International Journal of Food Science and Nutrition
ISSN: 2455-4898
Impact Factor: RJIF 5.14
www.foodsciencejournal.com
Volume 3; Issue 2; March 2018; Page No. 19-24

Preparation and pharmacognostic evaluation of *Sandesh*, an Indian sweet dairy product, using natural colorant from *Clitoria ternatea* (Aparajita) flower

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Abstract
The present study dealt with incorporation of natural color from *Clitoria ternatea* flowers into sandesh, a popular heat coagulated product of milk. It was done to improve the antioxidant capacity of the product as well as to make it presentable to consumers. Moreover, this ensured non-utilization of artificial colorants of foods. Sensorial evaluation was performed using nine-point hedonic scale with twenty semi-trained panelists. Antioxidant assays performed were ABTS and DPPH radical scavenging assays, determination of total phenolic contents, FRAP assay and crocin bleaching assay. The present study indicated that ABTS radical scavenging abilities of the test samples improved over the controls in both the products. Conversely, DPPH radical scavenging abilities of the test samples were not better over the controls. Since ABTS assay indicated activities of more polar biomolecules, it could be concluded that incorporation of flower colors to the products improved their contents of more polar bioactives. Significant improvement was observed in ferric reduction potential of the products after color addition, although abilities for crocin bleaching were not that much improved. This indicated that the antioxidants present in the flower colors followed electron transfer mechanism for their activities. Since the natural colorants have no specific flavor or odor, it did not produce any repulsive sensation towards the consumers as was observed in the sensorial evaluation.

Keywords: sandesh, dairy product, antioxidant, clitoria ternatea, polyphenols

1. Introduction
Sandesh is one of the heat-acid coagulated milk product available in India. It is a sweet product mostly produced in unorganized small-scale sectors wherein variations in quality between batches, days of production and shops are noticed [1]. Chhana, a heat-acid coagulated product of milk forms the base material for the preparation of sandesh. In the preparation of sandesh, milk is heated to 90-95°C, followed by cooling to 70°C. Sandesh represent the traditional Indian dairy product used as sweet dairy desserts, prepared by acid or heat coagulation of milk. It is popular throughout eastern part of India, especially in West Bengal. It has been estimated that the annual production of sandesh in West Bengal alone is 30,000 tons [2]. Recent studies reported that about 80% of chhana produced in Kolkata (West Bengal, India) is converted into sandesh [3]. It is a rich source of high quality animal protein, fat, minerals and vitamins.

Spices (clove, small cardamom, large cardamom, saffron, etc.) have been used as flavouring and also as colouring agent in sandesh preparation for centuries. Use of beet extracts in sandesh not only improved the lipid peroxidation inhibitory property of the components of the sandesh, but also imparted colors to the products [4]. Single components like betalain were also used to add visual tones to sandesh [5]. Recent studies reported that sandesh can be restructured with addition of bran for dietary fiber & oregano extract as a natural colorant antioxidant source. The color of the oregano extract played an important role by increasing the acceptability as it appealed more as compared to other sensory parameters. Even the sample containing oregano extract showed more radical scavenging activity as well as a synergistic effect [6].

Clitoria ternatea is commonly also known as Clitoria or butterfly pea. This plant is known as Aparajit (Hindi), Aparajita (Bengali) and Kokkattan (Tamil) in Indian traditional medicine [7]. The juice of flowers is reported to be used in insect bites and skin diseases [8]. Minor delphinidin glycosides and a few typical anthocyanins (i.e. ternatins and preternatins) were isolated from the young *Clitoria ternatea* flowers [9]. A malonylated flavonol glycosides were isolated from the petals of *Clitoria ternatea* with different petal colors [10]. The colorants present in petals are rich in anthocyanins and can be coined as alternative source of natural antioxidants [11]. It is used as a confectionary coloring in the food industry [12]. It has also been observed that sugar-free ice cream made with butterfly pea flower extract and petals showed good potential as a frozen dessert product with natural colouration, antioxidative properties and lower glycaemic response [13]. The present study was designed to use such natural colorant having potential antioxidative capacities in a popular Indian dairy product so as to minimize the use of synthetic coloring agents, which could be harmful to health. Antioxidative effects of Clitoria natural colorants, used in a popular dairy product - sandesh, were adjudicated by sensory evaluations as well as some common *in vitro* antioxidant assays.

2. Materials and methods
2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), ABTS, were obtained from Sigma, USA. 2,2′-Diphenyl-1-picryl

hydrazyl (DPPH) were obtained from Himedia, India. Folin-Ciocalteau reagent, gallic acid were obtained from Merck, India. 2,2′-azobis(2-Methylpropionamidine) dihydrochloride (AAPH) and 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) was procured from SRL, India. All other chemicals used were of AR grade. Deionized distilled water was used in the entire study.

2.1 Collection of fresh flowers and extraction of colour

Fresh Aparajita (*Clitoria ternatea*) flowers were collected from local market of Barasat, Kolkata. The flower samples were checked for dirt or any visible damages prior to the study. Such samples were discarded. A modified method for colorant extraction was used. Flower petals were separated and dried at 50±2ºC for 24 hours. About 5 gms of the dried flower petal powder were suspended in 50 ml water at 80±5ºC and kept for 15 minutes. The solution was filtered and the colored filtrate was used in sandesh preparation [14].

2.2 Preparation of Sandesh

A previously reported method was used with minor modifications [15]. Milk was heated to 90-95ºC. Heat coagulating agents like calcium lactate and lime juice were added to the heated milk in two separate sets. The precipitation of milk involved the formation of whey protein. Whey is drained out through a muslin cloth. Sugar was added to the partly dewatered product, known as chhana (cottage cheese) and smashed well. Smashed chhana was taken in a cooking pan and kheer was added to it and cooked over low flame. After 10 mins, the colored filtrate was added to the mixture and cooked over low flame with constant scraping until the mixture gets the desired consistency and flavor. Control products were prepared without using colored filtrate. Final product was prepared in various shapes to improve their acceptability. Sandesh prepared with calcium lactate precipitation was designated as CLS and those prepared from lime juice was designated as LJS.

2.3 ABTS radical decolorization assay

The ABTS assay was performed using a previously described procedure [16]. $ABTS^{-\bullet}$, the oxidant, was generated by per sulfate oxidation of 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid. This solution was diluted with phosphate buffer (pH 7.4) until the absorbance reached 0.7 to 0.8 at 734 nm in a Systronics spectrophotometer (model – 2202). The oxidant solution was mixed with the 60% ethanolic extracts of the sandesh in such a way that total volume of the solution reached 1 ml. The absorbance was read at room temperature, 4 minutes after mixing. Gallic acid was used as positive control and the results were expressed as Gallic acid equivalents (µg/gm sample).

2.4 DPPH radical decolorization assay

The DPPH assay was performed using a previously described procedure [16]. 1 ml DPPH solution (3 mg in 25 ml ethanol) was mixed with 0.5 ml of 60% ethanolic extracts of the sandesh and the decrease in absorbance of the mixture after 20 minutes of incubation in the dark was monitored at 517 nm in a Systronics spectrophotometer (model – 2202). Gallic acid was used as positive control and the results were expressed as Gallic acid equivalents (µg/gm sample).

2.5 Estimation of total phenolic contents

Total phenolic compound contents were determined by the Folin-Ciocalteau method [17]. 60% ethanolic extracts of the sandesh (0.5 ml) were mixed with Folin-Ciocalteau reagent (5 ml, of 1:10 diluted sample with distilled water) for 5 min and aqueous sodium carbonate (4 ml, 1 M) was then added. The absorbance of the reaction mixture was then measured at 765 nm with a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid was used as standard. The results were expressed in terms of mg gallic acid equivalent/gm sample.

2.6 Ferric reducing antioxidant potential: FRAP

Ferric reducing potentials of the samples were estimated with a previously established procedure with minor modifications [18]. Briefly, a maximum of 100 µl of 60% ethanolic extracts of the sandesh or standard was mixed with 1.9 mL of FRAP reagent and incubated at 37ºC for 30 mins. FRAP reagent was prepared by mixing 50 mL of 0.1 M acetate buffer (pH 3.6), 5 mL of 10 mM TPTZ solution and 5 mL of 20 mM $FeCl_3$ solution. After the stipulated time period, absorbance was measured at 593 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid is used as standard. Results are expressed as Gallic acid equivalents (GAE, µg/gm sample).

2.7 Crocin bleaching assay

Crocin solution was prepared from Saffron stigma in 80% methanol in water using an established procedure [19]. 60% ethanolic extracts of the sandesh (50-500 µl) were added to each test tube individually containing 4 ml of ethanol, 75 µl 0.5M AAPH and 425 µl crocin extract, to a final volume of 5 ml. These tubes kept incubated at room temperature for 60min. After incubation, absorbance of these reaction mixture tubes was measured at 443 nm. Control was prepared by mixing only ethanol in place of extract to a final volume of 5 ml, having 75 µl 0.5M AAPH and 425 µl crocin extract and absorbance was determined immediately. The percentage of crocin bleached by extract was calculated using the following formula: Bleached crocin % = $[\{(A_c-A_t)/A_c\} \times 100]$ where, A_c = absorbance of control and A_t = absorbance of test sample.

2.8 Sensory evaluation

Twenty panelists were selected from staff, faculty and students of the Department to evaluate the acceptability of the products, on the basis of color flavor and taste, in comparison to the control. They evaluated the samples on the basis of nine-point hedonic scale, ranging from 'like extremely = 9' through 'like or dislike = 5' to 'dislike extremely = 1' [20]. The panelists were satisfactorily trained to circumvent any biasing during the assessment of the sample. Each panelist assessed all the test samples as well as the controls. The entire experiment was repeated four times.

2.9 Statistical analysis

Experimental results are expressed as mean ± SD of four individual samples. The statistical analysis was done by using the software 'SPSS Statistics 17.0' (IBM Corporation, USA).

3. Results & Discussion

It has been observed in the past that herb extracts (including all types of natural sources) were used for preserving poultry, meat, beef, fish, lard, soyabean oil, etc., but there use in dairy products was limited. Milk itself can be used as source of antioxidants due to presence of compounds like urates, vitamins C and E, carotenoids or a few radical scavenging proteins [20]. However, treatments of milk for development of dairy products might reduce the levels of antioxidants in milk products. Earlier studies showed that herbs fortified dairy might serve as antioxidant supplements provided the antioxidant ability of the dairy product and herbs would not deplete through oxidation–reduction reactions upon mixing and storage of the products [21]. Addition of natural ingredients after heat treatments would be one of the strategies to maintain the quality of the dairy products, which has been followed in the present study. Also, a clear difference in the color of the products were observed with the sandesh prepared by the two different chhanas (Fig. 1). Sandesh developed from chhana, prepared using calcium lactate, showed more bluish tinge than the chhana prepared by lime juice. This might be due to the difference in the pH during preparation of chhana from milk. Most important thing was that the products did not develop any special flavour which might affect their acceptability to the consumers.

(a) (b)

Fig 1: Photographs of sandesh vis-á-vis control (without color). (a) Products developed from chhana prepared using calcium lactate, and (b) developed from chhana prepared using lime juice

The *in vitro* radical scavenging activities like ABTS and DPPH assays are generally used to indicate antioxidant potential of natural products. The assays are based upon polarities of the medium [16]. The present study indicated that ABTS radical scavenging abilities of the test samples improved over the controls in both the products (Fig. 3). Since ABTS assay system is based on aqueous medium, it could be indicated that the natural colorants, which were mostly polar biomolecules, were retained in the finished products.

On the other hand, DPPH radical scavenging abilities of the test samples were not better over the controls (Fig. 4). Since DPPH assay system is based on less-polar (ethanol) medium, it could be indicated that the natural colorants were devoid of less polar biomolecules.

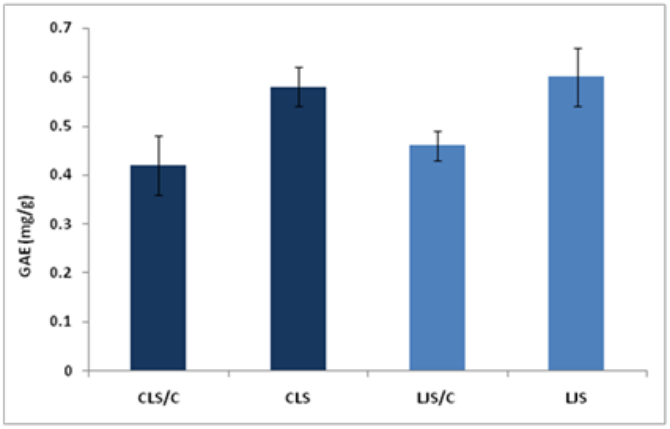

Fig 2: Comparative ABTS radical scavenging abilities of natural color supplemented sandesh vis-á-vis control. Results are expressed as Gallic acid equivalents (GAE, mg/gm). CLS/C= control sample prepared using calcium lactate, CLS= test sample prepared using calcium lactate, LJS/C= control sample prepared using lime juice, LJS= test sample prepared using lime juice

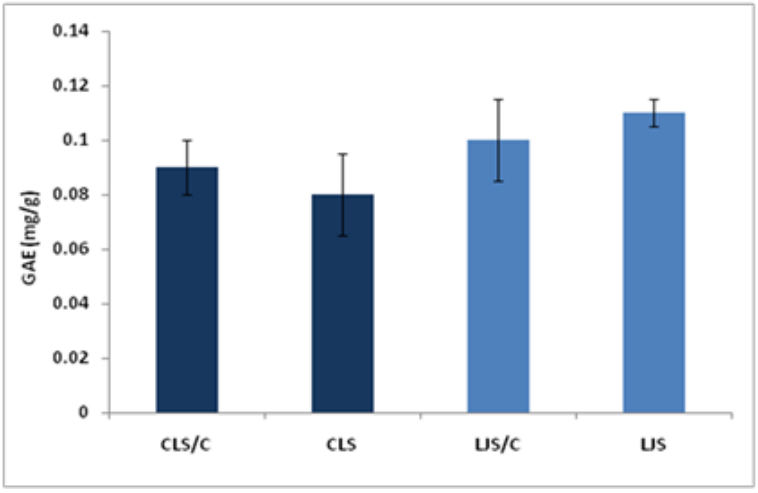

Fig. 3 Comparative DPPH radical scavenging abilities of natural color supplemented sandesh vis-á-vis control. Results are expressed as Gallic acid equivalents (GAE, mg/gm). CLS/C= control sample prepared using calcium lactate, CLS= test sample prepared using calcium lactate, LJS/C= control sample prepared using lime juice, LJS= test sample prepared using lime juice

Total phenolics contents of the test samples commensurate with the results of the radical scavenging abilities (Fig. 4). Since the results obtained from the study correspond to the ABTS radical scavenging study, it could be stated that the phenolics present in the Clitoria color were mostly polar in nature.

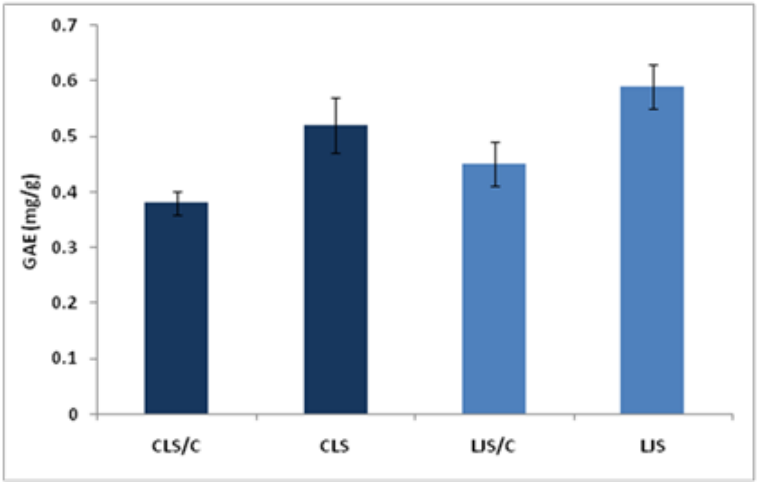

Fig 4: Comparative total phenolic contents of natural color supplemented sandesh vis-á-vis control. Results are expressed as Gallic acid equivalents (GAE, mg/gm). CLS/C= control sample prepared using calcium lactate, CLS= test sample prepared using calcium lactate, LJS/C= control sample prepared using lime juice, LJS= test sample prepared using lime juice

The present study also dealt with the mechanism of radical scavenging by the natural Clitoria color. Radical scavenging by natural products are usually accomplished by two mechanistic pathways - either by electron donation or by hydrogen atom donation to the experimental radicals by the antioxidants [22]. To adjudicate electron donation capabilities of the colorants, FRAP assay was performed, results of which was furnished in Fig. 5. Hydrogen atom donation capabilities were evaluated by crocin bleaching assay (Fig. 6). It was observed that there was significant improvement in the radical scavenging properties of the color supplemented sandeshes over their respective controls. This indicated that the colorant antioxidants were mostly electron donors. It was also observed that sandeshes prepared using lemon juice were significantly better than the sandesh prepared using calcium lactate. This might be due to the fact that lemon juice provided relatively lower pH to the products (data not shown). In lower pH, the antioxidants were mostly in their anionic state, which might help them to donate electrons easily to pair up the same in the radicals. On the other hand, there was improvement in the hydrogen atom donation abilities (in the form of crocin bleaching abilities at two different doses) of the two products albeit insignificantly. Even there was no significant difference between the test samples prepared by the two different methods. These indicated that the antioxidants present in the colorants were not potent hydrogen atom donors.

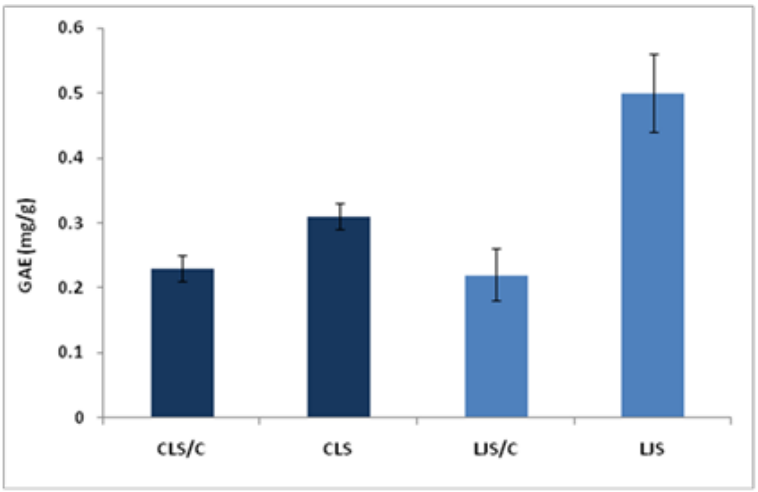

Fig 5: Comparative Ferric reducing antioxidant potential (FRAP) of natural color supplemented sandesh vis-á-vis control. Results are expressed as Gallic acid equivalents (GAE, mg/gm). CLS/C= control sample prepared using calcium lactate, CLS= test sample prepared using calcium lactate, LJS/C= control sample prepared using lime juice, LJS= test sample prepared using lime juice

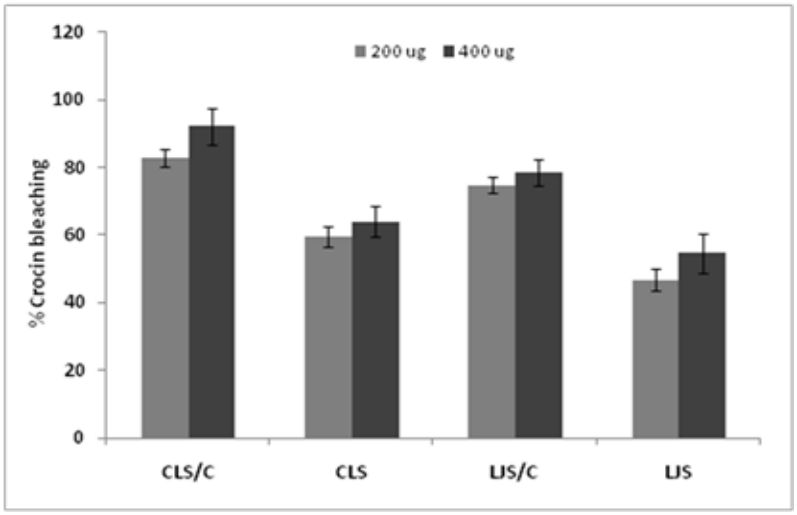

Fig 6: Comparative crocin bleaching potential of natural color supplemented sandesh vis-á-vis control. Results are expressed as Gallic acid equivalents (GAE, mg/gm). CLS/C= control sample prepared using calcium lactate, CLS= test sample prepared using calcium lactate, LJS/C= control sample prepared using lime juice, LJS= test sample prepared using lime juice

Phenolic compounds of plants having one or more aromatic rings with one or more hydroxyl groups can potentially quench free radicals by forming resonance-stabilized phenoxyl radicals which play a role in their antioxidant properties [23]. Natural colorants present in the sandeshes thus not only imparted colors to the finished products, but also provided antioxidant screen for the consumers. Since the natural colorants have no flavor or odor, it would not produce any hideous feeling towards the consumers. This was substantiated by the sensory evaluation of the products by 9-point Hedonic scale. Results were furnished in Fig. 7.

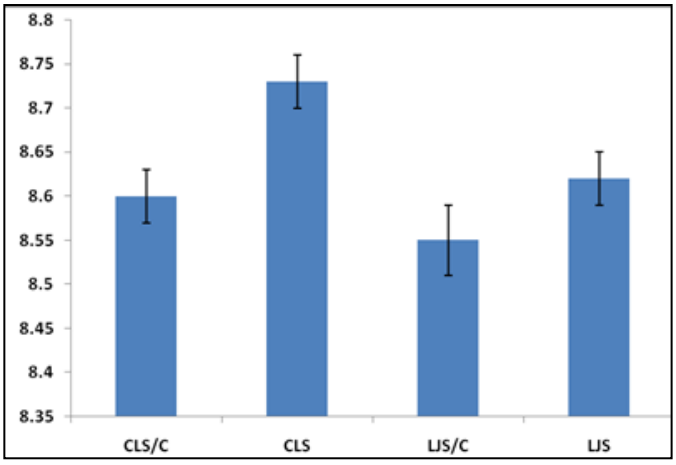

Fig 7: Sensory evaluation of natural color supplemented sandesh vis-á-vis control using 9-point Hedonic scale. CLS/C= control sample prepared using calcium lactate, CLS= test sample prepared using calcium lactate, LJS/C= control sample prepared using lime juice, LJS= test sample prepared using lime juice

4. Conclusion

Sandesh is a traditional Indian dairy product, used as sweet dairy desserts, and can act as rich source of high quality animal protein, fat, minerals and vitamins. It is prepared from Chhana, a heat acid coagulated product of milk that forms the base material. The present study dealt with the quality of the popular milk product, which could be improved by supplementation of natural colors. Natural colors not only avoided the probability of any toxic effect of use of artificial colors, they also endowed with antioxidant capabilities to the final products. ABTS radical scavenging abilities of the sandesh indicated that the natural colorants were mostly polar. it was also indicated that natural colorants mostly act as electron donating antioxidants, as was revealed by the FRAP assay. Since the natural colorants have no flavor or odor, it did not produce any hideous sensation towards the consumers, as revealed by sensory evaluations.

5. Acknowledgments

The authors are grateful to Sarada Ma Girls' College authority (under Ramakrishna Vivekananda Mission) for providing financial and infrastructural assistance.

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