 15 days of storage yoghurt probably due to unavoidable moisture losses.

There were no significant differences in the protein content in all samples of yoghurt treated with eugenol or in the untreated control yoghurt. Therefore, it could be concluded that the addition of eugenol had no effect on the yoghurt protein content.

The presence of eugenol (0.3, 0.6, 1.0 µl/ml) during yoghurt manufacture increased the antioxidant activity in all yoghurt samples treated with eugenol (61.40 - 66.75 %), (77.36 - 78.58%) and (86.22 - 91.69 %), respectively, compared to untreated control yoghurt (35.36 - 49.06 %) (Table 5 and Fig. 1). The results of antioxidant activity in all samples were increased significantly by increasing the ratio of eugenol added to the yoghurt samples. Increased in antioxidant activity was observed in all samples during storage period. The data also showed significant differences in the antioxidant activity in all yoghurt samples treated with eugenol and in the untreated control yoghurt. Phenolic content is the most influential factor to antioxidant activity (Shori et al. 2013). Eugenol has been previously reported to show antioxidant activity properties due the presence of its phenolic group (Guy et al. 2012). Additionally, milk protein proteolysis and organic acids production as a result of microbial metabolic activity during fermentation and refrigerated storage could be other sources of antioxidant activities (Shori et al. 2013).

Table 4. Changes in the chemical composition of yoghurt during storage period.

Treatments	pH	Acidity (%)	TS (%)	Fat /DM (%)	Protein (%)
Control					
Fresh	4.87^{A}	0.71^{F}	13.18^{ABC}	22.60^{A}	3.14^{D}
5 days	4.69^{C}	0.80^{E}	13.27^{ABC}	22.71^{A}	3.12^{D}
10 "	4.53^{ED}	0.85^{ED}	13.44^{ABC}	22.83^{A}	3.65^{BCD}
15 "	4.47^{E}	0.90^{CD}	13.75^{AB}	22.93^{A}	4.68^{A}
0.3 µl/ ml					
Fresh	4.69^{B}	0.80^{E}	13.05^{ABC}	22.68^{A}	3.20^{D}
5 days	4.56^{C}	0.85^{ED}	13.23^{ABC}	22.77^{A}	3.22^{CD}
10 "	4.37^{ED}	1.00^{BC}	13.39^{ABC}	22.96^{A}	4.00^{ABC}
15 "	4.35^{ED}	1.10^{B}	13.82^{A}	22.99^{A}	4.37^{AB}
0.6 µl/ ml					
Fresh	4.66^{B}	0.85^{E}	13.05^{BC}	22.63^{A}	3.15^{D}
5 days	4.56^{C}	0.90^{CD}	13.17^{C}	22.82^{A}	3.20^{D}
10 "	4.33^{ED}	1.00^{B}	13.54^{ABC}	22.94^{A}	4.20^{AB}
15 "	4.25^{E}	1.10^{A}	13.81^{AB}	23.02^{A}	4.79^{A}
1.0 µl/ ml					
Fresh	4.68^{A}	0.85^{E}	13.04^{BC}	22.59^{A}	3.00^{D}
5 days	4.59^{AB}	0.90^{CD}	13.27^{ABC}	22.70^{A}	3.14^{D}
10 "	4.35^{D}	1.00^{B}	13.62^{ABC}	22.96^{A}	4.21^{AB}
15 "	4.33^{E}	1.00^{B}	13.79^{AB}	23.12^{A}	4.70^{A}

Data expressed as mean of three replicates. Means with the same letter are not significantly different ($p \leq 0.05$).

From the Tables 6 and 7, it is clear that the significant effect of different concentration of the carnation essential oil on LAB population in yoghurt samples remained stable up to 10 days of storage and slightly reduced after 15 days but not completely suppressed during storage. The viable counts in both starter cultures slight decreased in the samples containing 1.00 µl/ml concentration of carnation essential oil compared with other samples. The count of *L. bulgaricus* and *S. thermophilus* in samples containing 1.00 µl/ml concentration of carnation essential oil decreased from 8.09 and 8.18 to 7.25 and 7.20 Log^{10} CFU/g, respectively at the end of storage. Present results confirmed by El-Nawawy et al. (1998) stated that the adding of some herbs in the production of yoghurt enhanced the counts of *S. thermophilus* and *L. bulgaricus* compared to control during storage. On the contrary, Bayoumi et al. (1992) established that mint oil is effective against *S. enteritidis* in low fat yoghurt. It inhibits the growth of yoghurt starter cultures at 0.05 - 5 µl/ml but cinnamon, cardamom and carnation oils are greatly effective.

Table 5. Antioxidant activity in yoghurt (inhibition %) during storage period.

Treatments	Storage period (days)			
	Fresh	5	10	15
Control	$36^{I}.35$	$0^{I}.37$	42.68^{H}	49.6^{G}
0.3 µl/ml	61.40^{F}	62.87^{EF}	63.71^{E}	66.75^{D}
0.6 "	77.36^{C}	77.95^{C}	77.11^{C}	78.58^{C}
1.0 "	86.22^{B}	88.27^{B}	90.75^{A}	91.69^{A}

Data expressed as mean of three replicates. Means with the same letter are not significantly different ($p \leq 0.05$).

Effect of different concentration of eugenol on the viability of *L. acidophilus* in yoghurt samples are shown in Table 8. From this study it indicated that the viable count of *L. acidophilus* decreased after 10 days of storage in all samples. Also, the viable count of *L. acidophilus* culture slight decreased in the samples containing 1.00 µl/ml concentration of eugenol compared with other concentrations and control. In generally, all different concentration slightly affected on the count of *L. acidophilus* using as probiotic strain to produce probiotic yoghurt product with long self-life which the counts of probiotic was more than log 10^7 CFU/g during storage period. Al Otaib and El. Demerdash (2008) suggested that the lactic acid bacteria were not inhibited by low concentrations of the several volatile oils.

The different concentration of eugenol contributed to increase the preservative of yoghurt samples up to 30 days compared to control which its spoilage after 15 days of storage, the viable counts of mold and yeast reached 1.80 Log^{10} CFU/gm in the control at 15 days of storage. Moreover, at the 30 days of storage appeared some mold and yeast in the sample contained 0.3 and 0.6 µl/ml, which the mold and yeast counts reached 1.35 and 1.15 Log^{10} CFU/g in these samples, respectively. Also all treatment samples were free from coliform bacteria counts during storage; this may be due to the pasteurization

process during manufacturing of yoghurt and eugenol found in yoghurt samples. Present results confirmed with Farag et al. (1990) who found that the essential oils obtained from thyme and cumin should be used to extend the shelf life of butter.

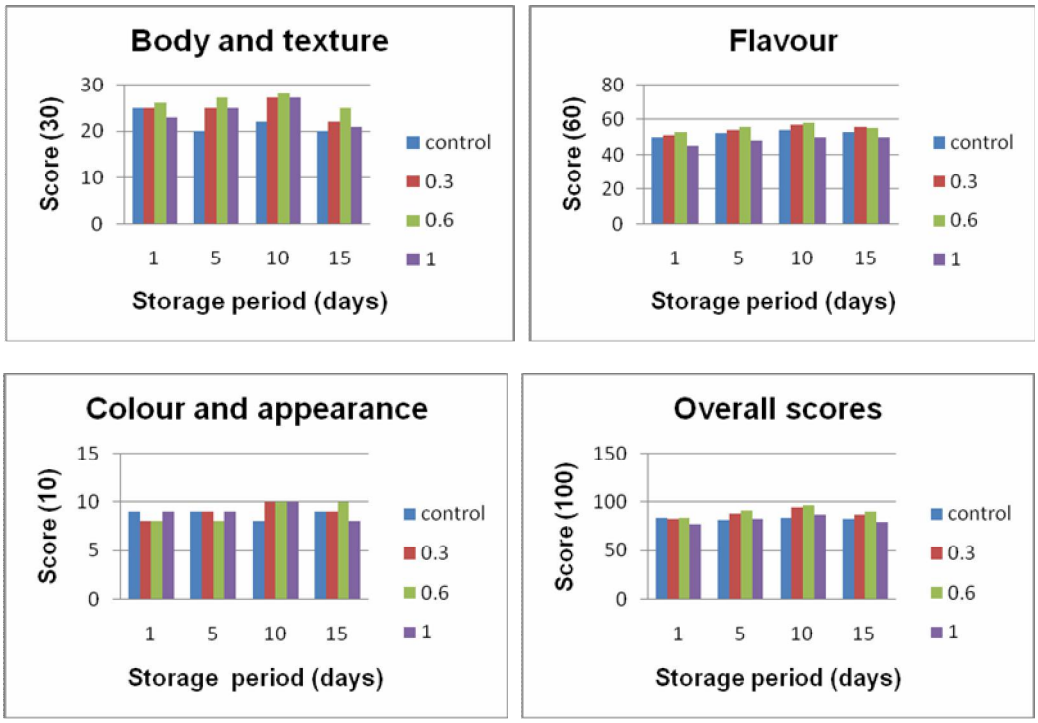

Fig. 1. Effects of storage period on the sensory properties of yoghurt.

Table 6. *Lactobacillus bulgaricus* count in treated yoghurt during storage period.

Treatments	Storage periods (days)						
	Zero	5	10	15	20	25	30
Control	8.05^{Cb}	8.17^{Ca}	8.19^{Da}	8.18^{Ca}	8.00^{Bb}	7.45^{Bc}	7.30^{Cd}
0.3 µl/ml	8.07^{Bd}	8.23^{Ac}	8.25^{Bb}	8.27^{Aa}	8.10^{Ad}	8.00^{Ad}	7.70^{Ae}
0.6 "	8.10^{Ad}	8.24^{Ac}	8.29^{Aa}	8.27^{A}b	8.15^{Ad}	7.50^{Be}	7.35^{Bf}
1.00 "	8.09^{Ac}	8.20^{Bb}	8.23^{Ca}	8.20^{Bb}	8.00^{Bc}	7.40^{Cd}	7.25^{Ce}

Data expressed as mean of three replicates. Means in the same row showing the same small letters are not significantly different ($p \leq 0.05$). Means in the same column showing the same capital letters are not significantly different ($p \leq 0.05$).

Cytotoxic activity of yoghurt sample contained 0.6 µl/ml eugenol in carnation on human cell line (RPE1: normal retina cell line) and (HCT116: Colon cell line) found in Table 9. The result revealed that yoghurt sample displayed a slim effect on colon carcinoma human cancer cell, this may be due to the little concentration found in the

yoghurt sample, but in the other researchers found that the different concentration of eugenol have anticancer effect. Jaganathan et al. (2011) showed that the eugenol has anti-proliferative effect against the colon cancer cells depending upon the concentration and the cell lines used. This anticancer effect due to eugenol induced apoptosis in colon cancer cells (Ghosh et al. 2005). The yoghurt sample did not have any effect on the normal human cell, therefore the addition of eugenol to dairy products contributed to extended the shelf life of these product without any effect on the human cell.

Table 7. *Streptococcus thermophilus* count in treated yoghurt during storage period.

Treatments	Storage periods (d)						
	Zero	5	10	15	20	25	30
Control	8.11^{Cc}	8.15^{Cb}	8.22^{Ca}	8.17^{Bb}	7.80^{Cd}	7.55^{Be}	7.25^{Cf}
0.3 µl/ml	8.15^{Bd}	8.25^{Abc}	8.27^{ABb}	8.29^{Aa}	8.10^{Bd}	7.95^{Ae}	7.50^{Bf}
0.6 "	8.20^{Ac}	8.24^{Ab}	8.29^{Aa}	8.30^{Aa}	8.20^{Ac}	8.00^{Ad}	7.75^{Ae}
1.00 "	8.18^{ABd}	8.23^{BCc}	8.25^{Bb}	8.28^{ABa}	8.10^{Bd}	7.65^{Be}	7.20^{Cf}

Data expressed as mean of three replicates. Means in the same row showing the same small letters are not significantly different ($p \leq 0.05$). Means in the same column showing the same capital letters are not significantly different ($p \leq 0.05$).

Table 8. *Lactobacillus acidophilus* count in treated yoghurt treatments during storage period.

Treatments	Storage periods (d)						
	Zero	5	10	15	20	25	30
Control	8.15^{Bc}	8.20^{Cab}	8.22^{Ba}	8.18^{Bb}	8.00^{Bd}	7.80^{Be}	7.55^{Bf}
0.3 µl/ml	8.18^{Ac}	8.23^{Bb}	8.27^{ABa}	8.23^{ABb}	8.15^{Ac}	8.00^{Ad}	7.75^{Ae}
0.6 "	8.19^{Ac}	8.25^{Ab}	8.28^{Aa}	8.24^{Ab}	8.00^{Bd}	7.70^{Ce}	7.60^{Bf}
1.00 "	8.17^{ABc}	8.24^{Ab}	8.29^{Aa}	8.25^{Ab}	8.12^{Ad}	7.75^{Be}	7.65^{Af}

Data expressed as mean of three replicates. Means in the same row showing the same small letters are not significantly different ($p \leq 0.05$). Means in the same column showing the same capital letters are not significantly different ($p \leq 0.05$).

Table 9. Cytotoxic activity of eugenol containing yoghurt sample on human cell line.

Cell line type	Yoghurt sample fortified with 0.6 µl/ml eugenol
Colon cell line	31.5% at 100 ppm
Normal human cell line	15.4% "
DMSO	5% "
Negative control	0 %

The organoleptic properties of the yoghurt treated with eugenol in carnation essential oil were studied and the results are presented in Fig. 1. There were substantial differences in the flavor of these treated samples as compared to the control yoghurt. The

yoghurt containing diverse concentrations of eugenol, was preferred once fresh and after 15 days of storage in comparing to the control yoghurt. But, yoghurt containing eugenol in essential oils at 0.6 µl/ml milk was the most preferred one. The overall scores of yoghurt containing eugenol reduced with an increase in the concentration of the eugenol higher than 0.6 µl/ml. Furthermore, in all cases the overall scores of the sensory evaluation reduced gradually during storage. It can be indicated that 0.6 µl/ml of eugenol can be utilized so as to prolong the storage time of yoghurt for more than 15 days at 4 ± 1°C with satisfactory flavor and good appearance without any signs of spoilage organisms.

The present study showed that, eugenol in carnation essential oil have biological activities, such as antimicrobial and antioxidant properties, antimicrobial effect against pathogenic strains like *E. coli* and *S. aureus*. It has any effect on the viable counts of different lactic acid bacteria found in milk and normal human cell line and has slight effect on human colon cancer cell line. Eugenol (0.6 µl/ml milk) contributed to increase the preservative of yoghurt samples up to 30 days with good taste compared to control after up to 15 days of storage. The addition of eugenol at 0.6 µl/ml milk has shown to extend the shelf life of yoghurt with acceptable taste, flavor and good appearance without any microbial spoilage compared with other two concentrations of eugenol. So, the concentration of eugenol that used as preservative in dairy products should not be more than 0.6 µl/ml milk. Finally it is suggested that carnation essential oil with eugenol can be used traditionally as natural preservative agent and antimicrobial material in dairy products.

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