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Optimization of omega-3 rich mixed fat table spread containing natural preservatives

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Abstract
Table spreads have tremendous market potential on account of their simplicity in use and being an excellent vehicle for nutritional ingredients. The aim of present study was to formulate mixed fat table spread containing all the natural ingredients. The carotenoid and omega-3 rich natural colour ant was extracted from carrot pomace-an agricultural bio-waste using flaxseed oil as extraction medium. Further, various combination of essential oil and MicroGARD™ -100 were used as natural preservative. The level of thyme essential oil (0 to 0.75%) and MicroGARD™-100 (0 to 0.75%) was selected based on 4 factorial experiment by two-way ANOVA using Proc GLM of SAS 9.3. The best combination of natural preservatives was achieved based on sensory, physicochemical (water activity, colour value, phenolic content and antioxidant value), and zone of inhibition (for *E. coli* and *Aspergillus niger*). The optimized level of essential oil and MicroGARD™-100 were 0.50% and 0.75%, respectively. The optimized combination yielded overall acceptability score of 7.14±0.69, while water activity, L*, a*, and b* values were 0.976±0.002, 78.9±0.17, 4.75±0.05 and 31.6±0.24 values, respectively indicating that the product had yellow colour with a tinge of red. The phenolic content, antioxidant activity, and zone of inhibition of optimized combination were 0.53±0.04 (mg GAE/g); 281.87±.93 (µg Trolox eq./mL); and 27.00±1.00 mm and 16.33±0.58mm for *E. coli* and *Aspergillus niger*, respectively. Two control spreads were also prepared with all ingredients; first control was without any preservative (CWP) and second one contained potassium sorbate (CPS) as preservative. The proximate composition of both control and test sample packed in PET (SPT) showed non-significant (p>0.05) difference having approximately 51.02% fat, 37.35% moisture, 4.17% protein, 62.65% total solid, and 1.96% ash. The control spread i.e. CWP and CPS and optimized spread were stored at 5±1°C in PET jar and analyzed for proximate composition.

Keywords: MicroGARD™-100, Thyme essential oil, Antioxidant, *E. coli* and *Aspergillus niger*

1. Introduction
Table/fat spread is nutritionally balanced and economical product possessing mild flavour and can be spread over food items into a thin layer (Patel, 1982; Patel *et al.*, 2015) [23, 24]. These are an interesting and effective food vehicle to be fortified with both water and lipid soluble compounds (Timon, 2010) [38].

Global fat spread market is forecasted to reach USD 28.9 billion by 2024 growing at a CAGR of 3.5% during 2019-2024. It is poised to grow by $ 7.60 bn during 2022-2026, progressing at a CAGR of 4.53% during the forecast period (Marketresearch, 2022) [18]. Sales value of spreads in India was USD 562.2 million in 2018 (Statista, 2019) and amounts to US$1,926.00m in 2022. The market is expected to grow annually by 6.28% (CAGR 2022-2027) (Statista, 2022) [35, 36]. Dr. Oetker India Pvt. Ltd., Cremica Food Industries Ltd., Agro Tech Foods Limited (Sundrop), Gujarat Cooperative Milk Marketing Federation Ltd., Parag Milk Foods Limited (Go), Britannia Dairy Private Limited are major players operating in India's spread market.

Preservatives (either natural or synthetic) are added to food products for increasing their shelf life and maintain quality and safety. According to Nielsen Global Health and Ingredient Survey (January to March, 2016), synthetic preservatives were avoided by up to 62% of consumers around the world (Simon *et al.*, 2017) [32]. Natural preservatives derived from plants, animals and other sources offer greater advantages over their synthetic ones due to their safety, low cost, non-toxic nature along with a wide range of health benefits. They also contain antioxidant, antimicrobial and anti-enzymatic properties (Kumari *et al.*, 2019) [17].

The different types of natural preservatives are available in market such as essential oils, oleoresins and bacteriocins.

The essential oils are steam distilled extract from various parts of spices like buds in case of cloves, fruits in case of pepper, etc. The oleo-resins are steam distilled followed by solvent extraction extract of the spices. Higher amount of phenolic compounds are present in essential oils such as eugenol (2 – methoxy – 4 - (2-propenyl) phenol), carvacrol, thymol, etc. depending upon the spice used for the preparation of extract. These possess strong antibacterial properties and are highly effective against Gram positive bacteria.

Thyme oil is highly effective against a wide variety of microorganisms, including Gram-negative bacteria *E. coli* O157:H7 and *Salmonella*, Gram-positive pathogenic bacteria *L. monocytogenes* and *S. aureus*, yeasts, and molds (Gutierrez *et al*., 2008; Iten *et al*., 2009) [11, 14]. Its properties have also been investigated previously against various pathogenic agents, including *Botrytis cinerea*, *Salmonella enteritidis*, *Yersinia enterocolitica*, *Salmonella typhimurium*, *Shigella exneri*, *Listeria monocytogenes*, *Shigella sonnei*, *Salmonella cholereasuis* and *Aspergillus niger* (Soylu *et al*., 2006) [34]. Juven *et al*. (1994) [15] studied the mechanism of action of thymol against *S. typhimurium* and *S. aureous* and proposed that thymol binds hydrophobically to membrane proteins using hydrogen bonding, thereby altering the membrane's permeability. Thymol and eugenol were found to be effective at 0.2 and 0.5 ml/ml MIC, respectively, against *Aspergillus flavus*, while 100 percent inhibition of aflatoxin B1 at their corresponding concentration was recorded to be 0.1 and 0.3 ml/ml, respectively (Mishra *et al*, 2013) [21]. The development of *Penicillium italicum*, *Penicillium expansum*, *Cladosporium*, *Rhizopus stolonifer* was significantly inhibited by Thymol and Carvacrol at 250 ppm (Camele *et al*., 2012) [7].

On the other hand, bacteriocins are ribosomally synthesized peptides or antimicrobial proteins produced by bacteria that kills or inhibits the growth of other bacteria. MicroGARD™ is a class of bacteriocin having bacterial peptide that usually but often closely are linked to produce strain inhibiting or destroying micro-organisms. The bacteriocin produced from Gram-positive bacteria involves the general killing mechanisms via pore formation and enzyme activity modulation or Quorum sensing (Gillor *et al*., 2007 and Turovskiy *et al*., 2007) [9, 40]. The biopreservative MicroGARD™ is synthesized by *Propionibacterium freudenreichii* subsp. *Shermanii* as a result of fermentation in pasteurized skim milk (Salih *et al*., 1985) [27]. It was approved for use in products such as cottage cheese and yogurt by the FDA. MicroGARD™ prevents the development of a variety of organism-causing spoilage and is commercially used for preservation in a range of dairy and nondairy products. It is active against gamma-negative bacteria (species of *Pseudomonas*, *Salmonella*, and *Yersinia*), selected yeasts and molds, but not grain-positive bacteria (Al-Zorekey *et al*., 1991) [1]. Al-Zoreky (1991) stated that MicroGARD™ performed optimally under pH 5.3. Even though 3 percent of MicroGARD™ gave complete inhibition against the test bacterium at pH 5.3, the concentration of 1 percent gave the same inhibition when cells were further diluted to give around 10^4 cfu/ml.

While the optimum level of different ingredients can be determined by changing one ingredient at a time during the development of any new food product, this approach is time-consuming, tedious, and sometimes fails to deliver the best combination and does not depict the combined effects of all factors involved. Therefore, four factorial experiment was implemented for the selection of level of thyme essential oil (0 to 0.75%) and MicroGARD™–100 (0 to 0.75%). Two-way ANOVA using Proc GLM of SAS 9.3 was used to achieve the best combination of natural preservatives based on sensory, physicochemical (water activity, colour value, phenolic content and antioxidant value), and zone of inhibition (for *E. coli* and *Aspergillus niger*). The table spread containing natural colourant extracted from carrot bio-waste in flaxseed oil was prepared by the method previously standardized in our laboratory (Tiwari *et al*., 2019; Kamble, 2019) [39, 16]. Butter and flaxseed oil were used as source of fat, flaxseed oil being outstanding source of omega-3 rich fatty acids. The aim of present investigation was to optimize level of essential oil & bacteriocin for extending shelf life of functional table spread using natural ingredients.

2. Material and Method

2.1 Ingredients

Carrots were procured from Vegetable Science Division, ICAR-IARI, Delhi. Flaxseed was purchased from the local market of Tilak Bazar Chowk, Delhi. WPC was procured from Milk Specialties Global. All other chemicals used were purchased from Sigma Aldrich Chemicals Pvt. Ltd, Fisher Scientific India Pvt. Ltd, HiMedia Laboratories Pvt. Ltd, or Easy Life Retailing Pvt. Ltd. MicroGARD™-100 was generously donated by DuPont (Danisco India Pvt. Ltd. Haryana); while thyme essential oil was procured from local market of Tilak Bazar Chowk, Delhi. Extraction of carotenoids from carrot pomace in flaxseed oil (CRE) was carried out according to protocol optimized by our research group (Tiwari *et al.* 2019; Kamble, 2019) [39, 16].

2.2 Preparation of Table spread

The preparation of table spread was carried out by mixing two phases i.e. aqueous and oil phase. Aqueous phase was prepared by mixing predetermined quantities of all dry ingredients like WPC, salt, TSC, emulsifier, stabilizer etc. in water and oil phase was prepared by mixing melted cow butter with carotenoid rich extract as standardized by Kamble (2019) [16]. The prepared oil phase was added into aqueous phase and blended for 10 min to obtained stable emulsion of table spread. The obtained emulsion was heated at 65 °C and homogenized at 1000 psi followed by pasteurization at 75 °C for 15 min and addition of natural preservatives at 16 different levels as indicated in Table 1. After pasteurization the prepared table spread was cooled to the room temperature and packed into PET jar and stored at 5±1 °C.

2.3 Sensory Analysis of Table spread

The prepared table spreads were evaluated by trained panelists on the basis of 9-point hedonic scale for flavour, body and texture, colour and appearance, spreadability, and overall acceptability. The samples were served in polystyrene cups along with bread and spoon for checking the spreadability.

2.4 Proximate composition analysis of Table spread

2.4.1 Estimation of protein

The protein content of table spread was determined by Semimicro Kjeldahl method (Menefee and Overman, 1940) [19]. The nitrogen content was calculated using the formula given below and the factor 6.38 was used for the conversion of percent nitrogen into protein content of table spread.

$$\%\ Nitrogen\ content = \frac{1.4007 \times (Vs - Vb) \times N}{W}$$

$$Protein\ (\%)\ = \%\ Nitrogen\ \times\ F$$

Where,
Vs = Volume in mL of the standard sulphuric acid used for sample
Vb = Volume in mL of the standard sulphuric acid used for blank
N = Normality of sulphuric acid (0.02N)
W = Mass of test portion (in g); expressed to nearest 0.1 mg
F = Conversion factor for nitrogen to protein i.e. 6.38

2.4.2 Estimation of Fat
The fat content of the spread was estimated as per the method given by Bligh and Dyer (1959) [5] with some modification. Twenty gram of sample was added to separating followed by addition of 50ml methanol and 25ml chloroform thereafter shaken gently. To the mixture, 27 ml water was added followed by shaking gently and adding 0.7 g NaCl. The mixture was held till separation of bi-layer. The chloroform layer containing fat was filtered through Whatman filter paper No.1 (with pinch of sodium sulfate placed on filter paper). The remaining residue was washed with the addition of 15ml ethanol and 15 ml chloroform. The mixture was shaken gently and held till separation of bi-layer. Fat rich chloroform layer was filtered through Whatman filter paper No.1 containing pinch of sodium sulfate. The chloroform was evaporated and fat content of table spread was calculated by following formula:

$$Fat\ (\%) = \frac{\text{Weight of the extracted fat (W2)}}{\text{Weight of the sample (W1)}} \times 100$$

2.4.3 Estimation of Moisture
The moisture content of the table spread was determined using the method described in I.S.I. Hand book of Food Analysis (Part XIII) – 1984) [13] and calculated by the following formula:

$$\text{Moisture } \% = \frac{\text{weight Loss in g of the material on drying (W1)}}{\text{Weight in g of the material taken for test (W)}} \times 100$$

2.4.4 Estimation of ash content
The ash content of table spread was estimated using the method as described in IS: SP: 18 (Part XI- 1981). The ash content was determined using the following formula.

$$\text{Ash } \% = \frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100$$

2.5 Determination of antimicrobial activity
2.5.1 Determination of zone of inhibition
2.5.1.1 Sample preparation
Ten percent of table spread were prepared in dimethyl sulphoxide (DMSO) followed by mixing on magnetic stirrer for 30 min (Seenivasan *et al.*, 2006). After that the sample was centrifuged at 4000 rpm for 10 min and the supernatant was collected in the Eppendorf tube and store at 4±1 °C until the use.

2.5.1.2 Procedure
Antimicrobial activity of table spread was determined by well assay method as described by Minj (2017) [20] against test organisms *Escherichia coli* and *Aspergillus niger*.

2.6 Estimation of antioxidant activity
2.6.1 Preparation of sample
Ten grams of table spread was weighed and 15 mL, absolute methanol was added to it. Centrifugation was done at 4000 rpm for 15 min. Thereafter, the supernatant was collected; 15 mL of 80% methanol was again added to this pellet and centrifuged for 15 min followed by collecting the supernatant. Next, 60% of 15 mL methanol was added to the pellet and centrifugation was done and supernatant was collected. All the supernatants were mixed and used as sample for analysis.

2.6.2 ABTS (2, 2-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) antioxidant activity
The ABTS antioxidant activity was measured by method given by Awika *et al*. (2003) [3]. 100 µL of sample extract was mixed with 2900 µL ABTS working solution and was allowed to react in dark for 30 min and absorbance was measured at 734 nm. Trolox was used as standard antioxidant.

2.7 Statistical analysis
All the experiments were carried out in triplicate. Results were expressed as mean ± standard deviation (SD). The data obtained during optimization was analyzed for two-way ANOVA using Proc GLM method of SAS 9.3. The optimized product was analyzed for proximate composition and compared with the positive and negative control using one-way ANOVA method in IBM SPSS Statistics 25 software as function of multiple comparison Tukey Test ($p< 0.05$).

3. Result and Discussion
The present work was aimed at incorporating the natural preservatives in the form of thyme essential oil and MicroGARD™-100 for checking the growth of micro-organisms. The table spread studied contained natural carotenoids as a colouring material (which were extracted from carrot pomace in flaxseed oil making the table spread omega-3 rich) as optimized by Kamble (2019) [16], while the combination of natural preservatives were optimized in the present study. Therefore, the aim of the present study was to have table spread prepared using all-natural ingredients. The natural preservatives generally include several herbs, essential oils, plant parts, fruits and vegetable extract. The criteria for the selection of preservatives as reported by Sethi (2017) [30] are optimum activity against targeted microorganism, active against microorganism for the desired shelf life and must not interfere with the quality and characteristics of the product.
Based on the preliminary trials, it was found that one essential oil (i.e. thyme oil, referred to as TEO) yielded the better desired flavor than other essential oils, while MicroGARD™-100 (MG) was used due to its reported properties of inhibition of gram-negative bacteria, some yeast and mold. Therefore, a combination of TEO and MG were used in this study. Further, the selection of appropriate level of combination of these preservatives was studied using 4^2 factorial experiment. These 16 treatments consisting of factorial combination of two preservatives each at 4 levels were analyzed for optimization.

3.1 Optimization of the preservative level
Table spread was prepared by the method suggested by Kamble, (2019) [16] with slight modifications and MG and TEO were used as the source of a natural preservative at the

rate of 0%, 0.25%, 0.50%, and 0.75% each. The combined effect of these preservatives was studied on zone of inhibition, antimicrobial activity, physicochemical properties and sensorial attributes for the selection of optimum combination of these.

3.1.1 Optimization based on antimicrobial activity and functional properties

3.1.1.1 Effect of combination of natural preservatives on Zone of inhibition (Agar well assay) of *E. coli* and *A. niger*

The zone of inhibition method was used to test the ability of preservative to inhibit the microbial growth for selection of the optimum combination of preservative for the long shelf life of the product. For this, *E. coli* and *Aspergillus niger* were used as representative microorganism as at the industrial level, *E. coli* is used as an indicator and index microorganism for fecal contamination of drinking water (Guentert & Linton, 2003) [10]. *Aspergillus niger* is one of the most common fungi of the *Aspergillus* genus and causes black mold disease in the fruit and vegetable and also it is lipolytic and a common contamination in food product (Sharma, 2012) [31]. Dimethyl sulfoxide (DMSO) is an organosulfur compound (Polarity Index 7.2) and it dissolves both polar and nonpolar compounds. Essential oils are highly soluble in DMSO. Seenivasan *et al*. (2006) used 10% solution in DMSO for the estimation of the antimicrobial activity of essential oil. Hence in this study, 10% solution in DMSO was used for the estimation of antimicrobial activity by the zone of inhibition method.

Table 1 shows all possible combination of TEO and MG interaction and it can be seen that significantly ($p<0.001$) lowest value of zone of inhibition for *E.coli* was obtained at 0% level each of TEO and MG, while significantly ($p<0.001$) highest value was obtained at 0.75% and 0.50% or 0.75% and 0.75% level of TEO and MG, respectively. It can be observed from Table 2, TEO and MG individually and in combination showed a highly significant ($p< 0.001$) difference on zone of inhibition. Further, Table 3 revealed that as the level of TEO and MG increased, the zone of inhibition also increased significantly ($p< 0.001$). However, for MG, it increased significantly ($p< 0.001$) from 0% to 0.50%, while it increased non-significantly ($p>0.05$) from 0.50% to 0.75%. From Table 3, it can be revealed that 0.75% and 0.50% of TEO and MG, respectively could be selected for further study as this combination yielded the highest zone of inhibition for *E. coli* (while the lowest zone of inhibition was obtained when level of both the preservative was 0%). Sienkiewicz *et al*., (2011) [32] reported that due to the presence of high amount of phenolic content, thyme oil has strong antimicrobial activity against *E. coli*. Also, Boskovic *et al*. (2015) [6] studied that thyme oil exhibited strong antimicrobial activity against *E. coil*. The significantly ($p< 0.001$) lowest zone of inhibition was obtained at 0% and 0%, while significantly ($p< 0.001$) higher zone of nhibition was obtained at 0.75% and 0.50% or 0.75% and 0.75% of TEO and MG respectively for *A. niger*. It was interesting to note from Table 2 that significant difference ($p< 0.001$) existed in the zone of inhibition due to TEO and MG individually and in combination. Further, Table 3 revealed that as the level of TEO and MG increased, the zone of inhibition for *A. niger* also increased significantly ($p< 0.001$). This increase was significant ($p< 0.001$) at all the treatment levels for TEO. However, for MG, it increased significantly ($p< 0.001$) from 0% to 0.50%, while it increased non-significantly ($p>0.05$) from 0.50% to 0.75% (Table 3). Further, Table 3 also revealed that 0.75% and 0.50% of TEO and MG, respectively could be selected for further study. However, with 0.75% MG, the increase in zone of inhibition was more than 1 unit, therefore 0.75% MG level could be selected from this parameter. Thus, 0.75% each of TEO and MG could be selected for further study based on zone of inhibition for *A. niger*.

Table 1: Effect of different levels of preservative on zone of inhibition, phenolic content, antioxidant, water activity, colour value and sensory parameters of Table spread

Treatments	Level (%) of natural preservative		Zone of inhibition (mm)		Phenolic content (mg GAE/g)	Antioxidant (µg Trolox eq./mL)	Water activity	Colour value			Sensory				
	Thyme essential oil	MicroGARD™-100	*E. Coil*	*Aspergillus niger*				L*	a*	b*	Colour and appearance	Flavour	Body and texture	Spreadability	Overall Acceptability
T_1	**0**	**0.00**	0.000^{f}	0.000^{h}	0.124	10.560	0.981^{e}	82.437^{b}	4.353^{i}	27.787^{m}	7.929^{ab}	7.143	6.643^{defgh}	7.000	6.786^{bcd}
T_2	**0**	**0.25**	16.667^{e}	16.333^{g}	0.137	14.188	0.986^{bc}	81.877^{c}	4.273^{j}	28.513^{k}	8.000^{a}	6.571	6.857^{bcdefg}	7.286	6.857^{bc}
T_3	**0**	**0.50**	15.66^{e}	16.667^{g}	0.139	10.963	0.989^{a}	81.447^{d}	4.180^{k}	28.393^{k}	7.786^{abcd}	7.071	7.143^{abcd}	6.357	7.000^{abc}
T_4	**0**	**0.75**	20.667^{d}	20.000^{ef}	0.140	13.180	0.983^{de}	82.397^{b}	4.550^{h}	28.010^{l}	7.857^{abc}	7.000	6.571^{efgh}	6.429	5.643^{f}
T_5	**0.25**	**0.00**	19.667^{d}	19.000^{f}	0.334	126.863	0.987^{ab}	83.863^{a}	4.533^{h}	29.497^{i}	7.357^{de}	6.857	7.286^{ab}	7.286	7.214^{ab}
T_6	**0.25**	**0.25**	20.333^{d}	21.000^{e}	0.335	126.258	0.981^{e}	83.743^{a}	4.673^{g}	29.583^{i}	7.571^{abcd}	7.143	7.286^{ab}	6.143	6.071^{ef}
T_7	**0.25**	**0.50**	27.000^{ab}	26.333^{bc}	0.333	131.297	0.981^{e}	80.227^{e}	4.647^{g}	31.023^{f}	7.000^{efg}	7.214	6.071^{i}	7.500	6.071^{ef}
T_8	**0.25**	**0.75**	24.333^{c}	28.333^{a}	0.330	128.274	0.982^{d}	79.547^{f}	4.747^{e}	29.877^{h}	7.500^{bcd}	6.643	7.286^{ab}	7.214	7.214^{ab}
T_9	**0.50**	**0.00**	24.667^{c}	24.000^{d}	0.519	273.199	0.984^{cd}	79.623^{f}	4.693^{fg}	30.610^{g}	7.357^{de}	7.429	7.214^{abc}	7.571	7.214^{ab}
T_{10}	**0.50**	**0.25**	26.667^{ab}	26.000^{c}	0.524	274.812	0.984^{cd}	78.993^{hi}	4.780^{e}	29.257^{j}	7.429^{cde}	7.071	7.143^{abcd}	7.714	7.429^{a}
T_{11}	**0.50**	**0.50**	27.000^{ab}	26.667^{bc}	0.514	278.440	0.981^{e}	79.280^{g}	4.680^{fg}	30.977^{f}	6.714^{fg}	6.643	7.071^{abcde}	7.429	6.857^{bc}
T_{12}	**0.50**	**0.75**	27.000^{ab}	26.000^{c}	0.533	281.867	0.976^{g}	78.900^{i}	4.747^{ef}	31.603^{e}	6.571^{gh}	6.714	7.000^{abcdef}	7.500	7.143^{ab}
T_{13}	**0.75**	**0.00**	26.333^{b}	26.667^{bc}	0.734	427.800	0.981^{e}	79.107^{h}	5.400^{c}	33.553^{c}	7.071^{ef}	5.857	7.357^{a}	6.714	6.500^{cde}
T_{14}	**0.75**	**0.25**	27.000^{ab}	27.333^{ab}	0.727	424.575	0.979^{f}	78.927^{i}	5.393^{d}	35.340^{a}	6.143^{h}	6.429	6.286^{h}	7.214	6.286^{de}
T_{15}	**0.75**	**0.50**	27.667^{a}	28.000^{a}	0.732	423.970	0.979^{f}	78.723^{j}	5.777^{a}	33.417^{d}	7.429^{cde}	6.143	7.071^{abcde}	7.000	6.000^{ef}
T_{16}	**0.75**	**0.75**	27.667^{a}	28.000^{a}	0.724	425.179	0.981^{ef}	78.493^{k}	5.513^{b}	34.393^{b}	6.214^{h}	6.000	6.357^{gh}	6.714	5.714^{f}

CD (Treatments) ($P< 0.05$) For *E. Coli*: 1.18; *Aspergillus niger*:1.08; Water activity: 0.002; L*: 0.14; a*: 0.07; b*: 0.13; Colour and appearance: 0.46; Overall acceptability: 0.51; Body and Texure: 0.52
CD (Treatments) (P>0.05) for Phenolic content, Antioxidant, Flavour, and spread ability: NS
Superscript$^{a\text{-}m}$: column wise

Table 2: ANOVA table for *E. coli*, *Aspergillus niger*, phenolic content, antioxidant, water activity, colour value and sensory parameters of Table spread

Source	DF	Mean Square												
		E.coli	*Aspergillus niger*	Phenolic content	Antioxidant	Water activity	Colour value			Sensory				
							L*	a*	b*	Colour and appearance	Flavor	Body and texture	Spreadability	Overall acceptability
T	3	488.410**	487.799**	0.777**	386792.100*	4.944×10^{-5}*	34.861**	3.039**	75.805**	7.145**	5.074**	0.705	3.282*	5.042**
M	3	130.187**	156.188**	3.742×10^{-5}	15.987	1.444×10^{-5}	6.000**	0.046*	0.984**	0.740	0.318	0.586	0.157	1.423
T*M	9	52.928**	49.706**	13.882×10^{-5}	20.599	3.159×10^{-5}*	3.590**	0.0516**	2.055**	1.316*	0.655	1.492*	1.453	1.857**

T: Thyme Essential oil; M: MicroGARD™-100
**($P< 0.001$)
*($P< 0.05$)

3.1.1.2 Effect of combination of natural preservatives on Phenolic Content

Natural ingredients are the main source of phenolic content in the food product. Phenolic content gives the antioxidant and antimicrobial activity in the product. The phenolic content of essential oil, carrots and flaxseed oil have been reported previously to be 783.81 mg GAE/l (Viuda-Martos *et al*., 2009), 26.6±1.70 µg/g (Oviasogie *et al*., 2009) and 14.23–16.64 mgGAE/kg (Herchi *et al.*, 2011) [12], respectively which also depends upon varieties of these commodities.

Table 2 revealed significant effect (P<0.001) of TEO individually on phenolic content of the table spread, while MG individually and in combination with TEO showed non-significant (P>0.05) effect. The phenolic content of different combinations of the product at varying treatment level of preservatives are shown in Table 1. From Table 3, it can be observed that the increase in concentration of TEO led to significantly (p<0.001) increased phenolic content of the product at all the treatment levels (i.e. 0, 0.25, 0.50 and 0.75%). Therefore, from Table 3, TEO at 0.75% could be selected for further study, while MG at lowest level (0%) should be selected based on phenolic content. But, considering the results of zone of inhibition for *Aspergillus niger* (as table spreads kind of product are more prone to spoilage by yeast & mold than bacteria), MG at 0.75% could be selected as MG did not contribute to the phenolic content (non-significant effect at P>0.05), but to inhibiting the microbial load of the product. Therefore, based on the results, TEO at 0.75% and MG at 0.75% could be selected.

3.1.1.3 Effect of combination of natural preservatives on Antioxidant activity

Essential oils give the antioxidant and antimicrobial activity mainly due to the presence of phenolic and flavonoid compounds. Gedikoğlu *et al*. (2019) evaluated *Thymus vulgaris* and *Thymbra spicata* essential oils and found that the hydrophilic fraction of essential oil had higher antioxidant activity. Further, in the present study, the prepared table spread was rich in carotenoid which is lipophilic compound (Tapiero *et al*., 2004) [37]. $ABTS^{\bullet+}$ is not affected by ion strength and it is soluble in both organic and aqueous solvent so it is used to determine both hydrophilic and lipophilic antioxidant activity of extract (Prior *et al*., 2005) [26].

$ABTS^{\bullet+}$ is a stable cation which gives a blue-green chromophore with maximum absorption at 734 nm. The antioxidants present in the food system scavenge $ABTS^{\bullet+}$. Thus, the intensity of the colour decreases in the presence of antioxidant due to neutralization of the radical cation $ABTS^{\bullet+}$ either by quenching of the hydrogen atom or donation of electrons. Table 2 revealed significant effect (p< 0.001) of TEO individually on antioxidant activity of the table spread, while MG individually and in combination with TEO showed non-significant (P>0.05) effect. Antioxidant activity value of the table spread prepared at different treatment interaction levels of the preservative is given in Table 1. It can be observed that a non-significantly (p>0.05) lower value of antioxidant obtained at 0% level each while non-significantly (p>0.05) higher value at 0.75% level each of TEO and MG (Table 1). From the Table 3, it can be observed that the increase in the concentration of TEO led to significantly (p< 0.001) increased antioxidant activity of the product at all the treatment levels (i.e. 0, 0.25, 0.50 and 0.75%). Therefore, based on these results TEO at 0.75% could be selected for further study, while MG at lowest level should be selected based on antioxidant activity. But, considering the results of zone of inhibition for *A. niger*, MG at 0.50% could be selected as MG did not contribute to the antioxidant activity (non-significant effect at P>0.05), but contributed in reducing *A. niger* load of the product. Therefore, based on the results, TEO at 0.75% and MG at 0.75% could be selected.

3.1.2 Effect of combination of natural preservatives on Physical properties of table spread

3.1.2.1 Water activity

The water activity value of table spreads at different treatment level of the preservative are shown in Table 1. Table 2 revealed the significant effect (p< 0.05) on water activity due to the treatment levels of TEO individually and in combination with MG, however, MG alone showed a non-significant (P>0.05) effect on water activity of table spread. It can be observed from Table 3 that as the level of TEO increased, the water activity decreased, this decrease was non-significant (P>0.05) between the treatment levels of 0% and 0.25%; 0.25% and 0.50%; and 0.50 and 0.75%. TEO and MG at 0 and 0.50%, respectively gave highest a_w, while 0.50% and 0.75% corresponding values resulted in lowest a_w (Table 3). Therefore, based on these observations, TEO and MG at 0.50% and 0.75%, respectively could be selected based on the basis of lowest a_w of this combination across Table 1.

3.1.2.2 Colour value: L*, a* and b*

The colour of the food has quite an impact on the selection of the food product and also influences the consumer perception of taste, flavor or odour.

L* value is a measure of lightness and darkness of a product and its range vary from 0 to 100, where L* value of 100 indicates the completely white body and 0 value indicates completely black body. The effect of the preservative was measured on the L* value and readings at different treatment level of the preservative is given in Table 1. It is revealed from Table 2 that TEO and MG individually and in combination had significant effect (p< 0.001) on L* value of table spread. From Table 1, for the combination of TEO and MG, significantly (p< 0.001) lowest value was obtained at 0.25% and 0% level, while significantly (p< 0.001) higher value was obtained at 0.75% and 0.75% level, respectively. Further, as the level of TEO and MG increased, the level of L* decreased significantly (p< 0.001) except between 0.50 and 0.75% levels of MG (Table 3) where a non-significant difference (P>0.05) was observed. The significantly (p< 0.001) highest L* value was obtained when TEO and MG were used at a level of 0.25% each, while significantly (p< 0.001) lowest value was obtained with the corresponding level of 0.75% each. Based on this, the TEO and MG that could be selected is 0.75% each on account of the lowest L*value across Table 3. Santora *et al.* (2018) [29] used thyme oil for the improvement of storage quality of peaches and nectarine and the researchers observed that L* value was increased but hue angle (h) decreased with the increasing level of TEO.

Table 3: Effect of preservatives and their levels on Zone of inhibition, Phenolic, Antioxidant, a_w, and colour value

	Preservative → / Level (%) ↓		Thyme essential oil				
			0	**0.25**	**0.50**	**0.75**	**Overall mean**
Zone of inhibition (*E.coli*)	**MicroGARD™-100**	**0**	0.0000	19.6667	24.6667	26.3333	17.6667^{r}
		0.25	16.6667	20.3333	26.6667	27.0000	22.6667^{q}
		0.50	15.6667	27.0000	27.0000	27.6667	24.3333^{p}
		0.75	20.6667	24.3333	27.0000	27.6667	24.9167^{p}
		Overall mean	13.2500^{d}	22.8333^{c}	26.3333^{b}	27.1667^{a}	
Zone of inhibition (*Aspergillus niger*)	**MicroGARD™-100**	**0**	0.00	19.00	24.00	26.67	17.42^{r}
		0.25	16.33	21.00	26.00	27.33	22.67^{q}
		0.50	16.67	26.33	26.67	28.00	24.42^{p}
		0.75	20.00	28.33	26.00	28.00	25.58^{p}
		Overall mean	13.25^{d}	23.67^{c}	25.67^{b}	27.50^{a}	
Phenolic content	**MicroGARD™-100**	**0**	0.1241	0.3341	0.5188	0.7341	0.4278^{p}
		0.25	0.1375	0.3348	0.5241	0.7268	0.4308^{p}
		0.50	0.1395	0.3335	0.5141	0.7321	0.4298^{p}
		0.75	0.1401	0.3301	0.5335	0.7241	0.4320^{p}
		Overall mean	0.1353^{d}	0.3331^{c}	0.5226^{b}	0.7293^{a}	
Antioxidant activity	**MicroGARD™-100**	**0**	10.5599	126.8630	273.1993	427.7997	209.6054^{p}
		0.25	14.1880	126.2583	274.8118	424.5747	209.9582^{p}
		0.50	10.9630	131.2974	278.4399	423.9700	211.1676^{p}
		0.75	13.1802	128.2739	281.8666	425.1794	212.1250^{p}
		Overall mean	12.2228^{d}	128.1731^{c}	277.0794^{b}	425.3809^{a}	
Water activity	**MicroGARD™-100**	**0**	0.9813	0.9873	0.9840	0.9813	0.9835^{p}
		0.25	0.9860	0.9807	0.9843	0.9787	0.9824^{p}
		0.50	0.9889	0.9807	0.9807	0.9793	0.9824^{p}
		0.75	0.9833	0.9823	0.9763	0.9813	0.9808^{p}
		Overall mean	0.9849^{a}	0.9828^{ab}	0.9813^{bc}	0.9802^{c}	
L* value	**MicroGARD™-100**	**0**	82.4367	83.8633	79.6233	79.1067	81.2575^{p}
		0.25	81.8767	83.7433	78.9933	78.9267	80.8850^{q}
		0.50	81.4467	80.2267	79.2800	78.7233	79.9192^{r}
		0.75	82.3967	79.5467	78.9000	78.4933	79.8342^{r}
		Overall mean	82.0392^{a}	81.8450^{b}	79.1992^{c}	78.8125^{d}	
a* value	**MicroGARD™-100**	**0**	4.3533	4.5333	4.6933	5.4000	4.7450^{q}
		0.25	4.2733	4.6733	4.7800	5.3933	4.7800^{q}
		0.50	4.1800	4.6467	4.6800	5.7767	4.8208^{pq}
		0.75	4.5500	4.7467	4.7467	5.5133	4.8892^{p}
		Overall mean	4.3392^{d}	4.6500^{c}	4.7250^{b}	5.5208^{a}	
b* value	**MicroGARD™-100**	**0**	27.7867	29.4967	30.6100	33.5533	30.3617^{r}
		0.25	28.5133	29.5833	29.2567	35.3400	30.6733^{q}
		0.50	28.3933	31.0233	30.9767	33.4167	30.9525^{p}
		0.75	28.0100	29.8767	31.6033	34.3933	30.9708^{p}
		Overall mean	28.1758^{d}	29.9950^{c}	30.6117^{b}	34.1758^{a}	

CD (TEO) (P<0.05) *E.coli*: 1.36; *Aspergillus niger*:1.25; Phenolic content: 0.05; Antioxidant: 2.60; Water activity: 0.002; L*: 0.16; a*: 0.08; b*: 0.16
CD (MicroGARD™-100) (P<0.05): *E.coli*: 1.36; *Aspergillus niger*: 1.25; L*: 0.16; a*: 0.08; b*: 0.16
CD (treatment) (P<0.05): *E.coli*: 1.18; *Aspergillus niger*: 1.08; Water activity: 0.002; L*: 0.14; a*: 0.07; b*: 0.13

The value of a* indicates redness and greenness in product and its value ranges from +60 to -60. The positive value represents redness of the sample, while a negative value represents greenness of product. The a* value of the products at different treatments level of the preservatives is mentioned in Table 1. From Table 1, it can be revealed that a significantly (P< 0.001) lower value obtained at 0% and 0.50% level, while significantly (P< 0.001) higher value obtained at 0.75% and 0.50% level, respectively. Table 2 depicts the significant effect (P< 0.001) on a* value due to the presence of TEO and MG individually and in combination (P< 0.05). Further, it can be noted from Table 3 that as the level of TEO increased, the a* value also increased significantly (P< 0.001) at all the levels (i.e. from 0% till 0.75%), while as the level of MG increased, the a* value showed an increasing trend, but a* value increased non-significantly (p>0.05) from 0 to 0.25% to 0.50%; and from 0.50% to 0.75% {at other values the increase was significant (P< 0.001)}. It can be observed from Table 3 that the highest a* value was obtained at 0.75% and 0.50% level of TEO and MG, respectively, while their corresponding level of 0% and 0.50% yielded lowest value of a*. Therefore, based on a* value, TEO and MG could be selected at the highest level of 0.75% and 0.50%, respectively.

The value of b* indicates yellowness and blueness in product and its value ranges from +60 to -60. The positive value represents yellowness of the sample, while a negative value represents blueness of the product. The b* value of the

product at different treatment level of the preservatives is mentioned in Table 1. From Table 1, it can be observed that a significantly (P< 0.001) lower value of b* obtained at 0% and 0.25% level, while significantly (P< 0.001) higher value of b* obtained at 0.75% and 0.25% level of TEO and MG, respectively. Table 2 depicts the significant effect on a* value due to the presence of TEO and in combination with MG (P<0.001), while MG individually showed significant effect at p<0.05. Further, it can be noted from Table 3 that as the level of TEO increased, the b* value also increased significantly at all the levels (i.e. from 0% till 0.75%), while as the level of MG increased, the b* value showed an increasing trend, but the increase was non- significant (P>0.05) from 0.50 to 0.75% level (at other levels the increase was significant). It can be observed from Table 3 that the highest b* value was obtained with 0.75% and 0.25% level of TEO and MG, respectively, while the lowest value was obtained in the combination that did not contain any of these natural preservatives. Therefore, based on obtaining highest b* value, TEO and MG at 0.75% and 0.25% level, respectively could be selected based on the values given across Table 3.

3.1.3 Effect of combination of natural preservatives on sensory properties

The 16 samples of table spread formulated with varying levels of both the natural preservatives were subjected for sensory evaluation to the trained sensory panelist for colour & appearance, flavor, body & texture, spreadability and overall acceptability using 9-point hedonic scale score-card. Table 1 contains the sensory score obtained for these 16 combinations for optimization of the level of preservative.

3.1.3.1 Colour and appearance

Colour and appearance is the first and most important attribute for the acceptance or rejection of the food product by the consumer. Sensory score of the table spread (at different treatment level of the preservative) is shown in Table 1 for colour and appearance. It is revealed from Table 2 that the presence of TEO (0%, 0.25%, 0.50%, and 0.75% treatments level) individually (P< 0.001) and in combination with MG (P< 0.05) had significant effect on colour and appearance of the product, while MG individually showed non-significant effect (P>0.05) on colour and appearance. From Table 4, it can be noted that as the level of TEO increased, the colour and appearance score decreased significantly (P< 0.001) from 0% to 0.25% to 0.50% to 0.75%, the minimum average score for color and appearance was obtained at 0.75% treatment level of TEO, while the maximum average score was obtained at 0% treatment level of TEO (Table 4). This could be due to increase in brownish colour in the samples with increasing level of TEO. It was observed that the lowest level of both the preservatives i.e. 0% each should be selected based on the highest score for colour and appearance. However, as discussed previously, this combination yielded very less zone of inhibition, therefore, a combination of 0.25% each of the two preservatives could be selected as it is evident from Table 4 that TEO and MG in combinations 0, 0%; 0, 0.25%; 0, 0.50; 0, 0.75% and 0.25, 0.25% were non-significantly different. Recently, Sethi (2017) [30] prepared fat spread by using TEO and wheat germ oil as a source of natural preservative and reported that the sensory score of fat spread for colour and appearance was decreased with increasing the level of TEO from 0.20% to 1.00% and the authors also found the same trend for wheat germ oil (from 0.05% to 1.50%).

Table 4: Effect of preservative and their levels on Sensory parameters

	Preservative → / ↓		Thyme Essential oil (TEO)				
		Level (%)	0	0.25	0.50	0.75	Overall mean
Colour and appearance	MicroGARD™-100	0	7.9286	7.3571	7.3571	7.0714	7.4286^{p}
		0.25	8.0000	7.5714	7.4286	6.1429	7.2857^{p}
		0.50	7.7857	7.0000	6.7143	7.4286	7.2321^{p}
		0.75	7.8571	7.5000	6.5714	6.2143	7.0357^{p}
		Overall mean	7.8929^{a}	7.3571^{b}	7.0179^{c}	6.7143^{d}	
Flavour	MicroGARD™-100	0	7.1429	6.8571	7.4286	5.8571	6.8214^{p}
		0.25	6.5714	7.1429	7.0714	6.4286	6.8036^{p}
		0.50	7.0714	7.2143	6.6429	6.1429	6.7679^{p}
		0.75	7.0000	6.6429	6.7143	6.0000	6.5893^{p}
		Overall mean	6.9464^{b}	6.9643^{a}	6.9643^{a}	6.1071^{c}	
Body and texture	MicroGARD™-100	0	6.6429	7.2857	7.2143	7.3571	7.1250^{p}
		0.25	6.8571	7.2857	7.1429	6.2857	6.8929^{p}
		0.50	7.1429	6.0714	7.0714	7.0714	6.8393^{p}
		0.75	6.5714	7.2857	7.0000	6.3571	6.8036^{p}
		Overall mean	6.8036^{a}	6.9821^{a}	7.1071^{a}	6.7679^{a}	
Spreadability	MicroGARD™-100	0	7.0000	7.2857	7.5714	6.7143	7.1429^{p}
		0.25	7.2857	6.1429	7.7143	7.2143	7.0893^{p}
		0.50	6.3571	7.5000	7.4286	7.0000	7.0714^{p}
		0.75	6.4286	7.2143	7.5000	6.7143	6.9643^{p}
		Overall mean	6.7679^{c}	7.0357^{b}	7.5536^{a}	6.9107^{d}	
Overall acceptability	MicroGARD™-100	0	6.7857	7.2143	7.2143	6.5000	6.9286^{p}
		0.25	6.8571	6.0714	7.4286	6.2857	6.6607^{p}
		0.50	7.0000	6.0714	6.8571	6.0000	6.4821^{p}
		0.75	5.6429	7.2143	7.1429	5.7143	6.4286^{p}
		Overall mean	6.5714^{b}	6.6429^{b}	7.1607^{a}	6.1250^{b}	

CD (TEO) (P<0.05) Colour and appearance: 0.35; Flavour: 0.41; Body & Texture: 0.39; Spreadability: 0.47; Overall acceptability: 0.59
CD (Treatments) (P<0.05) Colour and appearance: 0.46; Body & Texture: 0.52; Overall acceptability: 0.51

3.1.3.2 Flavour

Flavour of food product is the top preference of the consumer for selection of the product. Sensory score of the product at different treatment interaction of preservatives is shown in Table 1 for the flavour. Table 2 revealed significant effect ($P< 0.001$) of TEO individually on flavor of the table spread, while MG individually and in combination of TEO showed non-significant (P>0.05) effect on flavor of the table spread. From the Table 4, it can be observed that the TEO was non-significantly (p>0.05) different at 0 to 0.50% level of TEO, while significantly ($P< 0.001$) minimum average score was obtained at 0.75% treatment level of TEO. This could be due to increase in perception of flavor due to the TEO volatile component. Present in TEO. Further it can be observed that flavor score decreased non-significantly (P>0.05) with increasing level of MG. Therefore, based on flavor profile, TEO at 0.50% and MG at 0.25% could be selected, since beyond 0.50%, the score for flavor decreases significantly and prominently (Table 4).

Sethi, (2017) [30] prepared fat spread by using the wheat germ oil and TEO as natural preservative and observed that the sensory score of the flavour was increased up to 0.60% level of TEO and after that significantly reduced i.e. at 1% level of addition of TEO, also the score was significantly reduced with the increasing level of wheat germ oil.

3.1.3.3 Body and texture

Body and texture is an important characteristic that determines acceptability of several food commodities especially spread like products. Sensory score of the product at different treatment interaction of preservatives is shown in Table 1 for body and texture. It was interesting to note that TEO and MG individually showed non-significant (P>0.05) effect on the body and texture of the table spread, however their combination showed a significant effect ($P< 0.05$) (Table 2). From Table 4, it can be observed that TEO and MG individually showed non-significant effect (p>0.05) on body and texture of spread. Based on the score of body and texture given in Table 1, the values lying between 7.3571 to 6.8371 are non-significant (p>0.05) (as the CD value was 0.52). Therefore, the lowest combination of TEO and MG (i.e. 0.25%) could be selected based on the body and texture. Sethi, (2017) [30] reported that the score of body and texture was significantly increased up to 0.6% level of thyme oil after that the score was significantly decreased but there was no-significant increase up to 1.5% level of wheat germ oil.

3.1.3.4 Spreadability

Consumer gives more preference to those spreads that have good spreadability at refrigerated temperature. Sensory score of the product at different treatment interaction of preservatives is shown in Table 1 for spreadability. Table 2 revealed significant effect ($P< 0.05$) of TEO individually on spreadability of the table spread, while MG individually and in combination of TEO showed non-significant (P>0.05) effect on spreadability of the table spread. From the Table 4, it can be observed that the significantly ($P< 0.05$) highest score for TEO was obtained when its level was 0.50%. Therefore, based on this observation, level of TEO and MG that could be selected is 0.50% and 0.25%, respectively. Sethi, (2017) [30] reported that the score of spreadability significantly increased up to 0.6% level of thyme oil after which it decreased, however it increased non-significantly up to the 1.5% level of wheat germ oil.

3.1.3.5 Overall acceptability

The score of overall acceptability is given based on the perceptibility of all the parameters i.e. colour and appearance, flavour, body and texture and spreadability, etc. Sensory score of the table spread (at different treatment level of the preservative) is shown in Table 1 for overall acceptability. It is revealed from Table 2 that the presence of TEO individually and in combination with MG had significant effect ($P< 0.001$) on overall acceptability of the product, while MG individually showed non-significant effect (P>0.05) on overall acceptability. From Table 4, it can be noted that overall acceptability score was significantly ($P< 0.001$) highest for TEO at 0.50%, while at other levels i.e. 0%, 0.25% and 0.75%, it showed a non-significant (P>0.05) difference. Therefore, based on this observation, TEO and MG could be selected at 0.50% and 0.25%, respectively.

The optimized level of preservative on the basis of each of the parameters discussed so far is mentioned in Table 5. As per zone of inhibition, functional and physico-chemical properties, it is evident that TEO and MG could be selected at a level of 0.75% each. However, the final levels that could be selected will also be influenced by the sensorial attributes of the various combinations. As per these values, it is evident that TEO and MG could be selected at a level of 0.50 and 0.25%, respectively based on scores flavor, spreadability and overall acceptability. However, the selected optimized level of TEO and MG were 0.75% each, respectively based on parameters discussed in section of optimization based on antimicrobial activity and functional properties and optimization based on physical properties. Since flavor and overall acceptability of the product are very important parameters that influence the consumer acceptability of the product, therefore, level of TEO selected for further study was 0.50% as beyond this level the scores decreased significantly. However, since MG showed non-significant effect on all the sensorial attributes, therefore its final concentration was selected to be 0.75%. Therefore, the final product was prepared using TEO and MG at a level of 0.50% and 0.75% respectively.

Table 5: Optimized level of preservatives based on antimicrobial, functional and physicochemical properties

Parameter			Preservative (%)	
			Thyme essential oil	MicroGARD™-100
Zone of inhibition	*E.Coli*		0.75	0.50
	Aspergillus niger		0.75	0.75
Functional Properties	Phenolic content		0.75	0.75
	Antioxidant		0.75	0.75
Physico-chemical	Water activity		0.50	0.75
	Colour value	L*	0.75	0.75
		a*	0.75	0.50
		b*	0.75	0.25
Sensory	Colour & appearance		0.25	0.25
	Flavour		0.50	0.25
	Body & Texture		0.25	0.25
	Spreadability		0.50	0.25
	Overall acceptability		0.50	0.25

3.2 Proximate composition of the optimized table spread and control table spread

The study involved preparation of three samples for the purpose of storage, one of which was the optimized product packed in PET jar, while other two samples were control namely, one negative control (i.e. without any preservative) and second contained potassium sorbate as a preservative (i.e. chemical preservative; positive control). All the control samples were packed in the PET jar and contained same level of other ingredients and were stored at 4±1°C. The proximate composition of the table spread is given in Table 6. The cow milk butter and flaxseed oil contributed to the fat content, while whey proteins contributed to protein content of table spread. The prepared table spread had carotenoids extracted from carrot pomace as a source of natural colourant. It is revealed that from Table 6, that a non-significant ($p>0.05$) difference was observed for protein, fat, moisture, ash and total solid content of all the sample. But the test sample packed in PET jar contained non-significantly ($p>0.05$) highest protein content followed by positive control and negative control. Whey protein is the major source of protein content in fat spread and increased the nutritional value of spread. In the fat spread, the presence of milk protein imparts the creaminess besides increasing the consumer acceptability for eating the fat spread.

Table 6: Proximate composition of control and test table spread samples

Composition	Control sample without preservative	Control sample with potassium Sorbate	Test Sample stored in PET jar
Total protein (% by wt.)	4.13±0.25[a]	4.16±0.03[a]	4.21±0.12[a]
Total fat (% by wt.)	50.82±0.08[a]	50.94±0.11[a]	51.19±0.14[a]
Total moisture (% by wt.)	37.8±0.22[a]	37.33±0.17[a]	37.16±0.07[a]
Total ash (% by wt.)	1.93±0.06[a]	1.98±0.03[a]	1.97±0.04[a]
Total solid (% by wt.)	62.2±0.22[a]	62.67±0.17[a]	62.84±0.07[a]

CD ($P< 0.05$): protein: 0.13; fat: 0.42; moisture: 0.70; ash: 0.09; total solid: 0.83

The protein content imparts organoleptic, functional and nutritional properties as well as it provides the essential amino acids which is required for normal functioning and growth of body. Many researchers reported that the protein content enhance water holding capacity and also increased the viscosity of the product and enhanced the emulsion stability during processing and storage for long time. Positive control contains non-significantly ($p>0.05$) higher fat content followed by negative control and test sample. In this fat spread the fat content is mainly contributed by the cow milk butter and flaxseed oil used for preparation of product. Fat give a characteristic structure to the product as per the requirement also it contribute the flavour, energy and creamy flavour to product besides these fats is the major source of the fat-soluble vitamin and essential fatty acids. The ratio of fat to solid affect the physical properties of spread such as firmness, plasticity, spreadability and thixotropic behavior of product (Formo *et al.* 1979) [8]. The minimum moisture is required for long shelf life of the product. Test sample contain non-significantly ($p>0.05$) lower moisture contain followed by positive control and negative control. Mineral is required for normal functioning of organs and for strengthen the bones and muscles. Ash content is not a true representative of mineral but it contains major portion of mineral. The negative control contains non-significantly higher amount of ash content followed by test sample and positive control. Similarly test sample contained higher amount of total solid followed by negative control and positive control.

4. Conclusion

In this study, carotenoid was used as natural colorant and thyme oil and MicroGARD™ -100 were used as natural preservative to replace the chemical preservative. Butter and flaxseed oil were used as source of fat, flaxseed oil being an outstanding source of omega-3 rich fatty acids. The table spread was prepared using 16 different combinations of natural preservatives as per 4^2 factorial design experiments. The results indicated that Thyme Essential oil and MicroGARD™-100 individually or in combination showed a highly significant difference ($p< 0.001$) on zone of inhibition of both *E. coli* and *Aspergillus niger*, L* and b* value. TEO showed a highly significant difference ($p< 0.001$) on phenolic content and flavour and a significant difference ($p< 0.05$) on antioxidant activity and spreadability of fat spread, while MG individually and in combination with TEO showed non-significant ($P>0.05$) effect on phenolic, antioxidant, flavour and spreadability of table spread. Based on zone of inhibition, antioxidant and sensorial attributes the Essential oil and MG

were added to the final product at the level of 0.50% and 0.75%, respectively, as at this level no significant difference was observed on the flavour characteristics of the product compared to the control sample with highest antioxidant and antimicrobial property.

The protein content of control without any preservative (CWP), control containing potassium sorbate (CPS) as preservative and optimized sample was 4.13±0.25%, 4.16±0.03% and 4.21±0.12%, respectively; while the corresponding values for total fat, moisture, ash and total solid content were obtained to be 50.82±0.08%, 50.94±0.11% and 51.19±0.14%; 37.8±0.22%, 37.33±0.17% and 37.16±0.07%; 1.93±0.06%, 1.98±0.03% and 1.97±0.04% and 62.2±0.22%, 62.67±0.17% and 62.84±0.07%, respectively.

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