FORTIFICATION OF DAIRY PRODUCT SHRIKHAND WITH NATURAL ANTIOXIDANT FROM THE EXTRACT OF TAMARIND SEED

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Abstract
Shrikhand- an Indian dairy based dessert is high in fat content, hence susceptible to derogatory oxidative changes. In food industry and also in home based preparations, antioxidant compounds are added to readily oxidisable foods. In food industries commercial synthetic antioxidant usage is very common, whereas scientific reports are emerging indicative of the toxicity of these kinds of antioxidants. However due to food safety issues and consumer's preference on natural food ingredients, natural antioxidants are now being explored for food formulations. In this study antioxidant extracts from tamarind seed has been utilized in shrikhand. Functional, textural, physico chemical and organoleptic properties of the samples have been tested along with their changing profile over a 60 days storage period has also been monitored. The study has been concluded with the remarks that tamarind seed extract can be successfully utilized in food matrix as a source of natural antioxidant.

Keywords: HPLC, TLC, natural antioxidant, bioactive compounds

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1. INTRODUCTION

Tamarindus indica is a leguminous tree which produces pod like tamarind fruit- a widely utilized component of food processing industry. A single fruit contains multiple dark brown coloured seeds which were often considered as waste or by products of tamarind fruit industry, till recent years, when the seeds are being exhaustively studied as the source of natural antioxidants, hence their value as a source of therapeutic agent is increasing. Antioxidants are non-nutrient composites of human diet with some specific action in disease prevention, therefore, are valued as a principal constituent of functional food formulation (Rao and Mathew, 2012). Owing to the fact that, in recent years food industries are keen on developing value added products from the waste or by products generated by the food and agricultural processing industries and tamarind seed can show the potential to be a cost effective, yet valuable source of antioxidant in functional food formulation. Recently, researchers have developed (Oswell et al, 2018) tamarind seed powder enriched cookies and mango juice with greater antioxidant capability and higher phytochemical composition.

In India, 50- 55 % of total milk production is utilised for fermented milk product development. Shrikhand is one of the principal fermented milk product, however, due to the presence of milk fat, this food product is prone to oxidation, hence demands the addition of antioxidant for value added product formation. On the other hand, the quality of a food product does not only depend on its nutrient or nutraceutical property, but also is assessed by human sensory organs- as determined by 'sensory' or 'organoleptic' properties. Hence, the application of tamarind seed extract in shrikhand must be considered with a holistic approach- providing both nutraceutical benefits and sensory acceptance.

For application in food, the composition of tamarind seed must be studied. Previous studies have explored various antioxidant constituents of tamarind seed- some reported procyanidin polymers as the main bioactive component in tamarind seed with lesser amounts of epicatechin (Reiss et al, 2016),

other studies have found coumaric acid and gallic acid (Shlini et al, 2016) and epicatechin (Luengthanaphol et al, 2004), (Tsuda et al, 1994) as the principal bioactive compounds. Extensive research on the antioxidant composition of tamarind seed coat has revealed the presence of polyphenols including tannins, anthocyanidin, and oligomeric anthrocyanidins (Sinchaiyakit et al, 2011).
Hence, the presence of a wide range of antioxidant compounds in the seeds and also a lack of unanimity in the data on isolated compounds indicate that variation in antioxidant composition is a common occurrence for tamarind seed and it is necessary to explore the composition of the seed when an exhaustive study of antioxidants is taken into consideration. Hence, the study aimed at producing tamarind seed antioxidant fortified shrikhand with special emphasis for evaluation of the physico chemical, sensory and storage life analysis of the developed product.

2. MATERIALS AND METHODS

Chemicals and reagents

The chemicals used for the purpose of investigation were of analytical grade. Gallic Acid of S.d fine chem. Ltd Mumbai, India, 2,4,6-Tri(2--pyridyl)-S-triazine (TPTZ) of Himedia, Mumbai, India, Sodium Carbonate, acetic acid, Iron(III) chloride 6-hydrate, Iron(II) sulphate 7 –hydrate. Sodium Acetate, Ethanol, Hexane, Hydrochloric Acid, Folin–Ciocalteu's Phenol of Merck (Germany) were utilised for study. Silica gel 60- 120 mesh, silica gel G, hexane of analytical grade were supplied by Merck (Germany). HPLC grade solvents - Tri Fluoro Acetic acid (TFA), acetonitrile, methanol, water and HPLC grade standards namely, Gallic acid and Catechin - all were supplied from Merck (Germany)

Antioxidant extract preparation

The seeds of *Tamarindus indica* were pound to an average particle size of 0.25 mm. Antioxidant components from the seeds were extracted in their optimized condition as described in previous work by the authors (Sarkar and Ghosh, 2018).The tamarind seed (TSP) extract was lyophilized in a freeze dryer (made: Eyela FDU 1200, Japan) and stored at -20°C until used.

Column chromatography

The TSP extract was fractionated by silica gel column (1.0 cm diameter and 40 cm height, particle size of 60- 120 mesh**,** Pharmacia, Merck, Germany) chromatography technique (Amarowicz et al, 2003). The column was equilibrated with the lower phase of a chloroform- methanol water (65:35:10) mixture. 1 g sample of dried TSP extract was dissolved in 10 mL of methanol and was introduced to the top of the column and the same solvent mixture was used as eluent. Fractions (5 ml each) were collected at a flow rate of 20 ml / hour. Eluates were then evaluated for Total Polyphenol Content (TPC) determination by Folin- Ciocalteu method as described in our previous work (Sarkar and Ghosh, 2018).
Eluates were pooled into 7 major fractions, by combining the fractions with similar TPC values together. These 7 fractions were evaluated by Ferric Reducing Antioxidant Power (FRAP) (Sarkar and Ghosh, 2018) analysis to identify the fraction with highest antioxidant activity. The fraction with highest antioxidant activity was dried by evaporating the solvent and was further subjected for thin layer chromatography.

Thin Layer Chromatography

Glass plates (10×20 cm) were coated with silica gel G (for thin layer chromatography) paste. The plates were subjected to air drying and subsequent activation at 100° C for 30 minutes (Selvaraj et al, 2013). Silica plates were then cooled at room temperature (≈25^{0}C). The extract was separated by TLC in the ascending direction for 17 cm with the solvent system chloroform: methanol (1:1, v/v). The chromatograms were dried and colour was developed by spraying with ferric chloride-potassium ferricyanide solution. The spraying solution was prepared with equal volumes of

aqueous 1% solutions of each salt. The R_f values of separated bands were calculated. Standard solution matching to that R_f values were run on the plate to get confirmation on the result. The spots developed from the sample fractions were scraped out from the plates and extracted with HPLC grade methanol. The diluted mixture was then centrifuged at 10000 rpm for 10minutes at 4˚C and the supernatant was subjected for HPLC analysis.

High Performance Liquid Chromatography analysis

Reversed phase HPLC was conducted on Alliance Waters chromatograph, USA. Compounds 1 and 2 isolated from TLC, were filtered through membrane filter (Millipore, USA) and were injected (10µL) through the BDS Hypersil RP-C18 column (made: XTerra, Japan, pore size: 5µm, column dimensions: 250mm × 4.6mm) at column temperature of 25˚C. The mobile phase was composed of A: 0.1 % Tri Fluoro Acetic acid in phosphate buffer (30:70 vol. %) and B: acetonitrile (70:30 vol. % in phosphate buffer). The mobile phase (A: B, 70: 30 v/v) was eluted at a flow rate of 1ml/min for 20 minutes and the effluent was monitored at 280nm by UV detector (Selvaraj et al, 2013) (made: Waters 2487 dual lambda absorbance detector). The peaks were detected and compared with the standards.

Shrikhand preparation

Shrikhand was prepared from standardized cow milk (manufactured by Anand Milk United Limited, India, under the brand name of AmulMastiDahi), sugar and curd (Landge et al, 2011). Two sets of shrikhand were prepared, one with TSP as antioxidant additive in varied concentrations (0.5 – 2%, w/w) in chakka and the other without TSP extract and they were named as TEST (T) and CONTROL (C) respectively.

The schematic process of shrikhand preparation is given in Figure 1. The varied concentrations of TSP extract added are mentioned in Table 1 and the sugar level was adjusted to 40 % w/w of chakka.

Table 1: TSP content in different shrikh and varieties

Treatment	TSP extract (% w/w)
CONTROL (C)	0
TEST (T1)	0.5
TEST (T2)	1.0
TEST (T3)	1.5
TEST (T4)	2.0

TSP extract > 2% was not added as it resulted in undesirable flavor during preparation. The shrikhand samples were subjected to proximate analysis and texture analysis. The shrikhand samples after preparation were stored in refrigerator at 4 ±1° C for a period of 60 days. Organoleptic and chemical changes were studied during this storage period.

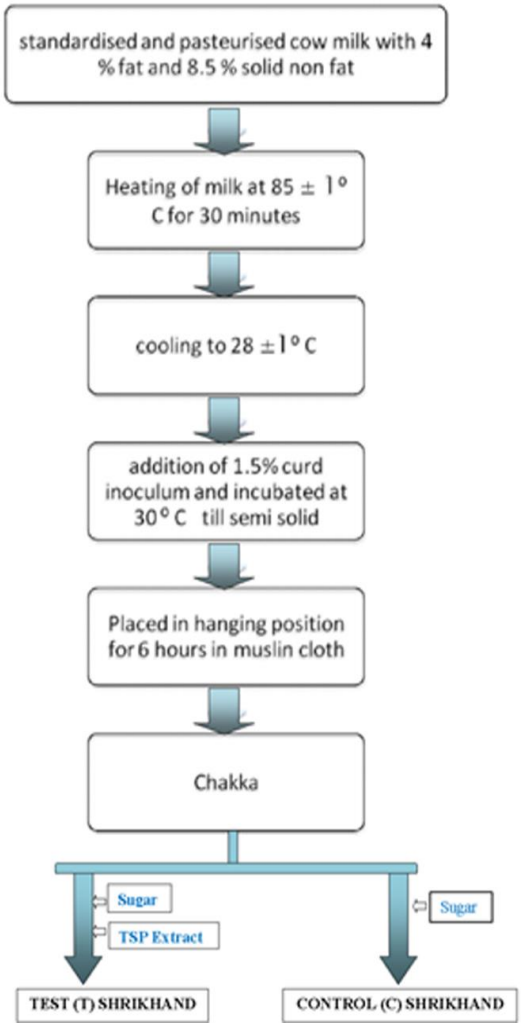


Figure1 : Preparation of Shrikhand

Proximate analysis an determination of total polyphenolic content and antioxidant power of shrikhand

Analysis of calorie value, total carbohydrate, protein and fat, moisture content and ash content (AOAC, 1975) of the shrikhand samples were estimated. Total Polyphenol Content (TPC) and FRAP (Ferric Reducing Antioxidant Property) were also determined.

Table 2: TPC values in different fractions collected from the column chromatographic separation of the crude antioxidant extract

Fraction no.	TPC (mg GAE/ g freeze dried extract)	Fractions pulled together into
1.	580.32± 8.65	Sub fraction I
2.	593.53± 7.33	
3.	587.54± 6.49	
4.	653.79±10.22	Sub fraction II
5.	630.53±12.44	
6.	649.83±6.87	
7.	659±6.97	
8.	879±7..4846	Sub fraction III
9.	885.51±5.33	
10.	863.42±5.60	
11.	883.95±5.15	
12.	865.39±9.31	
13.	789.44±7.43	Sub fraction IV
14.	784.62±8.75	
15.	778.53±5.65	
16.	786.65±8.36	
17.	910.42±8.40	Sub fraction V
18.	906.83±6.84	
19.	912.17±8.48	
20.	922.16±8.33	
21.	920.76±5.45	
22.	916.75± 6.49	
23.	914.77±6.72	
24.	921.58±9.41	
25.	983.77±5.76	Sub fraction VI
26.	962.59±6.73	
27.	964.36±6.93	
28.	987.68±2.65	
29.	967.13±4.96	
30.	976.74±3.87	
31.	970.33±3.95	
32.	981.83±5.88	
33.	982.60±1.46	
34.	749.27±5.66	Sub fraction VII
35.	734.62±5.22	
36.	728.71±4.96	
37.	726.32±5.77	
38.	730.38±7.84	
39.	723.79±4.86	
40.	714.18±6.27	
41.	712.74±8.86	
42.	723.85±4.56	

Values expressed are the average and standard deviation based on triplicate runs. (mean value ± standard deviation)

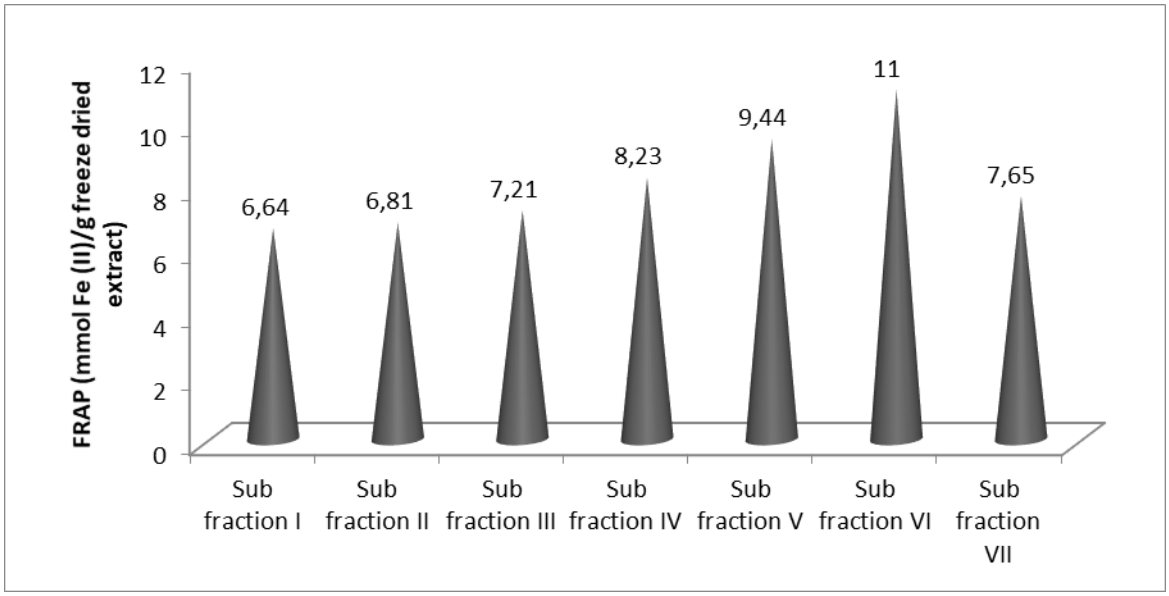


Figure 2: FRAP values of the sub fractions from the column chromatographic separation of the tamarind seed antioxidant extract

Texture analysis of Shrikhand sample

Texture analyser (made: TA.XT Express Enhanced Stable Microsystems, USA) equipped with Exponent Lite Express software was utilised for texture profile analysis (TPA) of shrikhand samples. The experiment was executed in threefold, where the different shrikhand samples were analysed in two consecutive cycles. In the first cycle, compression testing was done on 50 g sample using a 2 mm diameter cylinder probe with a 5 kg load cell run at a speed of 0.5 mm/s over 2 mm distance. Hardness of the sample was denoted in terms of the maximum force required for compression, which is again, equivalent to the maximum resistance offered by the sample surface against the compression of the probe.

At the second cycle, cohesiveness was measured by calculating the ratio of the areas under second and first compression.

Organoleptic evaluation

A panel of 15 semi trained judges was involved in a 9 point Hedonic sensory scale evaluation of the shrikhand samples. Prior to testing, each sample was coded and placed in random order to avoid biasness and ensure blind testing.

Statistical Analysis

Results of each analysis are shown in mean ± standard deviation of triplicate runs. The significance of difference between means was determined on the basis of 2 sample t test at significance level of $p< 0.05$. Minitab (version 17) software was used for statistical analysis.

3. RESULTS AND DISCUSSION

Separation and purification of major bioactive compounds from the crude antioxidant extracts of *Tamarindus indica* seeds

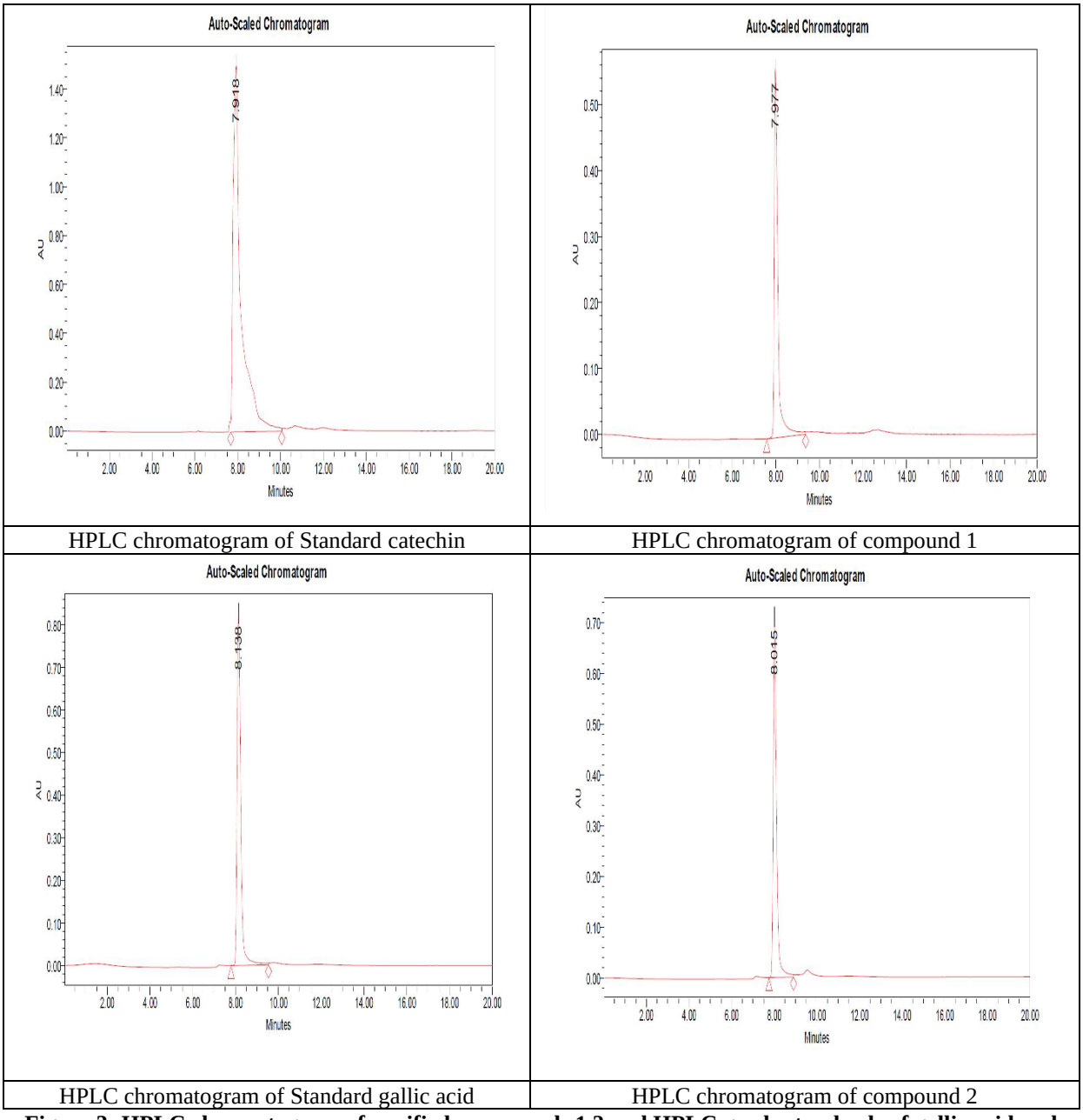
Table 2 presents the total polyphenol contents in different fractions collected from the column chromatographic separation of the crude antioxidant extract. The total of 42 fractions were pooled together and grouped into 7 sub fractions.

Among the seven fractions separated, fraction VI, showed the highest anti oxidative activity (Figure 2) and was used for further investigation by TLC technique.

TLC was done with Fraction-VI and the $R_f$values (compound-1:0.81; compound-2: 0.21) of two TLC spots almost matched with standard solution of Catechin and Gallic acid

with $R_f$values of 0.84 and 0.24 respectively. The Chromatographic analysis using HPLC indicated that compound-1 and compound-2 had almost the same retention time (Rt: 7.977 and 8.015 min) as the standards of Catechin and Gallic acid (Rt: 7.918 and 8.135 min respectively).Previous studies (Shlini et al, 2016) also have reported isolation of gallic acid, catechin as bioactive compounds from the seeds of *Tamarindusindica.*

The TPC and FRAP values of the freeze dried TSP extracts were recorded as 38± 1.4 mg GAE/ g sample and 220 ± 5.7 µmol Fe (II)/g sample respectively. The proximate composition of CONTROL and TEST shrikhand samples are given in Table 3.



HPLC chromatogram of Standard catechin	HPLC chromatogram of compound 1
HPLC chromatogram of Standard gallic acid	HPLC chromatogram of compound 2

Figure 3: HPLC chromatogram of purified compounds 1,2 and HPLC grade standards of gallic acid and catechin

Table 3: Proximate composition of untreated (CONTROL) and TSP extract fortified TEST (T1- T4) shrikhand samples

	C	T1	T2	T3	T4
Energy (Kcal)	311.75 ±9.13	312.22±7.63	312.31±5.83	312.48±6.94	312.56±5.29
Carbohydrate (%)	37.54±1.47	37.58±1.97	37.57±1.44	37.58±0.84	37.86±1.06
Protein (%)	6.47±0.76	6.50±0.64	6.51±0.54	6.51±0.52	6.52±0.58
Fat (%)	13.27±1.06	13.29±0.74	13.30±1.05	13.30±0.71	13.31±0.75
Moisture (%)	40.41±1.31	39.96±1.73	39.98±1.35	40.12±1.06	39.98±1.04
Ash (%)	0.65±0.23	0.63±0.21	0.66±0.03	0.65±0.06	0.67±0.02

Values expressed are the average and standard deviation based on triplicate runs. (mean value ± standard deviation)

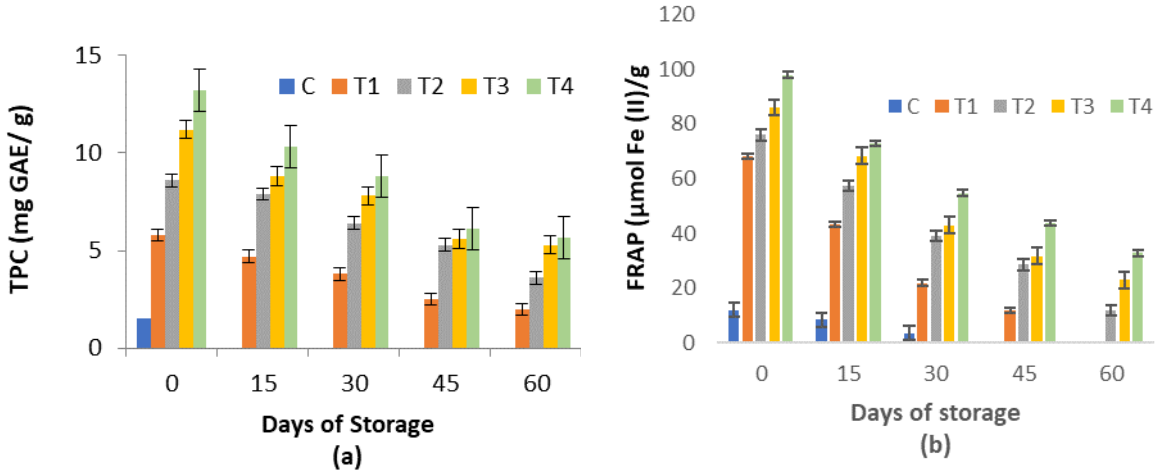


Figure 4: (a) TPC and (b) FRAP of Shrikhand samples during storage

Table 4: Sensory evaluation of CONTROL (C) and TEST (T1 –T4) shrikhand samples

Sensory parameters	C	T1	T2	T3	T4
Taste	8.13±0.63	8.09±0.38	8.18±0.43	7.12±0.39	6.33±0.89
Flavour	7.67±0.56	7.75±0.91	8.18±0.57	5.85±0.49	6.43±0.83
Colour	8.04±0.45	7.84±0.49	8.08±0.83	7.22±0.58	6.43±0.69
Overall acceptability	8.05±0.34	8.01±0.65	8.18±0.68	7.12±0.37	6.38±0.84

Values expressed are mean value ± standard deviation

It is noticeable from Table 3, that shrikhand is a source of high carbohydrate, fat and protein. The moisture content of around 40% and fat content of around 13 % makes shrikhand susceptible to rancidity and off flavour development. No significant ($p< 0.05$) change in proximate composition was noted upon addition of TSP extract to the CONTROL, i.e untreated shrikhand sample. Previous reports (Rajvir and John, 2019) are in agreement with these results of proximate composition of untreated shrikhand as indicated in table.

As indicated in table 4, T4 shrikhand sample with 2% TSP extract exhibited the highest TPC and FRAP values and mostly retained their phenolics and antioxidant activity on storage. However, a decline in TPC and FRAP was noticed in TPC extract when chakka was added (Figure 4). This was probably due to milk-polyphenol interaction as indicated by previous works (Han et al, 2019).

As indicated in Table 4, at the day of production, the shrikhand sample T2 showed the highest score in overall acceptability among all TEST and CONTROL samples (Table 5). T2 was noted as unacceptable after 45th day of storage. It is noticeable that, in shrikhand devoid of TSP extract, no significant antioxidant activity was noticed after 30th day, but the fortified shrikhand sample T2 showed

antioxidant activity beyond 30 days of storage. This highlights the benefits of TSP fortification in shrikhand samples. After 45th day texture scores were highly reduced. This may be due to natural syneresis, that the product lost their acceptability after 45th day of storage.

Texture profile parameters, namely, hardness, cohesiveness and adhesiveness were measured (Table 6). Texture profile measurement was carried out using double-bite compression tests, since, force and deformation are the two fundamental parameters for texture characterization. The peak force during the first compression cycle is defined as hardness or firmness; the ratio of the positive force area during the second compression cycle to that during the first compression cycle was expressed by the property of cohesiveness. The negative force area of the first compression cycle was denoted as adhesiveness. There was a decrease in hardness, though not statistically significant ($p< 0.05$) between samples C and T1. The decrease in hardness was significant ($p< 0.05$) in TEST samples with higher TSP extract (C and T2, T3, T4). This may be due to the interfering effect of TSP, that loosened the binding capacity of shrikhand, thereby reduced the firmness of the sample. Cohesiveness and adhesiveness increased a little upon addition of TSP extract, but with no significant difference ($p< 0.05$).

4. CONCLUSIONS

The bioactive components present in TSP extract were found to be gallic acid and catechin. No significant change in proximate composition was noted upon addition of TSP extract to the shrikhand, but the total polyphenolic antioxidant load increased significantly. Also, the storage life of shrikhand after fortification almost doubled. The formulated food was well accepted by sensory analysts. All these together indicated the successful fortification of shrikhand with TSP natural antioxidants.

5. ACKNOWLEDGEMENT

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Table 5: Sensory evaluation of CONTROL (C) and TEST (T1 –T4) shrikhand samples n storge

Parameters	Days of storage											
	5	10	15	20	25	30	35	40	45	50	55	60
Taste	8.13± 0.76	8.03± 0.18	7.95± 0.27	7.89± 0.83	7.11± 0.98	7.02± 0.82	6.31± 0.28	6.26± 0.83	5.79± 0.91	5.65± 0.51	-	-
Flavour	8.11± 0.35	7.89± 0.46	7.82± 0.21	7.78± 0.68	7.21± 0.58	7.15± 0.85	6.62± 0.85	6.54± 0.28	5.82± 0.21	5.76± 0.69	-	-
Colour	8.01± 0.53	7.82± 0.33	7.76± 0.67	7.73± 0.67	7.22± 0.68	7.16± 0.38	6.35±. 28	6.28± 0.49	5.72± 0.32	5.65± 0.44	-	-
Overall acceptability	8.12± 0.38	8±0.33	7.94± 0.63	7.89± 0.28	7.15± 0.47	7.07± 0.28	6.41± 0.48	6.35± 0.76	5.81± 0.45	5.72± 0.50	-	-

Values expressed are mean value ± standard deviation

Table 6: Texture profile analysis evaluation of CONTROL (C) and TEST (T1 –T4) shrikhand samples

Textural Parameters	C	T1	T2	T3	T4
Hardness	68.27±1.68^{a}	67.89±1.27^{a}	67.18±1.49^{b}	67.12±1.34^{b}	67.33±1.38^{b}
cohesiveness	35.07±1.27^{a}	35.15±1.03^{a}	35.18±1.29^{a}	35.25±1.25^{a}	35.33±1.42^{a}
Adhesiveness	32.04±1.45^{a}	32.14±1.26^{a}	33.18±1.32^{a}	33.22±1.28^{a}	33.23±1.29^{a}

Values expressed are the average and standard deviation based on triplicate runs. (mean value ± standard deviation).

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