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Preservative effect of marigold (*Tagetes erecta)* petal extract (MPE) on Indian cottage cheese and its storage study

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Abstract
The study was conducted to establish Marigold Petal Extract (MPE) as natural preservative in Indian cottage cheese. MPE was prepared with concentration 250mg/ml in ethanol and ZOI was studied for *S. cerevisiae*, *S. aureus*, *C. perfringens*, *P. aeruginosa*, *E. coli* and *S. enterica*, which were reported to be 1.3mm, 2.5mm, 2.9mm, 2.7mm, 1.8mm and 3.4mm respectively. Considering above results, MPE was used in three different concentrations to prepare Indian cottage cheese samples and its shelf life (at 4-7℃ for 15 days) was studied on basis of microbial and sensory analysis. The result showed a decrease in SPC from log 1.7160 cfu/ml to log 0.8450 cfu/ml on 6th day and log 2.0681 cfu/ml to log 1.6020 cfu/ml on the 15th day with increase in MPE concentration from 0% (control R) to 3%. No coliform reported in sample C (3% MPE). Sensory analysis showed the overall acceptability of sample B (2% MPE).

Keywords: Marigold petal extract (MPE), Zone of inhibition (ZOI), Indian cottage cheese, natural preservative, disc diffusion

1. Introduction
For centuries *Calandula officinalis* also known as pot marigold or scotch marigold is used as antifungal agent with antibacterial properties, marigold of the same family (*Asteraceae*) is grown in India in abundance. The flower is extensively used for decorative purposes, in every festival and in temples as offerings. The above usage is only for fresh flower and the waste generated is huge which is otherwise dumped for compost production.
Belonging to the same family (*Asteraceae*), marigold also bears the similar properties which was investigated by (Gupta & Vasudeva, 2012) [7]. He mentioned the bioactive compounds like thiophenes, flavonoids, terpenoids, carotenoids and phenols in marigold having biological activities like anti-microbial, antimycotic, anti-plasmodial, anti-oxidant, insecticidal etc. (Gupta & Vasudeva, 2012) [7]. Available reports indicate their broad spectrum against various spoilage and pathogenic microorganisms namely *Bacillus subtilis*, *Bacillus anthraces*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium digitatum*, *Candida albicans*, *Rhizopus stolonifer*, *Psedumonas agalactiae*, and more (Voon *et al*., 2012) [17]. The flavonoid possesses antibacterial activity against all tested strains and shows maximum zone of inhibition (ZOI) for *Klebsiella pneumonia* (29.50 mm) (Dixit *et al*., 2013) [4]. The dried flowers of marigold revealed the presence of phenolic compounds such as syringic acid in addition to various compounds such ethyl gallate and methyl- 3,5-dihydroxy-4-methoxy benzoate (Gupta & Vasudeva, 2012) [7], which have natural preservative properties (Shi *et al*., 2016) [13].
To establish the utility of marigold as a natural food preservative, experiments were conducted upon Indian cottage cheese, which has a limited shelf-life of only 6 days under refrigeration at 10°C due to high moisture content of about 55% (Khatkar *et al*., 2017) [9] and relatively high pH (~5.0) (Himabindu & Arunkumar, 2017) [8]. Food additives such as sorbic acid, potassium sorbates, solutions of H_2O_2 and brine, and other chemical preservatives have been tried successfully to increase the shelf-life of Indian cottage cheese (Buch *et al*., 2014) [3], but the potential hazards posed by these has encouraged to search stable natural preservatives acceptable to both food safety authority and consumers. The basic idea is to use a natural anti-microbial system to extend the storage life of Indian cottage cheese.
With present view, Marigold petal extract (MPE) was made by extracting the bioactive compounds in organic solvent (ethanol) and incorporating it in pasteurized milk used for Indian cottage cheese preparation.

2. Materials and methods

2.1 Materials: Fresh marigold flowers (*Tagetes erecta L.*) for the preparation of Marigold Petal Extract (MPE), polyvinyl chloride (cling film) used for packaging of Indian cottage cheese samples during storage and pasteurized Amul gold milk (6% fat, 9% SNF) for the preparation of Indian cottage cheese samples were purchased from the local market of Sipri market, Jhansi. Chemicals and reagents (analytical grade) used during the experimentation were precured from the chemistry and microbiology laboratories of Institute of Food Technology, Bundelkhand University, Jhansi.

2.1 Methodology

2.2.1 Preparation of Marigold Petal Extract (MPE): The extract was prepared following the process of maceration and centrifugation according to (Bissa & Bohra, 2011) [2] and (Tiwari *et al*., 2011) [15]. Marigold flowers were washed first by tap water and then by distilled water. Petals were separated from the base and washed again using distilled water and spread on a kitchen towel to remove excess water. 50g petals were grounded in 200ml ethanol and kept at room temperature for 24 hours to evaporate the solvent. The macerates were first squeezed through a double layered muslin cloth and then filtered through Whatman No.1 filter paper. The aliquot was centrifuged at the speed of 10000rpm for 20 minutes, followed by filtration of the supernatant through Whatman No.1 filter paper and then sterilized by passing through 0.2-micron disposable filters. The prepared extract (MPE) of concentration 250mg/ml was stored at 4°C until use.

2.2.2 Preparation of Indian cottage cheese samples: Indian cottage cheese was prepared in the laboratory similar to the procedure described by (De, 2013), using 500ml standardized milk (6% fat and 9% SNF) for each sample. For each sample, milk was initially heated to 80°C in a vat and maintained for 30 minutes12gfv followed by lowering the temperature to 70°C with occasional stirring to prevent skin formation. MPE was added at this time @ 1% (5ml MPE) in sample A, 2% (10ml MPE) in sample B and 3% (15ml MPE) in sample C, leading to final concentrations equal to 2.5mg/ml, 5.0mg/ml and 7.5mg/ml respectively (Singh & Immanuel, 2014) [14]. To separate casein and whey, which is the basis of Indian cottage cheese preparation, citric acid @ 0.25% (5ml) was added with continuous stirring (30-40 motions/min) till complete coagulation was achieved as evident from the clarity of whey (greenish white tinge, pH- 5.5-5.6) in about 60-80 seconds. After keeping the vat undisturbed for 15 minutes, whey was drained off and the coagulated mass was collected in a muslin cloth. Pressure was applied on the top of the coagulant mass by placing weight of approx. 2kg for another 15 minutes. This way extract (MPE) incorporated samples and a control sample without the incorporation of MPE (Sample R) were prepared. All the samples were stored at 4-7°C until the experiments were conducted.

2.2.3 Microbial analysis: All microbial tests were conducted on the 0^{th}, 3^{rd}, 6^{th}, 9^{th} and 15^{th} day according to the schedule followed by (Buch *et al*., 2014) [3] (Gautam & Siddiqui, 2020) [6].

2.2.2.1 Anti-microbial assay using disc diffusion method This method was used to check the anti-microbial activity of MPE. The discs were made using filter paper and kept in bio safety under UV light to sterilize them. These discs are now dipped in ethanol and MPE of 50% and 75% concentration. Then these discs were placed one by one on the previously solidified agar plates inoculated with the pure culture of test microorganism, namely *S. cerevisiae* (MTCC 170), *S. aureus* (MTCC 3160/NCTC 3756), *C. perfringens* (MTCC 450), *P. aeruginosa* (MTCC 424), *E. coli* (MTCC 1687) and *Salmonella enterica* (MTCC 170) separately, obtained from FICCI Research and analysis centre. They were then incubated at optimum temperature for each microbe until clear microbial growth was observed.
The area of zone of inhibition (ZOI) was calculated by measuring the diameter on the agar plates onto which discs dipped in ethanol and MPE were placed, taking the disc as the centre, until where no microbial growth is seen.

2.2.2.2 Standard Plate Count (SPC) & Yeast and Mold Count: The microbial tests were the basis to judge the effectiveness of MPE to retard microbial growth thereby increasing the shelf-life of the Indian cottage cheese samples. For this purpose, SPC, yeast & mold and coliform count tests were conducted upon Indian cottage cheese samples A, B, C and control sample R using pour plate technique. The SPC petri-plates were incubated for 5 days in an incubator at 30-35°C (Ledenbach & Marshall, 2009) [10] (Nwogu *et al*., 2012) [11]. For yeast & mold count potato dextrose agar (PDA) was used as a culture media and the inoculated Indian cottage cheese samples were incubated at 30-35°C for 5 days.

2.2.2.3 Coliform and E. coli: Coliform colonies were determined using Mac Conkey agar for presumptive coliform test at 37°C for 24 hours (Ahmed *et al*., 2016) [1].

After the incubation, the number of colonies appeared in the microbiological samples were incubated using the following formula (Aneja, 2018).

CFU/ml = CFU* dilution factor
Dilution factor =1/ diluent

2.2.3 Sensory analysis: The Indian cottage cheese samples A, B, C and R were evaluated organoleptically by serving in a random order to 10 semi-trained sensory panellists on a 9-point Hedonic scale on the basis of attributes like colour, flavour, texture, appearance, aroma and over all acceptability. The rating scale had points 9 to 1, corresponding to descriptions ranging from “like extremely” to “dislike extremely” respectively. The observations were recorded on the score-cards and the mean scores were calculated.

2.2.4 Statistical analysis: The effect of MPE on microbial shelf life of Indian cottage cheese samples was calculated statistically using ANOVA- two way (without replication) at 5% level of significance.

The impact of increase in MPE concentration on sensory attributes of Indian cottage cheese samples was analysed by measuring the correlation.
All statistical analysis was conducted using MS Excel (Windows 2013).

3. Results and Discussion

Microbial analysis: The prepared Indian cottage cheese samples A, B and C with MPE and R (without MPE) tested on microbiological parameters gave the following results:

3.1 Anti-microbial assay using disc diffusion method: The observed ZOI of the tested microorganisms showed that the antimicrobial effect of MPE was greater than that of ethanol. MPE with 75% concentration gave the best results with the highest ZOI in each case. The increase in the concentration of MPE from 50% to 75% showed a very little increase in ZOI being not more than 0.3mm for *S. cerevisiae, S. aureus* and *E. coli.* The increase in concentration of MPE showed maximum impact in ZOI of *C. perfringens, P. aeruginosa* and *Salmonella enterica* which was up to 1.5mm. Same tests performed by using double distilled water in place of ethanol or MPE gave no zone of inhibition for any of the microbe.
The ZOI against the tested microorganisms in ethanol and MPE are recorded in table 1.

Table 1: Zone of inhibition (ZOI) of selected microorganisms

Microbes	ZOI (mm)		
	Ethanol	MPE (50%)	MPE (75%)
S. cerevisiae	1.1	1.2	1.3
S. aureus	1.1	2.3	2.5
C. perfringens	1.1	2.5	2.9
P. aeruginosa	1.6	2.1	2.7
E. coli	1.1	1.6	1.8
Salmonella enterica	1.1	1.9	3.4

The reported ZOI clearly indicate the effectiveness of MPE in inhibiting the growth of the selected microorganisms. These findings were similar to the works of (Gupta & Vasudeva, 2012) [7], (Rhama & Madhavan, 2011) [12], (Verma & Verma, 2012) [16] and (Bissa & Bohra, 2011) [2] which supported that MPE has potent antimicrobial activity.

3.2 Standard Plate Count (SPC) and Yeast & Mold (Y/M) Count: SPC result showed continual decrease in microbial count from log 1.043 cfu/ml to log 0.3010 cfu/ml with increase in the concentration of marigold petal extract from 1% to 3% on the 3rd day and onwards. Whereas in Control sample R, there was 54% increase in microbial colonies on the 3rd day from log 1.1139 cfu/ml to log 1.7160 cfu/ml and 85% increase in microbial counts on the 15th day from log 1.1139 cfu/ml to log 2.0681 cfu/ml. In sample A, sample B and sample C, the increase in microbial counts from 3rd day to 15th day was 13%, 27% and 25%, which indicates the effectiveness of marigold petal extract in reducing the microbial growth. It is also evident that increase in concentration of MPE from 1% to 3% (sample A, B & C), there was 78% decrease in microbial count on 3rd day and 50% in numbers on 6th day, i.e., log 1.3979 cfu/ml to log 0.3010 cfu/ml and log 1.7160 cfu/ml to log 0.8450 cfu/ml respectively, while the decrease in microbial count was gradual and significant from 9th day to 15th day in all samples (A, B & C). Gupta & Vasudeva, 2012 [7], Rhama & Madhavan, 2011 [12], and Bissa & Bohra, 2011 [2] reported the similar trend and effectiveness of marigold plant extract in inhibiting microbial growth.
Yeast & Mold result showed the growth in all samples A, B and C on 3rd day with 23% decrease in Sample C as compared to sample A and Sample B, i.e., log 1.4771 cfu/ml to log 1.9444 cfu/ml. On 12th and 15th day also, Sample A showed 92% and 56% decrease in microbial (Y/M) count as compared to control Sample R from log 1.8450 cfu/ml to log 0.4771 cfu/ml and log 1.9444 cfu/ml to log 0.8450 cfu/ml respectively. In rest of the samples (A, B and C), no growth reported on 6th day, 9th day, 12th day, and 15th day, indicating the effectiveness of marigold petal extract as preservative in reducing Y/M colonies at temperature 4˚C for 15 days.

3.3 Coliform and E. coli: No significant growth in coliform counts were reported in Sample A, B and C till 12th day. On 15th day, Sample A and B showed few colonies which were very much below the permissible limit of FSSAI, i.e., log 1 cfu/ml to log 2 cfu/ml, as mentioned for Indian cottage cheese. No E. coli colonies were reported in any of the samples- Sample A, B and C, for 15 days including Control Sample R, at temperature 4-7˚C.

Table 2: SPC, Yeast and Mold and Coliform in cfu/ml

Days	Control (R)	Levels of MPE added in Indian cottage cheese samples		
A. Standard Plate Count (SPC)		**Sample A (1%)**	**Sample B (2%)**	**Sample C (3%)**
0	1.1139	0.4771	-ve	-ve
3	1.3979	1.0413	0.6020	0.3010
6	1.7160	1.4310	1.2041	0.8450
9	1.7781	1.5797	1.3424	1.2787
12	1.8976	1.6232	1.5440	1.4623
15	2.0681	1.7993	1.7075	1.6020
A. Yeast and Mold Count		**Sample A (1%)**	**Sample B (2%)**	**Sample C (3%)**
0	1.4471	-ve	-ve	-ve
3	1.5563	1.4771	1.3979	1.2041
6	1.6434	-ve	-ve	-ve
9	1.7634	-ve	-ve	-ve
12	1.8450	0.4771	-ve	-ve
15	1.9444	0.8450	-ve	-ve
B. Coliform Count		**Sample A (1%)**	**Sample B (2%)**	**Sample C (3%)**
0	-ve	-ve	-ve	-ve
3	-ve	-ve	-ve	-ve
6	0.3010	-ve	-ve	-ve
9	0.8450	-ve	-ve	-ve
12	1.1760	0.6020	-ve	-ve
15	1.3222	0.7781	0.4771	-ve

All above values are in log base 10

The SPC, Yeast & Count and Coliform count were within safe and acceptable limit in all the MPE incorporated samples (A, B and C) as well as control sample R as specified in (FSSAI, 2019) [5].

3.4 Sensory analysis: The sensory evaluation scores of Indian cottage cheese samples over the storage period of 15 days is stated as mean±SD in table 3.

Table 3: Sensory evaluation of sample R, A, B and C during the storage days

Rate of addition of Marigold Petal Extract (MPE) (%)	Sensory attribute				
	Color	Appearance	Flavor	Body and Texture	Overall Acceptability
Control (R)	7.68 ± 0.85	7.52 ± 0.97	7.28 ± 0.99	7.26 ± 1.01	4.81 ± 2.20
Sample A (1%)	7.70 ± 0.26	7.65 ± 0.49	7.39 ± 1.02	7.88 ± 0.42	7.5 ± 0.59
Sample B (2%)	7.44 ± 0.34	7.54 ± 0.29	7.58 ± 0.52	7.88 ± 0.42	7.52 ± 0.41
Sample C (3%)	7.01 ± 0.15	7.45 ± 0.17	7.01 ± 0.14	7.7 ± 0.03	7.38 ± 0.06

Each observation is mean±SD; n=3

Although the MPE extended the microbial shelf life of Indian cottage cheese samples, but its sensory attributes were also affected which is evident by the negative correlation.

The correlation coefficient(r) of color, appearance, flavor, body and texture and O.A.A. were calculated to be -0.990, -0.998, -0.654, -0.866 and -0.762 respectively.

The O.A.A. of the samples decreased with increase in MPE concentration due to the phytochemicals present in MPE. The negative correlation against color, appearance and flavor could be possibly due to the presence of yellow pigment lutein(xanthophyll) which may be responsible for color change and grassy flavor. From 9th day onwards the control was unacceptable (Khatkar *et al*., 2017) [9] on sensory parameters due to extreme sourness but the MPE incorporated samples were still palatable although they had a slimy appearance and gritty texture.

4. Conclusion

Above results indicate the antimicrobial properties of marigold petal extract (MPE) and its preservative effect on Indian cottage cheese to extend its shelf life for 15 days at temperature 4-7˚C. It showed a notable antimicrobial effect against lactose fermenting bacteria, yeast and *E. coli* but feeble antifungal activity (Gupta & Vasudeva, 2012) [7]. The product (Indian cottage cheese) complies with the FSSAI (2019) [5] standards for SPC log 5.176 to 5.544, Yeast & Mold log 1.699 to log 2.176 and coliform log 1 to log 2. On the basis of overall product performance, keeping in mind both sensory and microbial parameters, sample B (2%) was considered suitable as preservative in Indian cottage cheese/paneer at storage temperature of 4-7℃ for more than 15 days without any effect on sensory attributes. It showed suppressed microbial growth without effecting the color and flavor, which was a setback for sample C (3%).

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