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Bioactivity Analysis and Citral Content Estimation of Value Added Paneer Incorporated with Lemongrass Extract and Lemongrass Oil

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A B S T R A C T

Effect of lemongrass extract and oil on the bioactivity (antioxidant and antimicrobial property) and citral content of *paneer* was evaluated in this study. Lemongrass was incorporated in the following three ways: i) cut and crushed leaves were added to milk @ 0, 2, 4 and 6% (w/v) or to coagulant solution @ 20% (w/v) and extracted by heat treatment; ii) cut leaves were heat extracted in potable water followed by heat concentration, and chilling. The chilled water was used for soaking *paneer* blocks; iii) Lemongrass oil added to milk at 0, 0.015, 0.02 and 0.025% levels. The studies on anti-oxidant characteristics as evaluated by DPPH and FC assays revealed that, RSA activity was the highest for *paneer* added with lemongrass oil (8.77% inhibition) and the total phenolic compounds were found to be the maximum (0.0056 mg/g GAE) for *paneer* incorporated with crushed lemongrass leaf. The antibacterial studies of paneer samples revealed that the incorporation of lemongrass extract as well as oil did not impart any antibacterial effect. *Paneer* sample with the addition of lemongrass leaf-bits (4% w/v) into milk, was selected as the optimized sample based on organoleptic tests. In the gas chromatographic analysis, both isomers of citral, neral and geranial, were eluted out. It is concluded from the study, that lemongrass has fortified paneer with bioactive properties than control *paneer* and is a quite promising herb for the development of value added dairy products.

Introduction

Paneer represents a variety of Indian soft cheese, a base material for the preparation of a wide range of culinary dishes. It is highly nutritious and wholesome. *Paneer* consists of protein and usually all the fat, insoluble salts and colloidal materials, together with part of the moisture serum of the original milk, lactose, whey proteins, soluble salts, vitamins and other milk components (Kanawjia *et al.*, 1990). In recent years, consistent efforts have been made to create flavoured *paneer* with novel additives *viz.*, herbs and spices.

In food processing, herbs and spices have traditionally been incorporated to extend shelf life. Crude extracts of herbs and spices, and

other plant materials rich in phenolics are being viewed with increasing interest in the food industry as they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. Essential oils and their components are becoming popular as naturally occurring antimicrobial agents (Walker, 1994). The Food and Drug Administration (USA) considers most of the essential oils as GRAS (Generally Recognized As Safe). Many plant-derived antimicrobial compounds have a wide spectrum of activity against bacteria, fungi and mycobacteria and this has led to their usage as natural preservatives in foods (Farag*et al.*, 1989; Conner and Beuchat, 1984; Jagannath, 2012).

Lemongrass is an aromatic perennial tall grass and a native herb from India, with rhizomes and densely tufted fibrous root, termed by our ancients as a "sacred herb" that is widely used as an essential ingredient in Asian cuisines because of its sharp lemon flavour. Botanically, lemongrass belongs to Family: Poaceae (Gramineae) and species, Cymbopogon. There are two main species, East Indian, *Cymbopogon flexuosus* and West Indian, *Cymbopogon citratus*. It is an economically important plant that has been used for centuries as a medicine because of its wide-ranging therapeutic properties (Fenwick *et al.,* 1990).

Lemongrass contains an aldehyde namely citral, chemically known as 3,7-dimethyl-2,6-octadienal (mixture of two geometric isomers, geranial (citral A) and neral (citral B)) as its major component in a 70–85% concentration, which is responsible for the citrus aroma. Geranial and neral are light oily liquids. Geranial has a strong lemon odour while the lemon odour of neral is weaker but sweeter than geranial. Lemongrass also contains a volatile oil whose yield is about 0.5% from fresh grass (1 - 2% essential oil on a dry basis), characterized by its yellow or amber colour and lemon-like odour (also possesses an herbaceous verbena-like odour not possessed by lemon oil). Other major phytochemicals are borneol (5%), geraniol (2.6-40%), geranyl acetate (0.1-3%), linalool (1.2-3.4%) and nerol (0.84.5%) (Schaneberg and Khan, 2002; Carlson *et al.,* 2001). The bioactive compounds from lemongrass are extracted by different methods such as boiling of oven dried leaves (decoction), boiling of freshly ground leaves (infusion) and steam distillation (essential oil).

Citral possesses antioxidant activity, and it may serve as one of the antioxidant defences of the plant against harmful free-radicals or reactive oxygen species. In other studies, citral was demonstrated to serve as a plant defence against the damaging effects of microorganisms. According to studies by Vazquez- Briones *et al,* (2015), the essential oil of *Cymbopogon citratus* exhibited high citral concentration with high phenolic content (149.20 mg GAE per 100ml) and antioxidant capacity (44.06mg Trolox per 100ml of essential oil). Lemongrass has high antioxidant capacity and the free radical scavenging effect of hydro-alcoholic extract of *Cymbopogon citratus* was established (Rao *et al.,* 2009*)*.The comparative analysis of the antioxidant activities of methanolic and aqueous extracts of few selected herbs also proved the antioxidative potential of lemongrass leaves (Deepa Garg *et al.,* 2012). Ethanol extract of lemongrass leaves has a potential as antioxidant because of its inhibitory activity against free radical DPPH (2, 2-diphenyl-1-picrylhydrazyl) (Hasim *et al.,* 2015).

It has been established that lemongrass possesses antibacterial (Morris, *et al.,* 1979; Dube *et al.,* 1984; Onawunmi *et al.,* 1984; Onawunmi *et al.,* 1985; Elson and Underbakve, 1989; Ibrahim, 1992), antifungal

(Josper and Liguari, 1958; Rao and Narasimh, 1971), nematocidal (Sangwan, *et al.*, 1985), insect repellent (Jiang, 1993), antioxidant, antipyretic, anti thrombiotic and serum cholestrol lowering properties (Burger, *et al.*, 1986; Elson and Underbakve, 1989). Lemongrass oil possesses bactericidal and anti-fungal properties, comparable to penicillin in its effectiveness (Lutterodt *et al.*, 1999). Isam *et al.*, (2009) studied the antimicrobial activity of lemongrass leaf extracts and demonstrated a broad-spectrum of activity against both gram-positive and gram negative bacteria and fungi, possibly the acidic nature of the extracts (pH values ranging between 3-5) combined with bioactive components (saponins, tannins, alkaloids and flavonoids) enhanced the antimicrobial activity of the extracts especially against the bacteria. Gram positive organisms were found more sensitive to lemongrass oil as compared to gram negative organisms.

Citral showed potent antifungal activity against various fungi which cause severe postharvest diseases in fruits (Ben-Yehoshua *et al.*, 1995; Garcia *et al.*, 2008). Saponins, tannins, alkaloids and flavonoids are present in lemongrass extracts and are known to be bactericidal, pesticidal or fungicidal in nature thus conferring the antimicrobial property to plant (Rios *et al.*, 2005). Lemongrass oil and citral have a potent in vitro activity against Candida spp. (*C. albicans, C. glabrata, C. krusei, C. parapsilosis* and *C. tropicalis*) (Silva *et al.*, 2008). Among different essential oils, cinnamon, lemongrass, Japanese mint, ginger grass, geranium and clove oils were observed as most promising against *C. albicans*. Citral showed in vitro antifungal potential against strains of *C. albicans* (Leite *et al.*, 1986).

Herb extracts are known to preserve the quality of soybean oil, beef, meat, poultry, fish and lard. However, fortification by herbal extracts in dairy products would enhance the functionality of the product base, thus striving and aligning with the trend of increased consumption of natural remedies. Indian dairy industry should find ways to induce or to improve the functionality in traditional dairy products. Work reported about the study of bioactive properties of dairy products incorporated with lemongrass is meagre. Therefore, the present investigation was carried out to study the effect of incorporation fresh leaf extract of lemongrass as well as oil on bioactivities of the treated paneer.

Materials and Methods

For the preparation of *paneer*, fresh cow milk was procured from cattle yard of Southern Regional Station of National Dairy Research Institute, Bengaluru, filtered and standardized to 3.0% fat and 8.5% SNF. Fresh leaves - medium sized, matured and dark green coloured- of lemongrass (*Cymbopogon flexosus)* grown in the Institute campus was picked up for the project work. Freshly extracted lemongrass oil was obtained from the Horticultural Section, University of Agricultural Sciences, GKVK, Bengaluru.

Preparation of paneer

Paneer was prepared in the laboratory as per Bhattacharya *et al.*, (1971) with slight modification. Milk, standardized to 3.0% fat and 8.5% SNF was heated to 90°C without holding, cooled to 80 °C and coagulated by the addition of 2% citric acid solution at 80°C. The citric acid was added slowly to milk with continuous agitation till clear whey separated out. The curd was allowed to settle for 5 min and the whey was drained out by hanging the coagulum in muslin cloth. The curd was then collected and filled in wooden hoop lined with clean muslin cloth. Pressure was applied on the hooped curd @ 0.069

kg/cm^2 for 20-25 minutes. The *paneer* block was taken out from the hoop and then cooled by immersing in chilled water (4 to 6 °C) for 2 h for texturization. The *paneer* block was removed from chilled water and placed on wooden planks for 15 minutes for allowing the water to drain out. The *paneer* block was cut into 2 cm cubes and packed in LDPE pouches and stored in refrigerator (7 ± 2^{o}C) for further analyses.

Incorporation of lemongrass

Lemongrass was incorporated into *paneer* by the following methods:

(1) Fresh lemongrass leaves were washed, and added in both cut form (3-5 cm long cuts) and crushed form (cuts were crushed in a mixer) and added to milk @ 0 (control), 2, 4 and 6% by the weight of milk before heat treatment.
(2) Fresh lemongrass oil was added to milk @ 0 (control), 0.015, 0.020 and 0.025% (v/v) before heat treatment of milk.
(3) Extract from washed and cleaned lemongrass leaves (in crushed form) obtained by boiling the leaves in water (@ 10% by volume of water) was concentrated to half the volume, followed by cooling, chilling and was then used as dipping water for *paneer*.

Optimal levels of the ingredients used during standardisation of the process for preparation of lemongrass flavoured *paneer* were determined using sensory evaluation as given in the Table 1

In the addition of lemongrass oil into milk, 0.015% addition was selected, based on optimum sensory scores. Level of addition of 4% (w/v) was selected as the optimized sample for cut form and 2% (w/v) addition for crushed samples. For the method of dipping coagulum in concentrated and chilled lemongrass extract, the cut form was found to be good.

The optimized lemongrass flavoured *paneer* (4% w/v addition in milk) had a proximate composition as follows: Total solids – 44.74%; Fat -19.38 %; Protein – 21.25 %; Lactose – 2.47 % and Ash – 1.64%, which was found to be well within those reported in the literature.

The *paneer* samples are abbreviated as follows:

C: Control *paneer*; T1: Lemongrass extraction in milk (cut); T2: Lemongrass extraction in milk (crushed); T3: Dipping in lemongrass extract; T4: Lemongrass oil in milk

Bioactivity analysis

Antioxidant activity was measured using DPPH (2, 2-diphenyl-2-picryl hydrazyl) dye, as per the procedure described by (Shimada *et al.,* 1992). The amount of total phenolics in *paneer* samples was determined with the Folin-Ciocalteu reagent according to the method of Singleton *et al.,* (1999). Gas chromatographic analysis of *paneer* samples were performed as that reported by Aniruddha *et al.,* (2011).

Antimicrobial activity of lemongrass extracts and flavoured paneer

Test microorganisms used in the study

Bacterial strain *Escherichia coli* (MTCC 1698), *Staphylococcus aureus* (MTCC7443) and *Candida albicans* (MTCC 7315) were procured from MTCC, Chandigarh.

Inoculum preparation

24 hour old pure cultures of *E. coli* and *S. aureus* were used for the preparation of bacterial suspension as per Mac-Farland Nephelometer Standard. Suspensions of organisms were made in sterile isotonic

solution of sodium chloride (0.9% w/v). 0.5 McFarland standards (1.5 x 10^8 CFU/ml) were used as a reference to adjust the turbidity of microbial suspension (Singh and Jain, 2011).

Method of screening

Sterilization of media, peptone water, distilled water, petri-plates, L-shaped glass rod, micro-tips were carried out in an autoclave at 121°C for 15min. The sterilized Nutrient agar was poured into each petri-dish and allowed to solidify under aseptic conditions inside the Laminar Air Flow (LAF) chamber in a Class-II biosafety cabinet. Sterile paper disc of 6mm diameter was aseptically saturated with 30µl of the fresh lemongrass extract and dilute suspensions of the *paneer* samples(control *paneer* sample, lemongrass flavoured *paneer* with lemongrass leaves added in cut and crushed form and lemongrass oil added *paneer*) in 3 dilutions- 10^1, 10^2, 10^3 respectively. These discs, were allowed to dry for 1hour in Laminar Air Flow (LAF) chamber for complete absorption of the sample and later placed onto solidified nutrient agar [Hi Media (M002)] surface swabbed with30µl of respective test organism (~ 1.5 x 10^8 CFU/ml using 0.5 McFarland's standard)with the help of a sterilized forceps. The plates were incubated for 24 hours at 37°C. The results were recorded as three independent observations by measuring the zone of growth inhibition (mm) around the disc.

Statistical analysis

In order to select the method of incorporation of lemongrass, 3 replications were conducted for each trial. The values of each attribute under study were subjected to statistical analysis by one way ANOVA using SPSS Software 16.0. The significance of treatments effect was determined by Tukey test at 5% level of significance.

Results and Discussion

Optimization of the process of manufacture of lemongrass flavoured paneer

From various methods of incorporation of lemongrass, one sample has been selected based on sensory scores. When lemongrass was extracted into milk in cut form, 4% (w/v) addition was found to be having the best score, whereas in crushed form, 2% (w/v) addition was seen to be optimum.

Lemongrass when extracted into citric acid solution, the crushed form of addition obtained higher sensory scores than cut form, whereas for the method of dipping coagulum in concentrated and chilled lemongrass extract, the cut form was found to be good. In the addition of lemongrass oil into milk, 0.015% addition was selected based on optimum sensory scores, while for addition of oil into citric acid solution the effect of lemongrass oil on the sensory parameters of paneer were insignificant and hence the sample was not taken forward during further studies.

All the selected samples from each method of incorporation, was subjected to sensory evaluation by a panel of judges and the scores are tabulated in Table 1.

It is evident from the figures for sensory scores in the Table 1, that the extraction of cut lemongrass leaf in milk, obtained the highest score compared to all other samples, for most of the sensory parameters, including colour and appearance, flavour and overall acceptability, and the scores were statistically significant and comparable to that of control *paneer* sample.

Hence, the addition of cut lemongrass leaf (4% w/v addition) into milk was selected as the optimized solution for the preparation of lemongrass flavoured *paneer*.

Study of anti-oxidant properties of lemongrass flavoured paneer

Antioxidants are an important group of food additives that have the ability to protect against undesirable change of oxidizable nutrients and consequently extend shelf-life of foods. Antioxidants are receiving remarkable attention in the literature recently, due to their ability to preserve foodstuffs by retarding deterioration, rancidity and/or discolouration caused by oxidation. Plants are very good sources of natural antioxidants. These antioxidants are mostly produced via the secondary metabolism of plants and are referred to as secondary metabolites.

The results of radical scavenging activity (% RSA) of lemongrass extract as well as samples of *paneer* incorporated with lemongrass, expressed as % inhibition are presented in Table 2. In comparison to pure lemongrass extract with an RSA value of 72.62% inhibition, the RSA was found to be the highest for *paneer* added with lemongrass oil (8.77% inhibition) among all the experimental samples and the activity was found to be the least for *paneer* dipped in lemongrass extract (3.53% inhibition).

The results are in accordance with the findings reported in literature. Aqueous extracts of lemongrass were also found to inhibit oxidative stress particularly lipid peroxidation, as well as alteration of lipid membrane systems, caused by paracetamol (Ojo *et al.*, 2006).A study conducted byRao *et al.,* (2009) revealed that the extract of lemongrass at a concentration of 60 µg/ml resulted in a significant scavenging ability of 2,2-diphenyl-2-picryl hydrazyl (DPPH) (85%) and concluded that lemongrass has high antioxidant capacity; Deepa Garg *et al.*,(2012) carried out a comparative analysis of the antioxidant activities of methanolic and aqueous extracts of the selected leaves of herbs commonly used in Indian cuisine (lemongrass, mint, coriander and curry leaves) adopting various assays including DPPH assay. Among the herbs investigated, lemongrass exhibited the maximum content of phenols, leading to a more powerful radical scavenging effect and hence greatest antioxidant profile. According to Villalobos (2015), it has been observed during preliminary experiments of antioxidant that extracts of fresh and dried lemongrass plant samples possess antioxidant activity against ascorbic acid, which is a widely-used standard. Hence, in the present study, we could observe that the lemongrass extract is conferred highly with antioxidant activity and this property got transferred to *paneer* in small amounts, when added with lemongrass extract and oil.

Study of anti-oxidant properties of lemongrass flavoured paneer

Total phenolic constituents of pure lemongrass extract as well as *paneer* samples added with lemongrass were determined by experimental method involving Folin-Ciocalteu reagent (Singleton *et al.,* 1999). The range of phenolic content of all the samples expressed as mg/g Gallic Acid Equivalents (GAE) is presented in Table 3. It was found that the amount of phenolic compounds was high in the aqueous extract of lemongrass (1.7 mg/g GAE). The *paneer* samples also exhibited positive results with the highest amount obtained for *paneer* incorporated with lemongrass leaf in crushed form (0.0056 mg/g GAE), followed by *paneer* added with cut lemongrass leaf extract (0.0046 mg/g GAE) and *paneer* with lemongrass oil in milk (0.004 mg/g GAE) and the lowest concentration of 0.0037 mg/g GAE was obtained for *paneer* added with lemongrass oil. It is evident from the statistical analysis, that there was significant difference between the experimental samples ($P>0.05$) and lemongrass extract.

Evaluation of antimicrobial activity of Lemongrass flavoured paneer

The antimicrobial activity of the various lemongrass added samples was evaluated against species of *E.coli*, *Staph. aureus* and *Candida albicans* and the results tabulated in Table 4. The experimental samples added with lemongrass did not show any significant ($p \leq 0.05$) inhibition activity against any of the species tested (*E.coli*, *Staph. aureus* and *Candida. albicans)*, when compared with the lemongrass oil used as Standard Reference, which exhibited high antimicrobial activity against the same species under consideration, with a zone of inhibition of 17.00, 90.00 and 29.33 mm for *E. coli*, *Staph. aureus* and *Candida albicans*, respectively. The fact that the pure lemongrass oil has showed inhibition is evident from literature (Morris *et al.*, 1979) and amply demonstrated by the present experiment. Besides the bioactive components (phenols) present in the oil, the pH of the extract (pH 3-5) could also have been responsible for the inhibition. However, the low concentration of lemongrass oil (10^1 – 10^3) added to the *paneer* so as not to diminish the organoleptic appeal along with the acidic pH of the paneer might have contributed to whatever little effect observed against the bacteria and fungi.

Table.1 Effect of method of incorporation of lemongrass on the sensory acceptance of paneer

Sample	Colour and appearance	Flavour	Body and texture	Overall acceptability
C	8.24 ± 0.25^{bc}	8.20 ± 0.20^{cd}	8.07 ± 0.12^{a}	8.13 ± 0.12^{cd}
T1	8.60 ± 0.34^{c}	8.40 ± 0.10^{d}	8.04 ± 0.16^{a}	8.37 ± 0.24^{d}
T2	$7.86\pm0.32a^{b}$	7.67 ± 0.15^{bc}	7.97 ± 0.18^{a}	7.83 ± 0.11^{bc}
T3	7.53 ± 0.50^{a}	7.29 ± 0.25^{ab}	7.94 ± 0.12^{a}	7.46 ± 0.25^{ab}
T4	7.88 ± 0.10^{ab}	7.60 ± 0.26^{b}	7.85 ± 0.14^{a}	7.72 ± 0.19^{b}

Note: Figures are mean ± standard deviation of three replications. Values with different superscripts in a row are significantly different ($p\leq 0.05$)
C: Control *paneer*; T1: Lemongrass extraction in milk (cut); T2: Lemongrass extraction in milk (crushed); T3: Dipping in lemongrass extract; T4: Lemongrass oil in milk

Table.2 Radical scavenging activity of lemongrass added paneer samples against Std. Reference – GAE

Sample	%RSA
S	72.62 ± 0.49^{e}
T1	5.76 ± 0.39^{bc}
T2	4.76 ± 0.43^{b}
T3	3.53 ± 0.47^{a}
T4	8.77 ± 0.45^{d}

Note: Figures are mean ± standard deviation of three replications. Values with different superscripts in a row are significantly different ($p \leq 0.05$)
S: Pure lemongrass extract; T1: Lemongrass extraction in milk (cut); T2: Lemongrass extraction in milk (crushed); T3: Dipping in lemongrass extract; T4: Lemongrass oil in milk
RSA – Radical Scavenging Activity (% Inhibition)

Table.3 Effect of addition of lemongrass on the total phenolic content of lemongrass added paneer samples against Standard

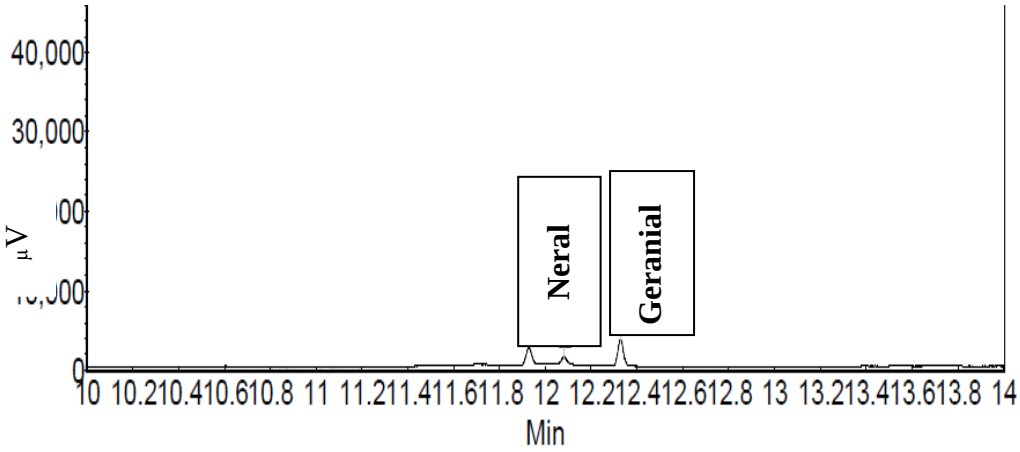
Sample	GAE (mg/g)
S	1.5 ±0.05[b]
T1	0.0046±0.0001[a]
T2	0.0056±0.0003[a]
T3	0.0040±0.0002[a]
T4	0.0037±0.0002[a]

Note: Figures are mean ± standard deviation of three replications. Values with different superscripts in a row are significantly different ($p \leq 0.05$)
S: Pure lemongrass extract; T1: Lemongrass extraction in milk (cut); T2: Lemongrass extraction in milk (crushed); T3: Dipping in lemongrass extract; T4: Lemongrass oil in milk
GAE – Gallic Acid Equivalents

Table.4 Antimicrobial activity of paneer samples against *E.coli, Staph. aureus and Candida albicans*

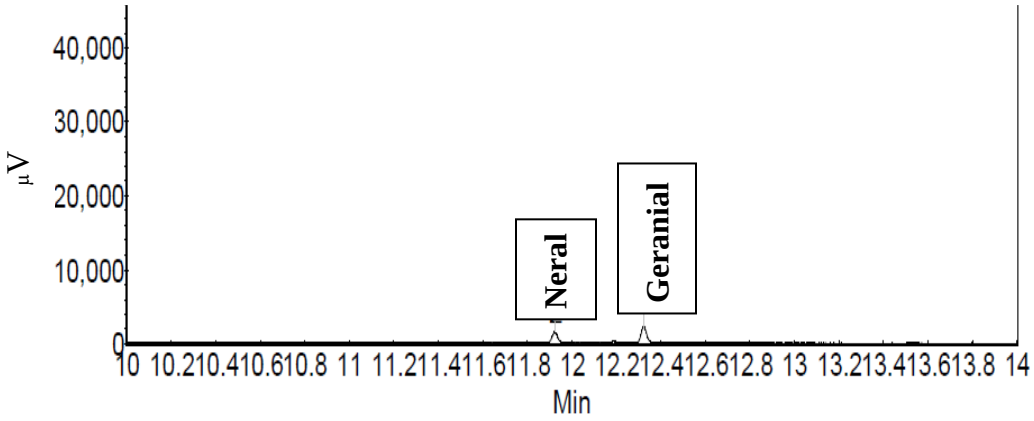
Microorganism tested	% Inhibition			
	Std.Lemongrass oil (mm)	Paneer with lemongrass oil	Paneer with cut leaf extract	Paneer with crushed leaf extract
E.coli	17.00±1.00	No inhibition		
S.aureus	90.00±0.00			
C.albicans	29.33±0.36	Slight inhibition		

Fig.1a Gas-Chromatography analysis report of lemongrass added samples against Standard (Lemongrass oil) a) Paneer added with cut lemongrass leaf (4% w/v) b) Paneer added with crushed lemongrass leaf (2% w/v) c) Paneer added with lemongrass oil (0.02%) and d) Pure lemongrass oil (Std. Reference



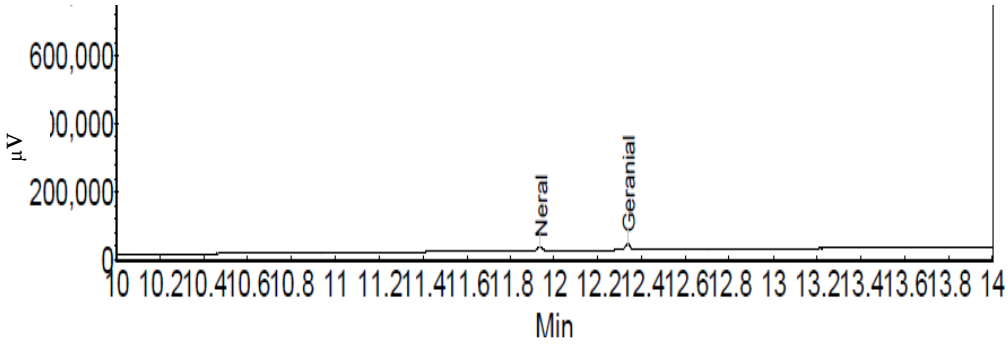
Neral: RT (min): 12.08; Area (%): 2.64. Geranial: RT (min): 12.33; Area (%): 6.81; RT-Retention Time

Fig 1(a)



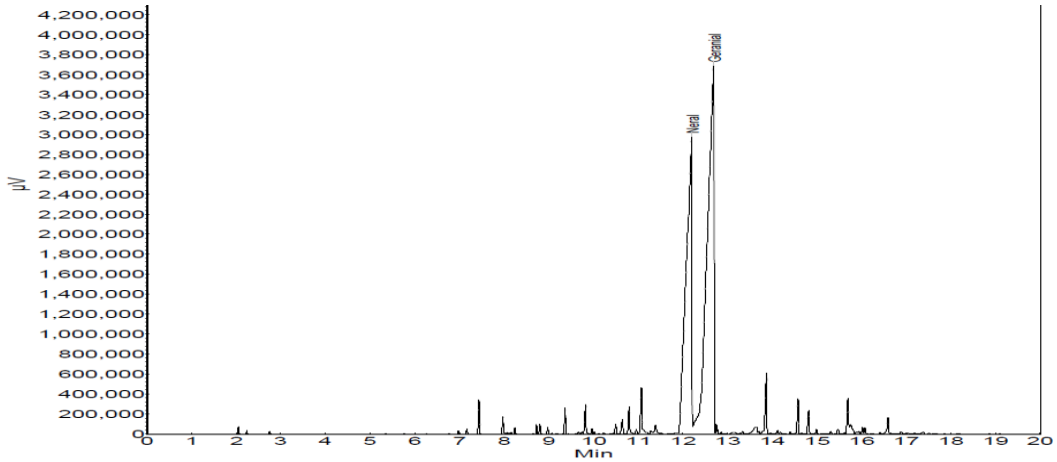
Neral: RT (min): 11.93; Area (%): 0.14. Geranial: RT (min): 12.33; Area (%): 0.26; RT-Retention Time

Fig 1(b)



Neral: RT (min): 11.94; Area (%): 8.96. Geranial: RT (min): 12.34; Area (%): 13.37; RT-Retention Time

Fig 1(c)



Neral: RT (min): 12.2; Area (%): 32.73. Geranial: RT (min): 12.69; Area (%): 50.07; RT-Retention

Fig 1 (d)

Estimation of citral content of lemongrass containing paneer samples

Lemongrass contains an aldehyde namely citral as its major component in 70–85% concentration, which is responsible for the citrus aroma as well as the bioactivity. It is a mixture of two geometric isomers, geranial (citral A) and neral (citral B).

The citral content of the lemongrass added *paneer* samples, was analysed using Gas-Chromatography and the results are shown in Figure 1. In the pure lemongrass oil (Std. Reference), neral and geranial corresponded to an area of 32.73% and 50.07% respectively. Both the isomers, neral and geranial, were eluted out, from all the experimental samples (*paneer* added with cut leaves, crushed leaves and lemongrass oil) when subjected to Gas-Chromatography, but with comparatively lesser area in the chromatogram.

The higher amount of citral was found in *paneer* added with lemongrass oil, while the lowest was obtained for *paneer* added with crushed leaves of lemongrass.

In conclusion, the present research has opened up possibilities of flavouring paneer with natural aroma. Paneer is a bland milk product and does not have any flavour of its own. The addition of herbs and spices to food and dairy products, due to their wide range of bioactivities along with enhancement of flavour and improved shelf life is increasingly being pursued. Being natural, herbs and spices appeal to consumers with regard to the safety of synthetic additives. The results show that use of the natural antioxidants occurring in herbs used in the Indian diet, or their extracts, is a viable option for the food industry as long as the organoleptic characteristics of the food product are not altered.

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