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In vitro elucidation of potential synergism in antioxidant and antimicrobial activity of novel bioactive compounds (curcumin, oregano, eugenol and α-tocopherol acetate for their application in meat product

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Abstract
Background: Purified four different antioxidants and antimicrobials were selected for assessment of potential synergism in antioxidant and antimicrobial activity (curcumin, oregano, eugenol and α-tocopherol acetate) for their suitability for application in processing of reformulated chicken seekh kebab (CSK).
Methods: Eight groups of chicken seekh kebabs (CSK) were prepared using lean spent hen meat as T_1 T_2 T_3 and T_4 containing 0.025, 0.04, 0.02, 0.05g of curcumin, α-tocopherol acetate, eugenol, oregano per 100 g of sample. Remaining four treatments the amount of active ingredient added was same but added in different combinations *viz*., T_5 = α-tocopherol acetate + Eugenol, T_6 = α-tocopherol acetate + Oregano, T_7 = Curcumin + α-tocopherol acetate + Eugenol and T_8 = α-tocopherol acetate + Eugenol + Oregano.
Result: Results indicated non-significant (p>0.05) effect on pH however, protein oxidation (metmyoglobin formation%), lipid stability, antioxidant activity, microbiological quality and sensory attributes were affected greatly (p<0.05). Protein oxidation, free fatty acid, peroxide value and TBARS number increased as the storage proceeded but remained lower than acceptable limits up to 20 days. Instrumental colour coordinates varied significantly (p<0.05) except *hue*. Significantly lower values of $ABTS^+$ and DPPH were observed in the curcumin, α-tocopherol acetate and eugenol treated samples during storage period. Significantly lower values of physicochemical parameters and better lipid and oxidative stability of curcumin, α-tocopherol acetate and eugenol treated sample was clearly indicative of potential synergism in the added compounds.

Keywords: Antioxidant, antimicrobial, curcumin, lipid stability, DPPH, $ABTS^+$

Introduction
Meat and meat products are a suitable medium for microbial growth since they are high in numerous nutrients. After slaughter, the imbalance induced by the conversion of muscle to meat among prooxidative and antioxidative systems predisposes it to oxidative degradation (Cunha *et al*., 2018) [8]. Meat and meat products are degraded by metabolites generated by microbial activities and oxidation products, which have a deleterious influence on human health if consumed. Microbial spoilage and lipid or protein oxidation are now regarded key causes in the deterioration of meat and meat products, reducing their shelf life even further. Because of oxidative changes, minced meat products are more susceptible to degradation (Amaral *et al*., 2018) [2]. To combat this, synthetic preservatives (antimicrobials and antioxidants) have been employed to preserve meat and meat products, although they have negative health effects (Cunha *et al*., 2018; Mohan *et al*., 2017) [8, 27]. Consumers are more likely to consume meat as a result of their significantly altered lifestyles and limited free time. Furthermore, customer demand for meat products that are safe, healthful, and have a longer shelf life has increased dramatically (Feng *et al*., 2017) [9]. As a result, research has shifted dramatically toward the use of natural preservatives to counteract the deterioration of meat products.
In last decade, although many studies were conducted on combination of advanced packaging techniques and natural preservatives (antimicrobials and antioxidants) but potential synergistic

activity of some novel preservatives like purified curcumin, α-tocopherol acetate, eugenol, oregano etc is yet to be explored. Curcumin is the active principle obtained from turmeric and proven for its antioxidant, anti-inflammatory, anticancer and antimicrobial actions (Naksuriya *et al*., 2014) [28]. α-tocopherol acetate stands as the first line of defence against lipid peroxidation because of its ability to quench radicals, donate a hydrogen atom to a free radical and prevents the cell membrane from free radical attack (Ahmad, 1996) [1]. Eugenol (4-allyl-2-methoxyphenol), the active substance, makes up 90-95g/100g of the clove oil (Briozzo *et al*., 1989) [5]. It is a potent antioxidant (Gülçin *et al*., 2012) [14] and antimicrobial compound (Briozzo *et al*., 1989) [5]. Oregano (O*reganum vulgare*) is a comm on spice of oreganum a genus of the mint family (Lamiaceae). Its antimicrobial activity is mainly attributed to carvacrol, thymol and rosmarinic acid. In this context, present study was undertaken to assess the potential synergistic effect by adding selected novel bioactive compounds in different combination on the keeping quality of chicken seekh kebab (CSK).

Material and Methods

Chemicals and raw material

All reagents and solvents were obtained from S. D. Fine Chemicals, New Delhi and Sisco Laboratory Ltd., Mumbai, India. 2-2- azinobis-3-ethylbenthiazoline-6-sulphonic acid ($ABTS^+$), 1, 1- diphenyl-2-picrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA), curcumin, α-tocopherol acetate and eugenol were purchased from Sigma Aldrich, USA. Spent hen meat was obtained from the experimental poultry processing plant of ICAR-Central Avian Research Institute, Izatnagar, Bareilly, India.

Processing of experimental samples

A total eight groups of chicken seekh kebabs (CSK) were prepared using lean spent hen meat as T_1 T_2 T_3 and T_4 containing 0.025, 0.04, 0.02, 0.05 g of curcumin, α-tocopherol acetate, eugenol, oregano per 100 g of sample. Remaining four treatments the amount of active ingredient added was same but added in different combinations *viz*., T_5 = α-tocopherol acetate + Eugenol, T_6 = α-tocopherol acetate + Oregano, T_7 = Curcumin + α-tocopherol acetate + Eugenol and T_8 = α-tocopherol acetate + Eugenol + Oregano. The curcumin and oregano were added in ice-water while α-tocopherol acetate and eugenol were added in oil. For processing, meat was cut in to small cubes, minced in Hobart meat mincer (Model 4812) and then transferred into the Hobart paddle mixer (Model N50G). The pre-weighed ingredients were added sequentially while paddle mixer was on run at low speed. For cooking, about 40 g of the meat batter was moulded in cigar-shaped kebab applying gentle pressure on skewer iron rods by wet fingers and palm. The kebabs were cooked in a hot air oven at 170 ºC for 10 min the core temperature of 75±2 ºC. After initial cooking for 5 min they were loosen from the skewer rod and then kept turning as and whenever required. The cooked samples were cooled, packed in LDPE bags and stored at 4±1 ºC for evaluation of various quality parameters at an interval of 5 days for 20 days.

Determination of pH

The pH value of various cooked CSK products (n=6) was determined (Trout *et al*., 1992) [37] using a benchtop digital pH meter (Eutech 2700) equipped with a glass electrode and automatic temperature sensors. 10 g of sample was homogenized with 50 ml of distilled water for 1 min using pestle and mortar. The electrode was dipped into the suspension and the pH value of the sample was recorded.

Lovibond tintometer colour

The colour profiles of cooked CSK were measured using Lovibond tintometer (Model F, Greenwich, U.K.). Four different samples of each product were analysed (n=12). The sample colour was matched by adjusting red (a) and yellow (b) units and the corresponding colour units were recorded. The 'Hue' and 'Chroma' values were determined by using formula, (tan-1) b/a (Little, 1975) [23] and $(a^2 + b^2)1/2$ (Froehlich *et al*., 1983) [11] respectively, where a, red unit; b, yellow unit.

Determination of protein oxidation (MMb formation %)

The metmyoglobin content was measured as per procedure described by Krzywicki (1979) [22]. Metmyoglobin (MMb) content was estimated based on principle of absorption maxima of myoglobin, oxymyoglobin and metmyoglobin at 525, 572 and 700 nm wavelengths. For this, 3.0 g of product sample thoroughly mixed with 30 ml 0.04 M cold phosphate buffer solution containing sodium dihydrogen orthophosphate (NaH_2PO4: H_2O) and disodium hydrogen orthophosphate or sodium pyrophosphate dibasic: dehydrate (Na_2HPO_4: $2H_2O$) (pH 6.8) (sample: buffer, 1: 10) for 20 sec. Then the sample homogenate was held at 4 ºC for 1 hour, centrifuged at 10,000 rpm for 5 min and finally the supernatant was filtered using Whatman filter paper No. 42. The OD of supernatant was measured at 525, 572 and 700 nm wavelengths and amount of metmyoglobin content was calculated as per following formula:

$$\text{MMb (g/100g)} = \left(1.395 - \frac{(OD572 - OD700)}{(OD525 - OD700)}\right) \times 100$$

Determination of antioxidant activity

The antioxidant activity (AOA) of product samples was determined by evaluating 2-2-azinobis-3-ethylbenthiazoline-6- sulphonic acid cation ($ABTS^+$) radical scavenging activity, 1,1- diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity expressed as percent inhibition relative to standard at different concentrations (Biswas *et al*., 2015) [4]. The $ABTS^+$ activity was performed using the principal of ability of antioxidant compounds to quench the long-lived $ABTS^+$ radical cation in the presence of a blue chromophore.

Determination of lipid oxidations

2-thiobarbituric acid reacting substances was used as index for determination of lipid oxidations of developed products (Witte *et al*., 1970) [38] and expressed as mg malondialdehyde (MDA) per kg of sample. Free fatty acid (FFA) and peroxide value (PV) were determined according to procedure of Koniecko (1979) [21], and the results were expressed as percentage of oleic acid and meq of peroxide oxygen per kg of fat, respectively.

Microbiological analysis

Conventional methods recommended by APHA (2001) were used to enumerate the microbiological quality of CSK samples. Samples (10 g) were excised from the packets with sterile scalpel and forceps and then homogenized with 90 ml of sterile 0.1 g/100 ml peptone water in a pre-sterilized mortar for 2 min. Standard plate counts were determined on plate

count agar, total coliforms count on violet red bile glucose agar, and *Salmonella* spp. were counted on Baird Parker agar. In all cases, the plates were incubated at 37±2 ºC for 48 h. Yeast and moulds were determined on potato dextrose agar and plates were incubated ate 25±2 ºC for 7 d. Pour plate methods in duplicate (n = 6) were used to analyse the samples. Cultural medias were obtained from HiMedia Laboratories Ltd., Mumbai, India.

Sensorial quality

Sensory quality of product sample was evaluated using 8-point descriptive scale (Keeton, 1983) [17], in which a ten members semi-trained panel of judges consisting of scientists, technical officers and postgraduate students of the institute were evaluated the samples. Either sex of males and females in the age group of 23 to 62 years were participated in sensory evaluation. Potable water at room temperature was provided to clean the palate between samples. The tests were carried out 2 h after the mid-day meal. Three sittings were conducted for each replicate at each storage time.

Statistical analysis

The statistical analyses of data generated in the experiment were carried out using standard software package as mentioned by (Snedecor and Cochran 1994) [34]. Duplicate samples were drawn for each parameter and the experiment was replicated thrice (n = 6). Sensory evaluation was performed by a panel of 7 member judges three times (n =21). Response generated were analysed using two-way ANOVA, and Duncan's Multiple Range Test (DMRT) for comparing the means to find the effects between treatment, between storage periods and their interactions. The statistical significance was expressed at $p< 0.05$.

Results and Discussion

pH

The pH values of CSK prepared with addition of different natural antioxidants and antimicrobials are presented in Table 1. Among all treatment groups, the pH values were within a narrower range during initial days of storage as indicated in Table 1. This might be attributed to presence of equal proportion of lean meat (64.36 g/100 g) which is sufficient to buffer the changes in pH due to treatments. The pH values of T_2 sample were numerically higher than other treatment groups ($p>0.05$). In present study a non-significant effect was observed within all treatment samples. Likewise, Zahid *et al*. (2020) [40] reported similar results in beef patties prepared with addition of acetic acid and clove extract individually. On the other hand, there was significant ($p<0.05$) increase in overall pH was observed with progressing storage interval. It could be due to the formation of ammonia and volatile aminies as sample was progressing towards spoilage (Kuswandi and Nurfawaidi, 2017) [20]. On the contrary, reduction of pH during storage was reported due to addition of turmeric powder (Sharma *et al*., 2012) [32], clove essential oil (Sharma *et al*., 2017) [33] and combination of sage, oregano and honey (Sampaio *et al*., 2012) [31] in meat and meat products.

Table 1: Effect of natural preservatives on pH, protein oxidation (metmyoglobin formation, %) and Lovibond tintometer colour of chicken seekh kebab

Storage Interval (days)/ Parameter	Treatments								Period Mean	SEM	P-Value		
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8			T	P	T*P
pH													
0	6.12	6.14	6.13	6.12	6.18	6.18	6.12	6.14	6.14^{Z}	0.006	0.066	0.001	0.955
5	6.18	6.20	6.21	6.17	6.19	6.19	6.18	6.20	6.19^{Y}				
10	6.25	6.25	6.25	6.24	6.23	6.22	6.21	6.23	6.23^{X}				
15	6.31	6.31	6.25	6.28	6.28	6.28	6.25	6.28	6.28^{W}				
20	6.35	6.38	6.33	6.35	6.35	6.34	6.30	6.31	6.34^{V}				
TM	6.24	6.26	6.23	6.23	6.25	6.24	6.21	6.23					
Protein oxidation (Metmyoglobin formation %)													
0	26.89^{defgh}	16.28^{h}	16.15^{h}	21.97^{fgh}	19.58^{gh}	15.91^{h}	31.21^{bcdefg}	23.25^{efgh}	21.41^{Z}	1.055	0.001	0.001	0.001
5	36.25^{bcdef}	32.13^{bcdefg}	29.44^{cdefgh}	32.24^{bcdefg}	34.16^{bcdefg}	22.72^{fgh}	31.43^{bcdefg}	29.44^{cdefgh}	30.98^{Y}				
10	38.25^{bcd}	40.80^{bcd}	30.76^{bcdefg}	63.48^{a}	34.84^{bcdef}	31.80^{bcdefg}	32.81^{bcdefg}	36.27^{bcdef}	38.63^{X}				
15	41.49^{bcd}	42.46^{bc}	38.79^{bcd}	64.43^{a}	41.14^{bcd}	43.95^{bc}	37.60^{bcde}	37.79^{bcde}	43.46^{W}				
20	45.41^{b}	43.26^{bc}	59.95^{a}	70.28^{a}	63.91^{a}	45.79^{b}	43.76^{bc}	45.41^{b}	52.22^{V}				
TM	37.66^{B}	34.98^{BC}	35.02^{BC}	50.48^{A}	38.73^{B}	32.03^{C}	35.36^{BC}	34.43^{BC}					
Lovibond tintometer colour*													
Redness (a- Value)													
0	2.53	2.51	2.53	2.53	2.55	2.54	2.55	2.58	2.54^{W}	0.007	0.038	0.001	1.00
5	2.51	2.49	2.53	2.52	2.54	2.53	2.53	2.57	2.53^{X}				
10	2.47	2.46	2.52	2.49	2.51	2.52	2.50	2.53	2.50^{WX}				
15	2.43	2.38	2.50	2.48	2.50	2.51	2.48	2.51	2.47^{X}				
20	2.39	2.37	2.48	2.47	2.36	2.43	2.45	2.45	2.42^{Y}				
TM	2.46^{BC}	2.44^{C}	2.51^{AB}	2.50^{ABC}	2.49^{ABC}	2.51^{AB}	2.50^{AB}	2.53^{A}					
Yellowness (b- Value)													
0	3.52	2.38	2.38	2.38	2.39	2.38	3.51	2.37	2.66	0.026	0.001	0.111	0.007
5	3.49	2.37	2.38	2.37	2.38	2.35	3.49	2.35	2.65				
10	3.45	2.35	2.34	2.36	2.33	2.35	3.45	2.29	2.62				
15	3.34	2.35	2.33	2.35	2.33	2.34	3.41	2.29	2.59				
20	3.31	2.34	2.32	2.23	2.27	2.28	3.37	2.24	2.54				
TM	3.42^{A}	2.36^{B}	2.35^{B}	2.34^{B}	2.34^{B}	2.34^{B}	3.45^{A}	2.31^{B}					
Hue													
0	0.95	0.76	0.76	0.76	0.75	0.75	0.94	0.74	0.80	0.004	0.021	0.211	0.675

5	0.95	0.76	0.76	0.75	0.75	0.74	0.94	0.74	0.80				
10	0.95	0.76	0.75	0.76	0.75	0.75	0.94	0.73	0.80				
15	0.94	0.78	0.75	0.76	0.75	0.75	0.94	0.74	0.80				
20	0.95	0.78	0.75	0.73	0.77	0.75	0.94	0.74	0.80				
TM	0.95^{A}	0.77^{B}	0.75^{AB}	0.75^{AB}	0.75^{AB}	0.75^{C}	0.94^{A}	0.74^{C}					
Chroma													
0	4.33	3.46	3.48	3.47	3.50	3.49	4.34	3.51	3.70^{W}	0.025	0.001	0.001	0.001
5	4.30	3.44	3.47	3.46	3.49	3.47	4.32	3.48	3.68^{WX}				
10	4.24	3.40	3.44	3.44	3.42	3.46	4.27	3.42	3.64^{XY}				
15	4.14	3.34	3.42	3.42	3.42	3.43	4.22	3.40	3.60^{Y}				
20	4.09	3.33	3.39	3.33	3.28	3.34	4.17	3.33	3.53^{Z}				
TM	4.22^{A}	3.40^{B}	3.44^{B}	3.42^{B}	3.42^{B}	3.44^{B}	4.26^{A}	3.43^{B}					

n= 6; *n=12; Mean bearing different superscript in capital letter (A-C) row-wise and column-wise (V-Z) differ significantly (P<0.05). Mean bearing different superscript in small letter (a-h) differ significantly (P<0.05); T_1=0.025% Curcumin; T_2=0.04% α-tocopherol acetate; T_3=0.02% eugenol; T_4=0.05% oregano; T_5=0.04% α-tocopherol acetate + 0.02% eugenol; T_6= 0.04% α-tocopherol acetate +0.05% oregano; T_7=0.025% Curcumin + 0.04% α-tocopherol acetate + 0.02% eugenol; T_8=0.04% α-tocopherol acetate + 0.02% eugenol + 0.05% oregano. TM= Treatment Mean.

Protein oxidation (MMb formation %)

Results indicated that MMb formation was increased with the increase of storage time irrespective of treatments (Table 1). It was found that disulphide bonds are formed if protein is oxidised, hence thiol group contents of protein are reduced (Jia *et al*., 2012) [16]. Since, Mb oxidation is related to lipid peroxidation process, a higher TBARS value resulted in lower thiol-group content simultaneously. In general, T_4 showed poor protein oxidation value could be due to oregano might have very little or no anti-oxidative power while potential inhibitory effect on lipid peroxidation showed by combination treatment using curcumin, α-tocopherol acetate and eugenol (T_7). In general, T_7 treated sample containing plenty of polyphenols showed better antioxidative effects than other treatment and thereby less protein oxidation (Chauhan *et al*., 2019) [6].

Lovibond tintometer colour

Results of different colour coordinates (redness *a*-value, yellowness *b*-value, *Hue* and *Chroma*) indicated that almost all colour coordinates except *Hue* of chicken seekh kebabs were significantly influenced (p<0.05) by addition of curcumin, α-tocopherol acetate, eugenol and oregano individually or in combinations (Table 1). In all samples, *a*-value decreased with the progress in storage time (p<0.05) similar as reported by Kumar *et al*., (2015) [19] and the lowest values of *redness* were obtained for T_2 sample at the end of the 20th day of storage. This decrease in *a*-value could be correlated with the increase in metmyoglobin formation (Fernandez-Lopez *et al*., 2005) [10]. In contrast, T_3 and T_4 sample showed significantly higher (P<0.001) *a*- value on that day. These results also suggested that the presence of potent antioxidant compounds curcumin, α-tocopherol acetate and eugenol in T_7 could retard the MMb formation in chicken seekh kebabs thereby abbreviated decline of *a*- value was observed.

It could be expected that treatment with curcumin, α-tocopherol acetate and eugenol would have higher redness values because they had the greatest antioxidant capacity. In all samples, yellowness values were little modified (p>0.05) by storage time. Therefore, the differences in *b*- values were observed amongst the treatments with the incorporation of antioxidant compounds can be attributed to oxidation processes and the presence of pigments in added components. Rojas and Brewer (2008) [30] have also reported a decrease in '*b*' values of beef patties containing natural antioxidants. Since, *Hue* and *Chroma* are secondary parameters of redness and yellowness, and are calculated values, significant observations were noted for these parameters due to variation of both *a*- and *b* values. Similar findings of colour changes were also reported by Gadekar *et al*. (2014) [12] in restructured goat meat product.

Antioxidant activity

It has been observed that overall $ABTS^+$ activity was significantly (P<0.001) greater for T_7 sample and lowest for T_2 and T_4 as compared to other treatment groups. However, the values were decreased with the increase of storage intervals, and thus greatest (P<0.001) $ABTS^+$ activity was found in all samples during initial days irrespective of treatment variations (Table 2). While compared the $ABTS^+$ activity period-wise, T_7 showed $ABTS^+$ activity of 68.43g/100g at initial day and 59.24g/100g at the end of the storage interval. This indicates that sample with curcumin + α-tocopherol acetate + eugenol (T_7) had highest $ABTS^+$ activity followed by α-tocopherol acetate + eugenol + oregano (T_8)> curcumin (T_1) treatments. Several researchers reported $ABTS^+$ activity of curcumin (Mancini *et al*., 2016), eugenol (Gülçin *et al*., 2012) [14] and α-tocopherol acetate (Biswas *et al*., 2015) [4]. The turmeric powder treated burgers showed the highest values of $ABTS^+$ activity at 0 and 7 day (Mancini *et al*. 2015) [24]. The values for DPPH free radical scavenging activity are shown in Table 2. The overall DPPH activity was greatest for T_7 while least for T_6 sample. During initial days of storage, there were significant variations in DPPH activity, amongst the treatments. After the end of 20 days storage study, curcumin + α-tocopherol acetate + eugenol treated sample (T_7) exhibited significantly higher DPPH activity followed by oregano (T_4)> α-tocopherol acetate (T_2)> curcumin (T_1) =T_5 = T_8 However, DPPH activity of all sample was declined very rapidly and significantly (P<0.001) at each storage interval until the end of the study. Similar findings were reported by Khare *et al*. (2014) [18] in eugenol-treated chicken noodles. The scavenging activity of curcumin may be due to the reaction between the free radicals and the residual free amino group to form stable macromolecule radicals or amino groups can form ammonium groups by absorbing hydrogen ions from the solution and then reacting with free radicals through an addition reaction (Xie *et al*., 2001) [39]. Whereas, Puangsombat *et al*. (2011) [29] observed that the DPPH scavenging activity of the turmeric was 92.5g/100g and its scavenging activity depended on the degree of deacetylation. The curcumin with a higher degree of deacetylation has better scavenging activity, thereby

suggesting the action of nitrogen at the C-2 position in the elimination of free radicals. Gülçin *et al*. (2012) [14] also reported higher DPPH radical scavenging activity of clove oil (eugenol).

Table 2: Effect on antioxidant activity

Storage Interval (days)/ Parameter	Treatments								Period Mean	SEM	P-Value		
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8			T	P	T*P
ABTS⁺ activity (2-2-azinobis-3-ethylbenthiazoline-6-sulphonic acid) % inhibition													
0	68.31^{a}	47.33^{defg}	59.29^{bc}	50.30^{de}	61.33^{abc}	58.03^{bc}	68.43^{a}	62.65^{b}	59.46^{W}	0.827	0.001	0.001	0.001
5	53.50^{cd}	44.33^{efghi}	58.07^{bc}	38.36^{hijk}	58.64^{bc}	45.05^{defgh}	67.38^{a}	59.57^{bc}	53.11^{X}				
10	49.31^{def}	34.02^{jkl}	47.02^{defg}	35.71^{jkl}	48.93^{def}	35.83^{jkl}	67.71^{a}	49.34^{def}	45.98^{Y}				
15	50.36^{de}	34.98^{jkl}	39.88^{ghijk}	34.79^{jkl}	40.88^{ghijk}	41.98^{fghij}	62.67^{b}	45.95^{defgh}	43.93^{Y}				
20	32.90^{kl}	33.45^{kl}	40.79^{ghijk}	33.19^{kl}	37.21^{ijkl}	30.76^{l}	59.24^{bc}	35.03^{jkl}	37.82^{Z}				
TM	50.88^{B}	38.82^{D}	49.01^{B}	38.47^{D}	49.40^{B}	42.33^{C}	65.08^{A}	50.51^{B}					
DPPH activity (1, 1-diphenyl-2-picrylhydrazyl) % inhibition													
0	57.62^{defg}	59.7491^{cd}	53.35^{hijk}	63.15^{ab}	57.62^{defg}	50.83^{k}	64.75^{a}	57.80^{def}	58.11^{V}	0.511	0.001	0.001	0.001
5	56.46^{efgh}	57.99^{def}	51.74^{ijk}	60.61^{bcd}	55.89^{efgh}	45.42^{no}	61.63^{bc}	54.29^{hij}	55.50^{W}				
10	55.32^{efgh}	54.75^{fghi}	46.80^{n}	55.93^{efgh}	51.43^{jk}	42.82^{op}	58.08^{de}	50.23^{klm}	51.92^{X}				
15	47.45^{mn}	50.16^{klm}	41.38^{pq}	50.12^{klm}	47.65^{lmn}	38.85^{qr}	54.42^{ghij}	47.40^{mn}	47.18^{Y}				
20	36.98^{rs}	45.13^{no}	35.56^{st}	46.86^{n}	41.16^{pq}	33.91^{t}	50.63^{kl}	44.88^{no}	41.89^{Z}				
TM	50.77^{D}	53.55^{C}	45.77^{E}	55.34^{B}	50.75^{D}	42.37^{E}	57.90^{A}	50.92^{D}					

n= 6; Mean bearing different capital letter (A-E) superscript row-wise and column-wise (V-Z) differ significantly (P<0.05). Mean bearing different small letter (a-t) differ significantly (P<0.05); T_1=0.025% Curcumin; T_2=0.04% α-tocopherol acetate; T_3=0.02% eugenol; T_4=0.05% oregano; T_5=0.04% α-tocopherol acetate + 0.02% eugenol; T_6= 0.04% α-tocopherol acetate +0.05% oregano; T_7 =0.025% Curcumin + 0.04% α-tocopherol acetate + 0.02% eugenol; T_8=0.04% α-tocopherol acetate + 0.02% eugenol + 0.05% oregano. TM= Treatment Mean.

TBARS value

Determination of TBARS value, which indicates the oxidative stability of products, showed that T_7 sample had significantly lower (p<0.05) overall TBARS value than other treatments (Table 3). During initial days of storage curcumin (T_1), α-tocopherol acetate (T_2) and T_7 treated samples showed significantly lower TBARS values, while at the end of the storage that was significantly lower for T_3, T_4, T_5 and T_7. Armenteros, Morcuende, Ventanas, and Estévez, (2016) also reported that mixture of garlic, clove, cinnamon and rosemary effectively inhibited increasing TBARS value over the storage period. Similarly, oregano + sage + honey was the most effective treatment for reducing lipid oxidation in the cooked chicken breast after 96 h (Sampaio *et al*., 2012) [31]. Lower TBARS value of samples treated turmeric powder than those treated with BHA; this difference was especially significant (p<0.05) after 60 days of storage time (Milon *et al*. 2016) [26]. However, in the present study, as storage interval progressed, the TBARS value also increased significantly (p<0.05) for all samples. Abbreviated increase of TBARS value with the increase of storage interval could be attributed to a strong pro-oxidant effect of salt and anti-oxidative effect of curcumin, α-tocopherol acetate, eugenol and oregano in chicken seekh kebabs.

Free fatty acid (FFA) contents shows that FFA content was increased during storage without compromising treatments (Table 3). It has also been observed that T_6 had significantly (p<0.05) higher overall FFA contents than the other treatment groups. Biswas *et al*. (2015) [4] also reported that the FFA in sodium ascorbates and α-tocopherol acetate treated aerobic packaged poultry meat wafers was increased due to the growth of some lipolytic microorganisms. Though during initial days of storage FFA content was near to similar for all samples, at the end of the storage the overall mean indicating that combination treatment *viz*., curcumin, α-tocopherol acetate, eugenol and oregano had synergistic effects, and for this, T_7 treated sample showed significantly (p<0.001) lower FFA contents than other treatments. Likewise, chicken meat treated with a combination of clove powder and garlic paste significantly lower FFA content (Tareq, Rahman, and Hashem, 2018) [35].

Table 3: Effect on lipid oxidation

Storage Interval (days)/Parameter	Treatments								Period Mean	SEM	P -Value		
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8			T	P	T*P
2-thiobarbituric acid reactive substances (TBARS) (mg malonaldehyde/Kg)													
0	0.35^{de}	0.31^{e}	0.26^{e}	0.44^{bcde}	0.37^{cde}	0.39^{bcde}	0.34^{de}	0.39^{bcde}	0.35^{Z}	0.023	0.012	0.001	0.001
5	0.39^{bcde}	0.45^{bcde}	0.31^{e}	0.44^{cde}	0.38^{bcde}	0.45^{bcde}	0.45^{bcde}	0.44^{bcde}	0.41^{YZ}				
10	0.49^{bcde}	0.52^{bcde}	0.46^{bcde}	0.50^{bcde}	0.52^{bcde}	0.47^{bcde}	0.49^{bcde}	0.54^{bcde}	0.49^{XY}				
15	0.52^{bcde}	0.53^{bcde}	0.56^{bcde}	0.53^{bcde}	0.59^{bcde}	0.48^{bcde}	0.53^{bcde}	0.66^{bcde}	0.55^{X}				
20	1.85^{a}	0.74^{bcd}	0.64^{bcde}	0.62^{bcde}	0.63^{bcde}	0.78^{bc}	0.55^{bcde}	0.79^{b}	0.82^{W}				
TM	0.72^{A}	0.51^{B}	0.45^{B}	0.51^{B}	0.49^{B}	0.51^{B}	0.47^{B}	0.56^{B}					
Free fatty acid (% oleic acid)													
0	0.09	0.08	0.07	0.09	0.10	0.14	0.08	0.10	0.09^{Z}	0.002	0.001	0.001	0.870
5	0.12	0.11	0.09	0.10	0.13	0.15	0.08	0.11	0.11^{Y}				
10	0.13	0.12	0.10	0.12	0.13	0.15	0.10	0.13	0.12^{X}				
15	0.14	0.12	0.11	0.12	0.15	0.16	0.11	0.14	0.13^{X}				
20	0.16	0.12	0.12	0.13	0.16	0.16	0.14	0.17	0.15^{W}				
TM	0.13^{B}	0.11^{C}	0.10^{C}	0.11^{C}	0.13^{B}	0.15^{a}	0.10^{C}	0.13^{B}					
Peroxide value (meq/kg)													
0	0.15^{ghij}	0.14^{ijkl}	0.10^{lm}	0.11^{jklm}	0.08^{m}	0.14^{hijk}	0.10^{klm}	0.09^{lm}	0.11^{Z}	0.007	0.001	0.001	0.001

5	0.15^{ghi}	0.16^{ghi}	0.12^{ijkl}	0.12^{ijklm}	0.09^{lm}	0.16^{ghi}	0.13^{ijkl}	0.11^{jklm}	0.13^{Y}				
10	0.19^{g}	0.18^{gh}	0.15^{ghij}	0.15^{ghij}	0.10^{lm}	0.15^{ghi}	0.12^{ijklm}	0.12^{ijklm}	0.14^{X}				
15	0.25^{f}	0.27^{ef}	0.26^{ef}	0.26^{ef}	0.25^{f}	0.34^{c}	0.29^{ef}	0.30^{de}	0.28^{W}				
20	0.30^{de}	0.34^{cd}	0.35^{c}	0.36^{c}	0.40^{b}	0.45^{a}	0.35^{c}	0.35^{c}	0.36^{V}				
TM	0.21^{CD}	0.22^{B}	0.19^{DE}	0.19^{DE}	0.18^{E}	0.25^{A}	0.20^{CDE}	0.19^{DE}					

n= 6; Mean bearing different superscript in capital letter (A-E) row-wise and column-wise (V-Z) differ significantly (P<0.05). Mean bearing different superscript in small letter (a-m) differ significantly (P<0.05). T_1=0.025% Curcumin; T_2=0.04% α-tocopherol acetate; T_3=0.02% eugenol; T_4=0.05% oregano; T_5=0.04% α-tocopherol acetate + 0.02% eugenol; T_6= 0.04% α-tocopherol acetate +0.05% oregano; T_7=0.025% Curcumin + 0.04% α-tocopherol acetate + 0.02% eugenol; T_8=0.04% α-tocopherol acetate + 0.02% eugenol + 0.05% oregano. TM= Treatment Mean.

Peroxide value (PV)

The peroxide values (PVs) in all samples were increased linearly with the increase of storage days, and greater values were noticed for T_6 at the end of the storage. Higher values noticed for T_6 could be attributed to metal ions from meats that catalyse the fat in the presence of molecular oxygen and heat. As per the data in Table 3, T_5 showed least (P<0.001) PV but did not differ significantly (p>0.05) from T_3=T_4=T_8<T_7 and <T_1. However, in T_7 sample, PV was nearly the same as least recorded value for T_5. Similar findings were reported by Tareq *et al*. (2018) [35]. So, in this study, curcumin, α-tocopherol acetate and eugenol revealed potent antioxidant effect against the lipid-mediated per-oxidation process, because PVs were expressed on a fat weight basis. Sharma *et al*. (2012) [32] reported lower PV for a sample which contained 5000 ppm of turmeric powder. The overall mean peroxide values for nitrite and ginger-garlic-turmeric paste treated minced chicken were significantly lower than that of control (Goswami *et al*., 2014) [13].

Microbiological quality

In the present study, it was observed that inclusion of natural preservatives significantly influenced the microbial quality of CSK (Table 4). Lowest counts were recorded for T_7 and highest for T_2 samples at each storage interval. These findings are in agreement with the results reported by Tareq, Rahman, and Hashem, (2018) [35]. Sharma *et al*. (2017) [33] reported a significant reduction in total plate count with 0.25 g/100g concentration of clove essential oil than control. Similarly, The Psychrotropic plate counts (PPC) were detected only after 10 days of storage studies, however, the intensity of growth of these bacteria was least in T_7 samples. Similar results were found by Thomas, Anjaneyulu and Kondaiah (2006) [36] who reported that the PPC in restructured goat meat product and buffalo meat nuggets appeared only after 10^{th} and 15^{th} day of storage. Mean count of PPC in T_7 was 1.36 $\log_{10}$ cfu/g after the end of this study. Total coliform counts, *Salmonella* spp. counts and yeast and mould counts were not detected at any storage interval. The absence of these groups of bacteria during storage might be attributed to hot air oven cooking and hygienic handling and packaging of products (Coma, 2008) [7].

Table 4: Effect on microbiological qualities*

Storage Interval (days)/ Parameter	Treatment								Period Mean	SEM		P- Value	
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8			T	P	T*P
Standard plate count ($\log_{10}$ cfu/g)													
0	1.12^{n}	1.18^{mn}	1.13^{n}	1.17^{mn}	1.12^{n}	1.14^{mn}	1.11^{n}	1.13^{n}	1.14^{Z}	0.014	0.001	0.001	0.001
5	1.19^{mn}	1.23^{lm}n	1.20^{mn}	1.17^{mn}	1.19^{mn}	1.21^{mn}	1.17^{mn}	1.19^{mn}	1.19^{Y}				
10	1.25^{klm}	1.44^{ghi}	1.39^{hij}	1.55^{efg}	1.36^{ijk}	1.56^{def}	1.20^{mn}	1.34^{ijk}	1.39^{X}				
15	1.36^{ijk}	1.52^{efg}	1.50^{fgh}	1.61^{cde}	1.44^{ghi}	1.66^{bcd}	1.33^{jkl}	1.45^{ghi}	1.48^{W}				
20	1.61^{cdef}	1.76^{ab}	1.60^{cdef}	1.74^{ab}	1.69^{bc}	1.81^{a}	1.50^{efg}	1.52^{efg}	1.65^{V}				
TM	1.30^{DE}	1.42^{B}	1.36^{C}	1.45^{AB}	1.36^{C}	1.47^{A}	1.26^{E}	1.33^{CD}					
Psychrotropic plate count ($\log_{10}$ cfu/g)													
0	-	-	-	-	-	-	-	-	-	0.045	0.001	0.001	0.001
5	-	-	-	-	-	-	-	-	-				
10	-	1.19^{h}	-	-	-	-	-	-	0.15^{Y}				
15	1.41^{d}	1.35^{f}	1.39^{de}	1.42^{d}	1.37^{ef}	1.30^{g}	-	-	1.03^{X}				
20	1.51^{c}	1.63^{a}	1.52^{c}	1.58^{b}	1.52^{c}	1.38^{ef}	1.28^{g}	1.36^{f}	1.47^{W}				
TM	0.58^{C}	0.83^{A}	0.58^{C}	0.60^{B}	0.58^{C}	0.54^{D}	0.26^{F}	0.27^{E}					

n = 6; " - " = Not detected; Mean bearing different capital superscripts (A-F) row-wise and column wise (V-Z) differ significantly (P<0.05). Mean bearing different small letter (a-n) superscript differ significantly (P<0.05). T_1=0.025% Curcumin; T_2=0.04% α-tocopherol acetate; T_3=0.02% eugenol; T_4=0.05% oregano; T_5=0.04% α-tocopherol acetate + 0.02% eugenol; T_6= 0.04 % α-tocopherol acetate +0.05% oregano; T_7 = 0.025% Curcumin +0.04% α-tocopherol acetate +0.02% eugenol; T_8 =0.04 % α-tocopherol acetate +0.02 % eugenol +0.05% oregano. *Total coliform count, *Salmonella* species and yeast and mould count were absent throughout storage interval.

Sensory evaluation

Results of sensory evaluation data for all attributes (Table 5) indicated that the overall sensory scores were diminished with the increase of storage time. The overall sensory scores for colour and appearance attribute were greater for T_7 followed by T_1> T_5>T_3>T_6> T_2> and T_8. At the end of the storage, the scores for colour and appearance, flavour, texture, juiciness and overall acceptability for T_7 samples were 6.17, 6.29, 6.00, 6.21 and 6.76, respectively. In general, sensorial scores of all attributes were decreased (P<0.05) with the increase of storage days and the results have coincided with the findings of TBARS value and antioxidant parameter. This study is supported with the earlier findings of Hakeem *et al*., (2016) [15] since a progressive and significant decline for all sensory attributes was observed during the period of storage in chicken seekh kabab treated with different levels of grape pulp. The significant decrease (p<0.05) in colour and appearance value might be due to rapid oxidation of myoglobin and increased loss of moisture from the seekh kebabs. The moisture reduction and fat oxidation could also

influence the texture; juiciness and overall acceptability parameters. The overall acceptability attribute for the seekh kebabs from the different treatments also followed the same pattern that observed for other sensory attributes.

The use of natural antioxidant and antimicrobial compounds has a positive impact on decreasing protein oxidation and lipid peroxidation products while improving $ABTS^{+}$ and DPPH activity of CSK, however, combination treatments showed greatest effects. Potential synergistic action was observed in CSK treated with curcumin (0.025 %), α-tocopherol acetate (0.04%) and eugenol (0.02%) and was most suitable as natural preservatives than individual compound or other combinations of tested natural preservatives.

Table 5: Effect on sensory qualities*

Storage Interval (days) / Parameters	Treatments								Period Mean	SEM	P- Value		
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8			T	P	T*P
Colour and appearance													
0	6.95	6.41	6.71	6.52	6.81	6.93	7.12	6.43	6.74^{W}	0.025	0.001	0.001	0.740
5	6.87	6.48	6.50	6.33	6.76	6.36	6.88	6.54	6.59^{X}				
10	6.79	6.48	6.55	6.12	6.67	6.64	6.95	6.45	6.58^{X}				
15	6.29	6.55	6.38	6.14	6.31	6.43	6.45	6.38	6.37^{Y}				
20	6.02	6.00	6.05	5.83	6.14	5.76	6.17	5.98	5.99^{Z}				
TM	6.58^{AB}	6.38^{BCD}	6.44^{BC}	6.19^{D}	6.54^{ABC}	6.42^{BC}	6.71^{A}	6.35^{CD}					
Flavour													
0	6.74	6.69	6.86	6.71	6.79	6.62	6.95	6.62	6.75^{W}	0.026	0.001	0.001	0.851
5	6.66	6.38	6.69	6.33	6.67	6.55	6.81	6.55	6.58^{X}				
10	6.64	6.33	6.48	6.48	6.62	6.52	6.76	6.50	6.54^{X}				
15	6.00	6.34	6.52	6.43	6.29	6.12	6.26	6.45	6.30^{Y}				
20	6.12	5.71	5.98	5.83	6.10	5.76	6.29	5.98	5.97^{Z}				
TM	6.43^{BC}	6.29^{C}	6.50^{BC}	6.36^{C}	6.49^{BC}	6.31^{C}	6.61^{A}	6.42^{BC}					
Texture													
0	6.43	6.76	6.71	6.71	6.67	6.48	6.95	6.50	6.65^{W}	0.026	0.001	0.001	0.488
5	6.58	6.24	6.50	6.33	6.36	6.36	6.76	6.52	6.46^{X}				
10	6.10	6.40	6.36	6.05	6.43	6.31	6.98	6.35	6.37^{X}				
15	5.93	6.40	6.33	6.05	6.17	5.98	6.48	5.91	6.15^{Y}				
20	6.07	5.91	5.86	5.83	5.91	6.00	5.97	5.88	5.93^{Z}				
TM	6.22^{B}	6.34^{B}	6.35^{B}	6.20^{B}	6.30^{B}	6.22^{B}	6.63^{A}	6.23^{B}					

Conti…

Juiciness													
0	6.76	6.79	6.86	6.81	7.02	6.67	7.10	6.79	6.85^{W}	0.028	0.001	0.001	0.308
5	6.53	6.33	6.64	6.48	6.76	6.64	7.00	6.76	6.64^{X}				
10	5.86	6.13	6.48	6.43	6.76	6.62	6.69	6.45	6.43^{X}				
15	5.98	6.00	6.48	6.43	6.52	6.17	6.19	6.12	6.24^{Y}				
20	6.12	5.41	5.98	5.98	5.86	5.91	6.21	5.93	5.92^{Z}				
TM	6.25^{CD}	6.13D	6.49^{AB}	6.42^{ABC}	6.59^{AB}	6.40^{BC}	6.64^{A}	6.41^{BC}					
Overall Acceptability													
0	6.83	6.76	7.07	6.79	6.71	6.71	7.14	6.93	6.87^{W}	0.028	001	0.001	0.287
5	6.87	6.29	6.57	6.10	6.52	6.74	7.00	6.81	6.61^{X}				
10	6.55	6.40	6.48	6.14	6.62	6.74	7.05	6.60	6.57^{X}				
15	6.19	6.16	6.52	6.21	6.21	6.21	6.55	6.64	6.34^{Y}				
20	5.95	5.64	5.98	5.60	5.86	5.71	6.76	6.14	5.96^{Z}				
TM	6.48^{BC}	6.25^{CDE}	6.52^{BC}	6.17^{E}	6.39^{CD}	6.42^{BCD}	6.90^{A}	6.62^{B}					

n= 21; Mean bearing different capital letter (A-E) superscript row-wise and column-wise (W-Z) differ significantly ($P<0.05$). T_1=0.025% Curcumin; T_2=0.04% α-tocopherol acetate; T_3=0.02% eugenol; T_4=0.05% oregano; T_5=0.04% α-tocopherol acetate + 0.02% eugenol; T_6= 0.04% α-tocopherol acetate +0.05% oregano; T_7 =0.025% Curcumin + 0.04% α-tocopherol acetate + 0.02% eugenol; T_8=0.04% α-tocopherol acetate + 0.02% eugenol + 0.05% oregano. *Based on 8-point descriptive scale, where 8=extremely desirable and 1+ extremely undesirable.

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