

***In vitro* and *in vivo* evaluation of *Prosopis cineraria* (khejri tree) leaves for their preservative potential in minced mutton**

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Abstract

With an objective to extend the shelf life with improved microbial safety by natural means without compromising sensory attributes, lyophilised *khejri* tree leaf extract (LKE) was evaluated *in vitro* and *in vivo*. The LKE was subjected to preliminary scrutiny followed by FTIR mediated analysis and RP-HPLC and then its antioxidant activity was accessed. Five batches of minced mutton were prepared as C-1 (negative control with no LKE or BHA), C-2 (positive control with 200 ppm BHA), T-1 (0.05% LKE), T-2 (0.1% LKE), and T-3 (0.5% LKE). FTIR analysis showed C=O-CH₃, C=C, C-O, and O-H peaks at 1028, 1609, and 1445 cm⁻¹ wave numbers, respectively. The LKE was found to have six phenolic compounds namely *p*-hydroxybenzoic acid, gallic acid, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid. In minced mutton, LKE was found to be significantly ($p \leq 0.05$) more effective preservative than BHA at 0.5% level of incorporation in terms of TBARS value, tyrosine values, and microbial quality. During storage, the decrease in ERV and increase in total plate count were at a slower rate in treated samples and were devoid of coliform counts. However, sensory attributes at 0.5% LKE incorporation level were negatively affected and imparted a greenish tinge to minced mutton. The present work concluded that LKE might be incorporated without compromising sensory attributes. Further, preservative effects at 0.1% level were at par with BHA (200 ppm) and extended the minced mutton's shelf life up to 9 d at refrigerated temperature.

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Introduction

Meat has been an important component of the human diet since ancient times. Meat quality is a broad term and is mainly defined by nutritional, sensorial, and microbial qualities. During storage of meat, it is very important to have minimal deteriorative changes to maintain the meat quality. Loss of bloom, rancidity, and product safety are the key responsible factors that rigorously affect the viability of raw meat industry in terms of limited shelf life. Product attributes like appearance, flavour, and

keeping quality have an upper hand in the meat market as far as consumers' preferences are concerned. However, free radical-mediated oxidation of muscle myoglobins and lipids along with microbial growth are the major contributors to limited shelf life (Sharma *et al.*, 2021). These issues further become more prominent if meat is in a minced form because of greater exposure of the surface area to the environment.

In order to improve keeping quality, preservatives like antioxidants and antimicrobials have been used. Meat lipids can undergo oxidative

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changes during storage. There are a number of antioxidants available for use, but few of them have been associated with untoward effects like cancer and cell damage (Felter *et al.*, 2021). Therefore, antioxidant like butylated hydroxyanisole (BHA) is no longer used in the food industry, and there is a paradigm shift toward the use of more natural food additives (various plant parts' extract/powder). Some have already been studied by researchers like apple peel extract, crude aloe vera gel, and grape seed extract (Jairath *et al.*, 2016; Singh *et al.*, 2018) which have shown positive effects on the storage stability of muscle foods owing to their phenolic compounds. However, many remain unexplored and underutilised.

In the modern era of 'go natural' theme, consumers are more conscious about the food quality, and mostly prefer food with no/minimal preservatives. For researchers, it is, therefore, a challenge to find suitable natural, safe, cost-effective preservative with multiple benefits like antioxidative and antimicrobial properties. *Khejri* tree (*Prosopis cineraria*) of the family Fabaceae, also commonly known as "kalpatru" (the king of the desert), was selected to be used in the present work as they abundantly grow in arid and semi-arid areas, especially in the Rajasthan state of Western India, and have been reported to have food, feed, and medicinal values for every part (Pareek *et al.*, 2015). For instance, dry pods of the *khejri* tree are helpful in averting protein and mineral deficiency in humans (USNAS, 1980), and different parts of *khejri* tree have also been traditionally used for treating several ailments (USNAS, 1980; Chogem *et al.*, 2007). Though the studies on antimicrobial and antioxidative properties of *khejri* leaves have been documented (Preeti *et al.*, 2017; Asati *et al.*, 2021), these are not only scarce and preliminary, but also limited to *in vitro* evaluation at basic level. Therefore, the present work aimed to gain insight into polyphenolic compounds present in the leaves that make them a potent bioactive candidate, and to optimise their level of incorporation as lyophilised extract in meat to validate its antioxidant and antibacterial activities under *in vivo* conditions, with the purpose of extending shelf life. To the best of our knowledge, this report would be the first of its kind to study the antioxidant and antibacterial effects of lyophilised *khejri* leaves extract (LKE) under *in vivo* meat model system.

Materials and methods

Raw meat and chemicals

Randomly, 12 rams from the institute flock (breed = Malpura; weight = 22 - 30 kg; age = 12 - 15 months) were sacrificed at the institute abattoir following standard guidelines with due consideration for extant animal ethics laws after IAEC and CPCSEA approval. Twelve muscle samples of *longissimus thoracis* and *lumborum* muscles were then minced together. Chemicals, ready-made growth media, and HPLC solvents were purchased from different registered suppliers like CDH Chemicals, Merck's India, and HiMedia India.

Preparation of khejri tree leaf extract (LKE)

The *khejri* leaves were collected from the herbal garden of the ICAR-CSWRI, Avikanagar Institute. The leaves were separated, washed, and oven-dried at 50°C in a pre-heated hot air oven for 48 h, and then finely ground into powder. The obtained fine powder was mixed with 80% methanol at the ratio of 1:9, the mixture was kept at 25°C for 4 h at 150 rpm in an orbital shaker (orbital shaker incubator, Gallenkamp), filtered (Whatman filter paper 1), and then subjected for orbital shaking for another 4 h after adding 80% methanol. The pooled filtrate was subjected to evaporation using a vacuum rotatory evaporator (HeidolphHei-VAP Platinum 4 Rotary Evaporator) at 50°C and 120 rpm to evaporate the solvent. The extract obtained was vacuum lyophilised into fine powder in a freeze drier (Hetosicc HT50 freeze dryer, Netherland) at -70°C under vacuum, and stored at -20°C until further use in an airtight amber-coloured bottle. The pressure development was as per the pressure being developed during vaporisation.

Preparation of minced mutton

After overnight thawing at refrigeration temperature ($4 \pm 1^\circ\text{C}$), the leg muscle was minced (Mado meat mincer, Junior, MEW-510-2, Germany; 3 mm plate) and divided into five groups (250 g each) for each day (Day 1, 3, 5, 7, and 9) *viz.* C-1 (negative control with no LKE or BHA), C-2 (positive control with 200 ppm BHA), T-1 (0.05% LKE), T-2 (0.1% LKE), and T-3 (0.5% LKE), in total 25 packets, and were aerobically packaged and stored at refrigeration temperature ($4 \pm 1^\circ\text{C}$). The samples were analysed at the interval of two-day for nine days *i.e.*, 1, 3, 5, 7, and day 9 for various quality parameters.

Characterisation of LKE

Phytochemical screening of LKE

The working solution from the lyophilised powder was formulated by sonicating on an ice bath using Soniprep 150 (Model MSS150.CX3.5; UK). Next, 20 mg of LKE was suspended in 80% methanol (10 mL), and was kept on an ice bath. It was then given ultrasonic treatment for 3 min (both setting and output control at 50). Then, beaker was cooled for 2 min on ice bath to avoid heat-mediated denaturation. The treatment was repeated for a total of 12 min. Then, it was centrifuged at 5,000 rpm for 10 min at 4°C, and filtrate was used as a working solution of LKE which was screened for phytochemicals like saponins, phenols, tannins, terpenoids, flavonoids, and glycosides as per methods described by Teli *et al.* (2018).

Fourier-transform infrared spectroscopy (FTIR) of LKE

The LKE powder was analysed by observing transmittance spectra at the wavenumbers 500 - 4000 cm^{-1} through attenuated total reflectance attachment in Fourier-transform double beam infrared spectrophotometer (Bruker, model- Alpha).

RP-HPLC analysis

The phenolic compounds were analysed through HPLC equipped with a UV detector, a binary pump of 3000 series, and an autosampler (Analytical Technologies Ltd.). Synchronis C₁₈ reversed-phase analytical column of dimensions 250 × 4.6 mm, diameter 5 μm (Thermo® Scientific) (column temperature: 28°C), at a solvent flow rate of 1.5 mL/min, and 20 μL injection volume. Next, 1% acetic acid (mobile phase A) and pure HPLC grade methanol (mobile phase B) were mixed online in 80:20 ratio. The stock solutions of seven standards (gallic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid) were prepared by dissolving 10 mg in 10 mL pure methanol. The working standards of concentrations 50 $\mu\text{g}/\text{mL}$ in a mobile phase were prepared for the study. A standard mixture of phenolic compounds containing 50 μg each in a mobile phase was prepared. Dry LKE was diluted to 150 mg/10 mL of the mobile phase. RP-HPLC was run after filtering both samples and solvents with a microfiber filter (0.2 μm). Based on the retention time (RT) and area, the compounds were detected and quantified.

In vitro analysis

Total phenolic contents

The phenolic content was quantified in terms of mg gallic acid equivalent (GAE) per 100 mg of extract (Porter *et al.*, 1985). Various concentrations of gallic acid (2 - 20 $\mu\text{g}/\text{mL}$) were prepared for the standard curve to estimate the content in test samples.

Total flavonoid contents

The aluminium chloride colorimetry method with slight modifications was employed to quantify the TFC (milligram quercetin equivalent per 100 mg sample) (Vador *et al.*, 2012). Quercetin in 80% methanol was used as a standard (5 - 25 $\mu\text{g}/\text{mL}$). The AlCl_3 reagent was prepared by dissolving 133 mg AlCl_3 and 400 mg sodium acetate in 100 mL of methanol (80%). The extract was first diluted in a 1:7 ratio with 80% methanol, and then 2 mL of the solution was mixed with 1 mL of AlCl_3 reagent and 400 μL of DW. The absorbance was recorded after vortex against blank containing no AlCl_3 at 430 nm using a UV-Vis spectrophotometer (Model-UV-160A, Shimadzu Analytical India Pvt. Ltd.).

Antioxidant activities

The antioxidant activity of LKE was evaluated through antioxidant assays like ferric reducing antioxidant power (FRAP) assay, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, and ABTS^+ radical cation decolourisation assay as described by Dudonné *et al.* (2009).

In vivo analysis

pH of mutton samples

The pH of meat samples (1:5 ratio in DW) was recorded (Troutt *et al.*, 1992) by immersing the electrode of a digital pH meter in the sample.

Oxidative stability

In vivo oxidative stability was evaluated by measuring the extent of lipid oxidation through thiobarbituric acid reacting substances (TBARS) value assay (Witte *et al.*, 1970), and the extent of protein oxidation through tyrosine value assay (Strange *et al.*, 1977). Before performing TBARS and tyrosine assays, filtrate was prepared by homogenising one part of sample with five parts of 20% trichloroacetic acid solution prepared by dissolving 200 g/L of trichloroacetic acid in 135 mL/L phosphoric acid solution, followed by filtration through Whatman filter paper number 1.

Thiobarbituric acid reacting substances are produced as the by-products of lipid oxidation, and determined through the TBARS assay. For the assay, an equal volume of sample filtrate and 1 mM aqueous TBA solution was mixed and incubated at 100°C for 30 min in water bath. After cooling under running water, absorbance was recorded at 532 nm using a UV-Vis spectrophotometer. The TBARS values (mg malonaldehyde per kilogram (MDA/kg) were calculated from a standard curve prepared by taking different concentrations of standard, *i.e.* 1,1,3,3 tetraethoxy propane in glacial acetic acid.

For tyrosine value (mg tyrosine/100 g of sample), 1 mL of the prepared filtrate was pipetted in a test tube to which same amount of DW, 4 mL of NaOH (0.5 N) and 1.2 mL FC reagent (1FC: 2 D.W.) were added, and absorbance at 660 nm was recorded after mixing and 15 min incubation. Blank was prepared by replacing sample with 10% TCA, and standard was prepared by taking different concentrations of pure tyrosine.

Microbial stability

Microbial stability was determined in terms of extract release volume (ERV), and microbial counts (total plate count and coliform count) of mutton samples on days 1, 3, 5, 7, and 9 of storage. The spoilage assessment of meat through ERV was also performed as per the method described by Pearson (1968), in which the quantity of filtrate obtained in 15 min at room temperature of 21°C was reported as mL of ERV. The microbial quality was assessed following prescribed methods (APHA, 2001).

Evaluation of sensory attributes

Six pre-trained panellists evaluated the sensory attributes of all samples kept under refrigeration temperature on days 1, 3, 5, 7, and 9 of storage. The consent was taken from all participants before conducting the sensory evaluation, and during each trial, six panellists conducted a sensory evaluation and panel members were the same each time. The samples were served in containers with a lid at room temperature. The seven-point hedonic scale was briefed to the panellists. The panellists were requested to score for odour and colour on the numerical scale of 1 to 7; where 1 indicated extremely offensive, 2 as very offensive, 3 as moderately offensive, 4 as slightly offensive, 5 as appreciable, 6 as barely appreciable, and 7 indicated inappreciable

rancid odour. For colour, 1 indicated extremely dark brown, 2 as very dark brown, 3 as dark brown, 4 as dark red, 5 as slightly dark red, 6 as cherry red, and 7 as light cherry colour.

Statistical analysis

A completely random design model was applied in the present work. Three trials were conducted independently, and each time, each parameter was done in duplicate. Means were compared by two-way analysis of variance, homogeneity test, and critical test at 5% level ($p \leq 0.05$) of statistical significance. *In vitro* data was analysed via unpaired *t*-test (Snedecor and Cochran, 1967). After appraising the data with Tukey's honest significant difference test, it was tabulated and interpreted.

The data for colour and odour (responding variables) were analysed using the general linear model procedure of SPSS (SPSS 16.0). The fixed factors used were treatment and time of evaluation, whereas panellists were used as a random variable.

$$Y_{ijkl} = \mu + T_i + D_j + (T_i * D_j)_k + Z_p P + e_{ijkl}$$

where, Y_{ijkl} = the responding variable, either colour or odour; μ = overall mean; T_i = fixed effect of i^{th} treatment; D_j = fixed effect of j^{th} day of evaluation; $D_i * T_j$ = interaction effect of treatment and day; $Z_p P$ = random effect of panellist P with incidence matrix Z_p ; e_{ijkl} = random error NID ($0, \sigma^2$) of associated Y_{ijkl} .

The differences between the least-squares mean for subclasses under a particular effect were tested by Duncan's multiple range test (Kramer, 1957).

Results

Characterisation of LKE

Phytochemical screening of LKE

The qualitative/subjective phytochemical analysis of LKE revealed the presence of saponins, phenol, tannins, flavonoids, and glycosides.

FTIR analysis

FTIR spectrum of LKE (Figure 1) showed characteristic peaks corresponding to bioactive compounds as revealed by phytochemical analysis. The peaks were detected at 1028, 1200, 1445, 1515, 1609, 2933, and 3749 cm^{-1} wave numbers.

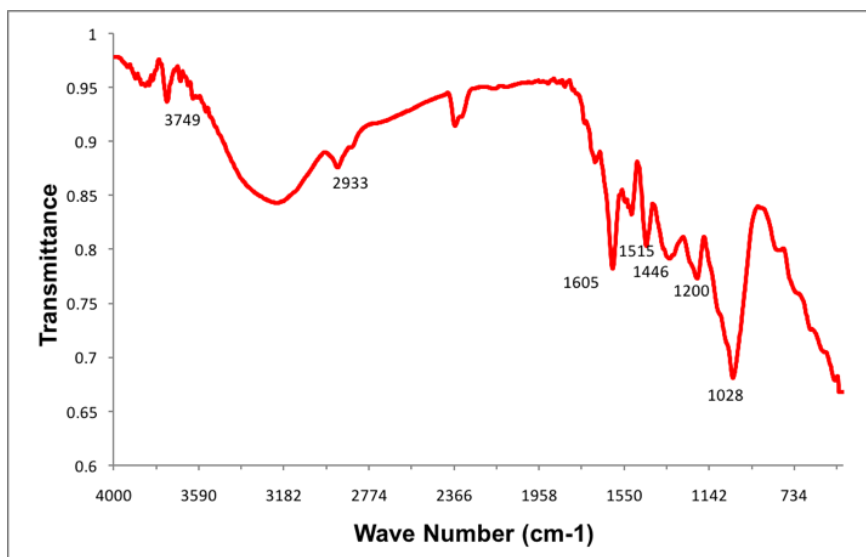


Figure 1. FTIR analysis of *khejri* tree leaf extract (LKE).

RP-HPLC analysis

The retention time (RT) of RP-HPLC (Figure 2) of LKE revealed the presence of *p*-hydroxybenzoic acid, gallic acid, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid in the extract. However, vanillic acid was not detected in LKE. Para-

hydroxybenzoic acid was found to be in a higher amount (2.45 mg/gm of LKE) amongst all, followed by gallic acid (1.89 mg/gm of LKE), syringic acid > *p*-coumaric acid > ferulic acid and sinapic acid. RP-HPLC of extract confirmed the presence of six phenolic compounds in LKE.

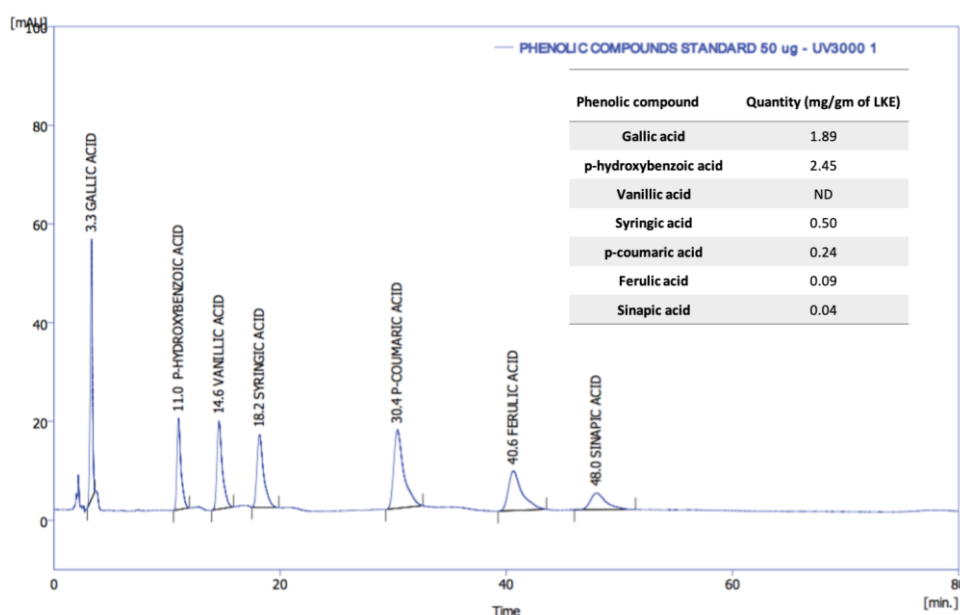


Figure 2. Retention time of seven standard compounds through RP-HPLC analysis and quantity of phenolic compounds found in lyophilised *khejri* tree leaf extract (LKE).

In vitro analysis

Total phenolic contents

Total phenolic content of LKE (7.57 ± 0.36 mg GAE/100 mg) calculated from the standard curve ($R^2 = 0.998$) was almost 50% less than that of synthetic BHA (14.42 ± 0.09 mg GAE/100 mg) (Table 1).

Total flavonoid contents

Total flavonoid content (Table 1) was 74% higher in LKE (0.464 ± 0.01 mg quercetin equivalent per 100 mg) calculated from standard curve ($R^2 = 0.998$) in comparison to BHA (0.124 ± 0.01 mg quercetin equivalent per 100 mg).

Table 1. Total phenolic and flavonoid contents of LKE, and its antioxidant activities in comparison to BHA.

Parameter	BHA	LKE	t-value
Total phenolic and flavonoid content			
TPC (mg GAE/100mg of sample)	14.42 ± 0.09 ^b	7.57 ± 0.36 ^a	0.000
TFC (mg quercetin equivalent per 100 mg)	0.124 ± 0.01 ^a	0.464 ± 0.01 ^b	0.000
Antioxidant activity			
FRAP (µM Fe ²⁺ /g)	69.30 ± 0.22	67.35 ± 0.79	0.077
DPPH (% inhibition)	91.50 ± 1.56 ^b	86.05 ± 1.56 ^a	0.013
ABTS ⁺ (% inhibition) - 10 times diluted	20.59 ± 1.43 ^b	16.52 ± 0.90 ^a	0.031

Means ± S.E. followed by different lowercase superscripts in the same row differ significantly ($p \leq 0.05$).

Antioxidant activities

The antioxidant activities were measured in terms of FRAP, DPPH, and ABTS⁺ scavenging activities (Table 1) which assess the percentage of free radicals scavenged by the antioxidant in question. The reducing ability (FRAP) of BHA and LKE was 69.30 ± 0.22 and 67.35 ± 0.79 µM Fe (II)/g, respectively. The H⁺ donating ability of LKE was significantly ($p \leq 0.05$) lower (86.05 ± 1.56) than BHA ($91.50 \pm 1.56\%$) at same concentration. The ABTS⁺ scavenging potential of BHA was significantly ($p \leq 0.05$) higher ($20.59 \pm 1.43\%$) than that of LKE (16.52 ± 0.90) at same concentration.

In vivo analysis

pH

The effect of LKE on the minced mutton was quite visible from the first day of storage (Figure 3).

The pH of minced mutton was observed to be very stable in T-3. On day 1, the pH of all the samples was significantly ($p \leq 0.05$) lower in comparison to T-3. Throughout the storage period, pH increased, irrespective of treatment up to day 5. However, the rate of increase was slow in the treated sample in comparison to BHA and control.

Oxidative stability

The TBARS values of minced mutton were influenced by both antioxidant treatment and storage time, and their interaction effect determined the malonaldehyde concentration in the samples (Figure 3). C-1 samples showed significantly ($p \leq 0.05$) higher, and T-3 had significantly ($p \leq 0.05$) lower TBARS values among the treatments. Though the incorporation of antioxidants significantly aided in the reduction of TBARS values since day 1, these

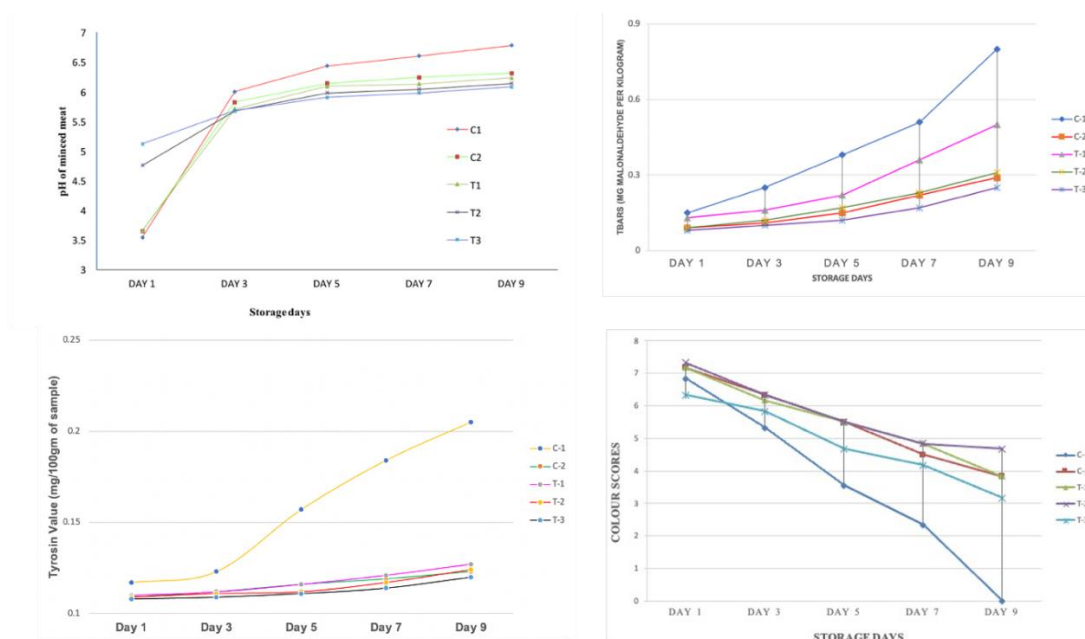


Figure 3. Comparative effect of LKE and BHA on the pH, TBARS, tyrosine, and colour profile of minced mutton under aerobic packaging stored at $4 \pm 1^\circ\text{C}$. $n = 6$, C-1 = negative control with no LKE or BHA, C-2 = positive control with 200 ppm BHA, T-1 = 0.05% LKE, T-2 = 0.1% LKE, and T-3 = 0.5% LKE.

values increased in all samples correspondingly with storage duration. But this rate of increase remained significantly ($p \leq 0.05$) lower in treatment batches. This rate of increase in all samples followed a particular decreasing trend as C-1 > T-1 > T-2 > C-1 > T-3, where the samples attained corresponding values as 0.80, 0.50, 0.31, 0.29, and 0.25 mg MDA/kg on the last day (day 9).

Tyrosine values as depicted in Figure 3, revealed the significant interaction between storage period and treatments, and noticed throughout from day 1 to day 9 of storage period. The tyrosine values of LKE-treated products were comparable to that of BHA-treated products, irrespective of the concentration of LKE on day 1. However, the tyrosine values were recorded significantly ($p \leq 0.05$) lower in T-3 on day 9 of storage period than in others.

Microbial stability

The data of microbial stability in terms of ERV and microbial profile (total plate count, coliform count) indicated the significant impact of LKE incorporation in minced mutton (Table 2). The release volume of all the samples was found at par during the initial period of storage. However, with time, C-1 had significantly ($p \leq 0.05$) lower ERV among all and significantly higher for T-3. Though, with storage, ERV decreased significantly ($p \leq 0.05$) in all, irrespective of type of treatment, the rate of decrease was slower in T-2 and T-3 samples in comparison to others.

TPC was comparable in all the samples on day 1. Though the total plate count increased with the increase in the storage period, the microbial load was quite higher in negative control samples (C-1) as compared to the rest of the other batches, and the same trend was exhibited during the entire period of storage. Among the LKE-treated samples, T-2 and T-3 samples had significantly ($p \leq 0.05$) lower bacterial load in comparison to BHA, which indicated the higher antimicrobial potential of LKE at 0.1 and 0.5% levels under *in vivo* conditions in comparison to 0.05% LKE and BHA. Coliform counts were not detected throughout the storage period.

Sensory evaluation

During the sensory evaluation, all samples showed a declining trend in average scores of colour and odour throughout 9-d storage period (Table 2 and Figure 3). However, it was done for 7 days in control

group. The colour scores of T-2 were the highest ($p \leq 0.05$) amongst all on day 9. Similar to the colour scores, odour scores also followed the same trend.

Discussion

Characterisation of LKE

Phytochemical screening of LKE

The results corroborated with Khandelwal *et al.* (2016). Kulshreshtha *et al.* (2019) also documented the presence of alkaloids, tannins, flavanoids, proteins, terpenoids and saponins in the methanolic extract of *P. cineraria* leaves. These bioactive compounds have the potency to scavenge the free radicals, and are effective against many microorganisms, thus resulting in antioxidant and antimicrobial activities (Baba and Malik, 2015; Preeti *et al.*, 2017). This preliminary screening was further validated by FTIR analysis.

FTIR analysis

C=O-CH₃ ester group present in saponins was detected at a 1028 cm⁻¹ wave number, as depicted by a sharp peak (Sharma and Paliwal, 2013). Besides, C=C and C-O stretching at 1609 cm⁻¹ peak were suggestive of saponins (Ahmad *et al.*, 2013). The condensed tannins were depicted at 1445 cm⁻¹ wave number showing the characteristic peak of the O-H bending and the aromatic ring vibration characteristic to condensed tannin. At wave number 1515 cm⁻¹, a small peak was observed, confirming the presence of C=C stretching of the aromatic ring system (Pandey *et al.*, 2018). The peak at 1200 cm⁻¹ depicted esters, representing the C-O single bond vibration, however, that at 3749 cm⁻¹ indicated the presence of tannin. The C-H stretching in alkanes associated with the terpenoids was recorded at 2933 cm⁻¹, depicted as a small peak in the FTIR spectrum (Ricci *et al.*, 2015; Vanaja and Kavitha, 2016).

RP-HPLC analysis

Being the first report, a thorough phytochemical analysis could only identify the active phenolic compounds, therefore, RP-HPLC was performed to validate the presence of phenolic compounds in *khejri* tree leaves. However, the simultaneous presence of many other phenolic compounds could not be ruled out. The results further reiterate the fact that LKE has the potency to exhibit antioxidant and antimicrobial actions.

Table 2. Comparative effect of LKE and BHA on the ERV, TPC, coliform count, and odour of minced mutton under aerobic packaging stored at $4 \pm 1^\circ\text{C}$.

Treatment/ Day	Day 1	Day 3	Day 5	Day 7	Day 9
ERV (mL of exudate)					
C-1	36.67 \pm 0.88 ^{Ae}	27.33 \pm 1.20 ^{Ad}	20.00 \pm 0.58 ^{Ac}	16.00 \pm 0.57 ^{Ab}	12.00 \pm 0.58 ^{Aa}
C-2	39.00 \pm 0.58 ^{Ae}	35.33 \pm 0.33 ^{CDd}	28.67 \pm 0.33 ^{CDc}	23.67 \pm 0.33 ^{BCb}	17.67 \pm 0.88 ^{BCa}
T-1	37.67 \pm 0.33 ^{Ae}	30.00 \pm 0.58 ^{ABd}	24.00 \pm 0.58 ^{Bc}	18.33 \pm 0.33 ^{Ab}	14.33 \pm 0.33 ^{ABa}
T-2	38.33 \pm 0.33 ^{Ae}	32.33 \pm 0.88 ^{BCd}	26.67 \pm 0.33 ^{Cc}	22.33 \pm 0.88 ^{Bb}	17.33 \pm 0.88 ^{BCa}
T-3	39.33 \pm 1.20 ^{Ac}	36.00 \pm 0.57 ^{Dc}	29.33 \pm 0.33 ^{Db}	25.67 \pm 0.33 ^{Cb}	20.67 \pm 1.20 ^{Da}
TPC (log₁₀ CFU/g of sample)					
C-1	5.08 \pm 0.06 ^{Aa}	5.90 \pm 0.01 ^{Ab}	6.54 \pm 0.03 ^{Ac}	6.92 \pm 0.09 ^{Ad}	7.63 \pm 0.03 ^{Ae}
C-2	4.97 \pm 0.02 ^{Aa}	4.97 \pm 0.04 ^{CDb}	5.42 \pm 0.06 ^{CDc}	5.95 \pm 0.06 ^{BCd}	6.71 \pm 0.07 ^{BCe}
T-1	4.79 \pm 0.03 ^{Aa}	5.18 \pm 0.04 ^{ABb}	5.86 \pm 0.05 ^{Bc}	6.59 \pm 0.06 ^{Ad}	7.07 \pm 0.05 ^{ABe}
T-2	4.49 \pm 0.03 ^{Aa}	4.77 \pm 0.04 ^{BCb}	5.46 \pm 0.09 ^{Cc}	5.88 \pm 0.05 ^{Bd}	6.65 \pm 0.09 ^{BCe}
T-3	4.21 \pm 0.07 ^{Aa}	4.42 \pm 0.06 ^{Db}	4.88 \pm 0.05 ^{Dc}	5.59 \pm 0.06 ^{Cd}	6.36 \pm 0.6 ^{Da}
Coliform (log₁₀ CFU/g of sample)					
C-1	ND	ND	ND	ND	ND
C-2	ND	ND	ND	ND	ND
T-1	ND	ND	ND	ND	ND
T-2	ND	ND	ND	ND	ND
T-3	ND	ND	ND	ND	ND
Odour¹					
C-1	7.00 \pm 0.00 ^{Ac}	6.33 \pm 0.33 ^{Ac}	4.33 \pm 0.33 ^{Ab}	2.67 \pm 0.33 ^{Aa}	-
C-2	7.00 \pm 0.00 ^{Ad}	6.83 \pm 0.17 ^{Ad}	5.83 \pm 0.17 ^{Bc}	4.67 \pm 0.33 ^{Bb}	3.16 \pm 0.17 ^{Aa}
T-1	7.00 \pm 0.00 ^{Ac}	6.83 \pm 0.16 ^{Ac}	5.83 \pm 0.17 ^{Bb}	4.83 \pm 0.44 ^{Ba}	4.50 \pm 0.29 ^{Ba}
T-2	7.00 \pm 0.00 ^{Ab}	6.83 \pm 0.16 ^{Ab}	5.67 \pm 0.33 ^{Ba}	5.5 \pm 0.29 ^{Ba}	4.83 \pm 0.44 ^{Ba}
T-3	7.00 \pm 0.00 ^{Ad}	6.83 \pm 0.17 ^{Ad}	6.16 \pm 0.16 ^{Bc}	5.33 \pm 0.16 ^{Bb}	4.83 \pm 0.17 ^{Ba}
Panellist [#]	NS	NS	NS	NS	NS

$n = 6$, C-1 = negative control with no LKE or BHA, C-2 = positive control with 200 ppm BHA, T-1 = 0.05% LKE, T-2 = 0.1% LKE, and T-3 = 0.5% LKE. Means \pm S.E. followed by different lowercase superscripts in the same row differ significantly ($p \leq 0.05$). Means \pm S.E. followed by different uppercase superscripts in the same column differ significantly ($p \leq 0.05$). ND = not detected. ¹Rancid odour scoring scale: 1 = unacceptable, 2 = very strong, 3 = moderately strong, 4 = slightly strong, 5 = perceptible, 6 = barely perceptible, and 7 = none. [#]Panellist was used as a random variable in the analysis. NS = non-significant ($p > 0.05$).

In vitro analysis

Total phenolic contents

Total phenolic contents of LKE were analysed in comparison to BHA to have a clear view on the comparative efficacy of these compounds under *in vitro* and *in vivo* conditions. BHA is a synthetic pure phenolic antioxidant (Brewer, 2011) used as a preservative in the food industry. Phenolic compounds tend to act as antioxidants due to their redox potential, and OH groups of these phenolic compounds are the key scavengers of free radicals (Soobrattee *et al.*, 2005). Antimicrobial activities of

phenolic groups are mediated either by damaging the outer membrane or interaction with the nucleic acids (Puupponen-Pimiä *et al.*, 2001).

The above quantitative analysis of *P. cineraria* was further refined through RP-HPLC to unmask individual phenolic compounds responsible for antioxidant activities.

Total flavonoid content

Flavonoids, the secondary metabolites of plants, consist of free OH groups, especially 3-OH and flavones, flavanols, and condensed tannins (Baba

and Malik, 2015). The flavonoids exhibit both antimicrobial and antioxidant activities (Eddine *et al.*, 2016).

Antioxidant activities

FRAP is a widely used assay in the antioxidant world to evaluate antioxidant potency (Dudonné *et al.*, 2009). The reduction potency of the samples to form TPTZ-Fe (II) by reducing TPTZ-Fe (III) complex is the basic principle for assessing the antioxidant potential of the same. The FRAP values for LKE were lower than that of BHA, stamping the fact of having more phenolic content in BHA, as shown in Table 1. The DPPH scavenging activity assay assesses the capability of the sample/antioxidant component to donate H⁺ to a free radical to neutralise radical's reactivity. The values of the antioxidant potential obtained from LKE were found to be at par or higher than the earlier reports. Asati *et al.* (2021) has reported 78.85% of radical scavenging activity in the crude extract of *P. cineraria* pods/seeds (200 µg/mL). The study further emphasised the use of methanol as an excellent solvent to extract antioxidants from different floral parts. As the scavenging potential of extracts was very high, they were diluted ten times before performing the ABTS+ decolourisation assay. Though the antioxidant activities of LKE were significantly lower than BHA at the same concentration, they were enough to protect the meat from oxidative damage. The higher flavonoid and phenolic contents of LKE make it competent to BHA in terms of antioxidant activities, as the phenolic and flavonoid contents are positively correlated to antioxidant activities (Oktay *et al.*, 2003). Moreover, this also explained the ability of *khejri* tree to sustain/flourish in the water-scarce ecosystem and its ability to withstand stress as phenolic and flavonoid contents make the plants more stress-tolerant (Baba and Malik, 2015).

In vivo analysis

pH

The possible justification for lower pH in the sample could be the release of H⁺ ions due to drip loss, which consequently resulted in low instrumental pH or alternatively higher solute concentration in the muscle tissue due to loss of fluid, which in turn resulting in a decrease in pH (Leygonie *et al.*, 2012). The extract might have properties to buffer the pH of the meat, thus resulting in stable minced meat pH.

The antibacterial and buffering ability of LKE probably resulted in a slower rate of increase in treated samples followed by BHA-treated and control samples. The possible reason for increased pH during the storage period was the increased microbial growth and protein decomposition. With microbial growth, metabolites start accumulating, and protein decomposition results in the formation of ammonia and other products of amino acid decomposition, which increase the pH (McDowell *et al.*, 1986). The slow increase in pH in LKE-treated products in comparison to C-2 might be attributed to the higher antimicrobial activity of LKE than BHA. Spoilage microorganisms metabolise the basic nitrogenous compounds, and generate secondary metabolites which increase the pH. Therefore, the higher the antimicrobial activity, the slower the microbial growth, and the slower the rate of pH increase (Zhang *et al.*, 2016).

Oxidative stability

TBARS assay depicts the oxidative stability of foods, and is based on the principle of measuring secondary products of oxidation which are mainly responsible for oxidative rancidity. The TBARS data signified the potential of *khejri* tree leaves as an efficient antioxidant against lipid oxidation in raw meat system. This observed efficacy of LKE to maintain the oxidative stability of meat may be attributed to its constituent phenolic compounds detected through RP-HPLC as mentioned earlier (Figure 1). The phenolic compounds possess the antagonistic ability against radical-mediated chain reaction which progresses during the oxidation process (Negi and Jayaprakasha, 2003).

Tyrosine value is the indicator of the degree of autolysis and bacterial proteolysis in meat. The assay quantitatively determines the amino acids level (tyrosine and tryptophan) present in meat extract. The values were found to increase with the progression of storage. Studies indicated the commencement of autolysis and bacterial-mediated autolysis as microbial load increased with storage period. Proteolytic enzymes are produced during the later phase of logarithmic microbial growth that may be responsible for proteolysis, thus resulting in higher tyrosine values (Dainty *et al.*, 1975).

Microbial stability

Microbial spoilage can be physically identified by measuring ERV. The threshold limit of ERV for

product acceptability is greater than 15 mL of extract release. Jay *et al.* (2008) documented ERV relation and validity to assess the spoilage of beef. The ERV showed a similar trend of pH, inferring a positive correlation. ERV values gave clear indication of antibacterial activities of LKE in minced mutton as the amount of extract release was inversely related to the spoilage-causing bacterial load.

The lower TPC in LKE-treated minced meat indicated the antibacterial potential of LKE. The presence of phenolics, flavonoids, and tannins well explained and justified the antibacterial activity of the *P. cineraria*. Jayashree *et al.* (2014) also proposed the role of flavonoids and tannins in antimicrobial activity, and reported the antibacterial effect of ethanolic and aqueous extracts of *P. cineraria* against *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. Preeti *et al.* (2017) has also concluded that the methanolic extract of *P. cineraria* leaves is effective against a number of spoilage-causing and pathogenic bacteria like *E. coli*, *S. aureus*, *B. subtilis*, and *S. griseus*. Liu *et al.* (2012) has also documented the antimicrobial activity of methanolic extract of *Prosopis* (stem bark) at 250 µg/mL against almost all pathogens. Absence of coliform count indicated the good hygienic practices adopted during meat processing and sampling.

Sensory evaluation

The sensory evaluation of the control group was aborted on day 7 due to the detection of foul odour, which is one of the probable indicators of the start of meat spoilage. The lower colour scores of T-3 were probably due to the slightly greenish colour imparted by LKE powder, which led to the conclusion that a 0.5% concentration of LKE negatively affected the consumers' acceptability. A decrease in odour scores directly suggested an increase in rancidity or lipid oxidation. The odour scores were at par in all treatments, though higher ($p \leq 0.05$) than that of control, thus indicating the inhibitory effect of LKE on rancidity. Therefore, a combined study of sensory evaluation and oxidative stability indicated the role of LKE in extending the shelf life of minced mutton, comparable with BHA-treated samples.

Conclusion

The present work highlighted the presence of six phenolic compounds in LKE, exhibiting high antioxidant and antimicrobial potentials. At 0.5%

level of LKE incorporation, the minced meat was found to be more oxidative and microbial stable than controls with BHA while maintaining its sensory attributes. For sensory acceptability, 0.1% level of LKE was concluded to be the optimal limit of incorporation in minced meat. Therefore, LKE may be exploited as a promising alternative for BHA in muscle food items, and experimented with many other food products. This may further aid to meet out the consumer demands for healthy natural food products. Consequently, large scale extraction of antioxidants from *Prosopis cineraria* tree leaves could represent a promising future perspective in the natural antioxidant world. Further, the individual fractions of LKE exhibiting antioxidant and antimicrobial activities may be separated for further analysis.

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