Original Article

Aqueous Phytal extracts as source for staining in gel based protein separation techniques

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Abstract

PAGE (Polyacrylamide gel electrophoresis) has been considered as best and easy gel based protein separation technique among rapidly growing array of proteomic technologies. The post electrophoresis procedures usually comprise staining techniques to identify the separated proteins using CBB, Silver nitrate, fluorescent stains etc. Chemical stains used in staining methods are non eco-friendly and non-degradable. Natural plant extracts used as stains for the development and identification of protein bands is a novel idea. Current study focuses on the development of new staining techniques using natural ingredients like tea, coffee, henna and beetroot which are easily available. The results found to be 100% reliable, more ecofriendly, cost effective. Application of natural stains in the routine proteomic studies could be a step towards ecofriendly approaches in science.

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Introduction

Natural colorants have been in use since ancient times for dying and coloring purpose for eg., wall painting of Ajanta, Ellora. The leaves of *Lawsonia inermis* has been well known example in the Indian subcontinent for decorating hands, soles, and dyeing beard and hair to impart beautiful shades of dark red color. Dyes are also obtained from beetroot, cranberry; milkwort flowers (yellow dyes), stigmas of saffron flowers (orange dyes) and indigo (Blue).

Staining procedures play very important roles in bio-molecular studies. It has been used to stain cell nucleus, cell membrane, protein, DNA, etc. The use of stains has been very predominant in molecular biology studies such as in proteomics for identification, characterization and further processing of bio-molecules.

Proteins are the primary functional agents of biological system and regulate metabolic processes, signal transduction, small molecule/ion transport, cell replication, and apoptosis. Proteome examination is a critical way to analyze how a cell responds to its environment. Polyacrylamide gel electrophoresis (PAGE) remains the well accepted, widespread, and successfully implemented technique for the assessment, high-resolution separation, and characterization of these critical molecules[1]. Protein separation by one or two dimensional electrophoresis (1D/2D) is largely used in proteomic approaches because of both high resolution and the availability of powerful image analysis software for gel comparison and compatibility with subsequent protein characterization by mass spectrometry[2]. For these various aspects, the selection of the protein staining procedure is of major importance[3]. Coomassie blue has been the most widely used non-covalent dye for post-electrophoretic protein staining[4]. However, it suffers from a low sensitivity in protein detection, including in the improved colloidal version[5]. In contrast, the other classical protein stain, silver nitrate, displays an excellent sensitivity but could interfere with protein analysis by mass spectrometry[6]. Different fluorescent dyes have been introduced[7] recently, for eg. Sypro Ruby[8], and Ruthenium red-based dyes[9]. However, their use remains relatively limited, probably due to their cost and/or technical difficulties. These stains are neither easily biodegradable nor environment-friendly. CBB is toxic and its use necessitates specialized disposal efforts.

Current day's need is to develop a new stain which is natural, efficient, effective and sharp in characterizing the bio-molecules without harming the environment. Use of aqueous extracts natural phytal origin for the development and identification of protein bands is a novel idea. Phytal extracts can excel over standard stains by simplicity and non toxicity.

Earlier studies indicated that for the preparation of Henna (*Lawsonia inermis*) extractions, chemical based combinations of CaOH and 50% ethanol[10] or acidic aqueous [11] were used as extraction media. These henna extractions were used as stains in protein staining techniques. Aqueous henna leaves extract (cold or hot) oxidized with potassium permanganate was used as a substitute to the usual counter stains used in Gram staining[12] During the current research an attempt was made to develop stain by using henna in combinations with 1.beetroot and 2. coffee and tea and its application in the post –elelctrophoretic staining.

Materials and Methods

Sample: Blood Serum and Bovine Serum Albumin were considered. Each was mixed with appropriate quantity of gel loading dye.

Reagents: 1.5 M Tris-HCl (pH 8.6), 10% APS , TEMED, Tris glycine buffer, Gel loading dye

Gel electrophoresis: The gel used was 10% polyacrylamide. Standard Protocols were followed for the preparation of PAGE gel using acrylamide:bisacrylamide (ratio 29:1) solution, 10%APS, Tris HCl buffer (pH 8.6), and TEMED,. The Tris Glycine buffer (pH8.3) was used as tank buffer (25mM Tris base, 0.2 M Glycine, pH 8.6). Prior to electrophoresis, the samples were heated in the presence of sample buffer (70 mM Tris–HCl (pH 6.8), 11.4% glycerol and 0.01% bromphenol blue) at 100°C for 30 seconds in a boiling water bath. Protein samples were loaded into the individual wells, and electrophoresis processed using 100 Volt of current[13].

Preparation of staining solutions:

- Preparation of Standard Staining solution (CBB R 250): CBB R-250 (0.25 g) was dissolved in mixture of methanol: D/W: Glacial acetic acid in ratio of 45:45:10.

- Preparation of phytal stains: The plant leaves of henna (Lawsonia inermi, Family: Lythraceae), root of Beetroot (Beta vulgaris, Family: Amaranthaceae), dried leaves of tea (Camellia sinensis, Family: Theaceae) and dried seed
- Powder of coffee (Coffeea Arabica, Family: Rubiaceae) were considered for the preparation of herbal stains. Henna had been considered as main ingredient along with carrot and beetroot or coffee and tea.
- Combination 1: Equal quantity of 5gm of tea and 5gm of coffee powder were boiled in 50 ml of distilled water and 4% henna extraction was made in decoction and filtered. This was considered for staining purpose
- Combination 2: A 4% decoction was prepared in D/W of equal quantity of henna powder and beetroot. It was then filtered and used for staining purpose.
- A 4% decoction was prepared in D/W of equal quantity of henna powder and beetroot. It was then filtered. Used for staining purpose

Protein staining:

Standard solution: CBB stain was used as control: After separation, the gels were carefully transferred to a steel tray filled with distilled water. The staining solution was added to the gel and kept at room temperature for 1 hour. The gel was washed with D/W and destaining was carried out at room temperature for 2 hours using destaining solution.
Test solution: After separation, the gels were carefully transferred to a steel tray filled with distilled water. Gels were stained with individual stains (HB and HCT) immediately after removing distilled water considering different temperature conditions (Room temperature, 37°C and 120°C) and time variations (30/60/90min). Destaining was carried out for 1 hour using 6% acetic acid/Vinegar without the need of fixation step.
Phytochemical Screening: The preliminary phytochemical tests were performed for testing different chemical groups present in extracts.
Alkaloids: 100 mg of extract was added to small quantity of Wagner's reagent [Solution of iodine in potassium iodide]. The presence of reddish brown precipitate indicates the presence of alkaloids[14]. 100 mg of extract was added with small quantity of Hager's reagent [saturated solution of Picric acid]. The presence of alkaloids is indicated by yellow precipitate[14,15]
Glycosides: 2 mL of extract was treated with 0.4 ml of glacial acetic acid containing a trace amount of $FeCl_3$. 0.5 ml of concentrated H_2S0_4 was also added by the side of the test tube. Persistent blue color appeared in the acetic acid layer indicates the presence of cardiac glycosides[14]
Tannins: 5 ml of extract was added with few drops of 5% $FeCl_3$. Deep blue black colour indicates the presence of tannins[14].
Flavonoids: 5 ml of extract was added with few drops of NaOH solution. An intense yellow color, which turns to colorless on addition of few drops of dil.H_2SO_4 indicates the presence of flavonoids[11].
Terpenoids: 5 ml Extract was treated with 5 ml $CHCl_3$ and few drops of conc. H_2SO_4. Shake well and allow it to stand for some time. Formation of yellow colored lower layer indicates the presence of terpenoids[14,15]
Phenols: A few drops of extract was taken in a test tube and added with few drops of 1% $FeCl_3$. The formation of a red, blue, green, or purple coloration indicates the presence of phenols[14].
St. Sabouraud's agar containing dextrose and peptone was used to isolate the fungus grown on the phytal extract as the high concentration of dextrose and acidic pH (pH 5.6) make this medium selective for fungi.
Gel Analysis techniques: MyImage Analysis software of Thermo Scientific was used to analyse the gel characteristics with respect to Rf value of bands, relative quantity and purity of the bands. The data was used to assess the similarity between the world wide used CBB and the current phytal aquatic staining techniques.

The statistical analytical methods such as correlation and student t test were performed to assess the significant similarity between the different staining procedures and their protein binding expressions.

Result

The study has revealed the presence of phytochemical constituents. Important phytochemicals such as terpenoids, flavonoids, alkaloids, phenols, glycosides, tannins and steroids were present in the phytal extracts. The phytal extract of henna and beetroot contained steroids, terpenes, glycosides, tannins, phenol, flavonoids, and alkaloids

whereas phytal extract of henna showed absence of steroids, terpenes, and flavonoids. Tannins were absent in beetroot. Similarly, the phytal extract of tea and coffee contained steroids, terpenes, glycosides, tannins, phenol, flavonoids, and alkaloids whereas phytal extract of coffee showed absence of terpenes. Thus, the combinatorial effect may have shown the staining ability. The phytochemical constituents of the phytal extracts found to be different at their individual levels to that in combination (Table-1). Analysis of plant extracts revealed the presence of flavonoids, glycosides, phenols, saponins, steroids and tannins in most of the selected plants which could be responsible for the observed binding property with the protein and staining property.

Table 1: Phytochemical analysis of phytal extracts of Tea, Coffee, Henna, Beetroot and in combinations of Tea and Coffee, Henna and beetroot, henna.

Stains	Flavonoids	Steroids	Alkaloids	Phenols	Tannins	Glycosides	Terpenes
Tea	+	+	+	+	+	+	+
Coffee	+	+	+	+	+	+	-
Henna+ Tea+Coffee	+	+	+	+	+	+	+
Henna	-	-	+	+	+	+	-
Beetroot	+	+	+	+	-	+	+
Henna+Beetroot	+	+	+	+	+	+	+

Phytal stains and PAGE Gel:

Staining and destaining methods of standard CBB R 250 and phytal extract showed remarkable results. In our effort to develop and optimize a sensitive protein detection procedure using henna, beetroot, tea and coffee aqueous extract, a number of conditions for staining process were tested.

Temperature: Temperature is important factor for gel staining protocol and expression of protein. Staining at 37°C for 60 minutes was found to be enough to obtain a clear band for both phytal extracts of tea and coffee. Room temperature showed equal efficacy as that of 37°C of HCT. On the contrary, staining at 120°C for 60 minutes indicated no clear bands (Fig.1).

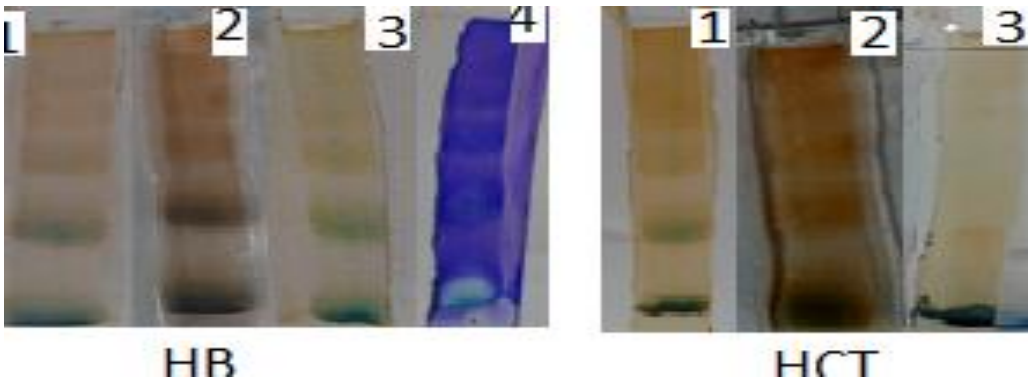

Fig.1: Efficiency of Natural stains' (Henna Beetroot (HB) and Henna, Coffee and Tea (HCT)) staining with respect to temperature on Protein band expression. Serum was separated using PAGE and stained with staining solutions at RT (1), 37°C (2), and 120°C (3), and CBB stain (4) for 60 minutes.

Time variation: Time is a very important factor for protein detection in protocol. Gel staining under different staining times at 37°C indicated the impact on binding efficacy of the phytal stains. Visual observations of 60 min time variations indicated similar results in the case of both types of aqueous stains.

Staining for 60 minutes was found to be enough to obtain a clear band. However, staining for 30 minutes was less effective, and no clear bands were obtained. The color of the bands and backgrounds became more intense and unclear as the staining time increased. for both types of aqueous stains (Fig.2)

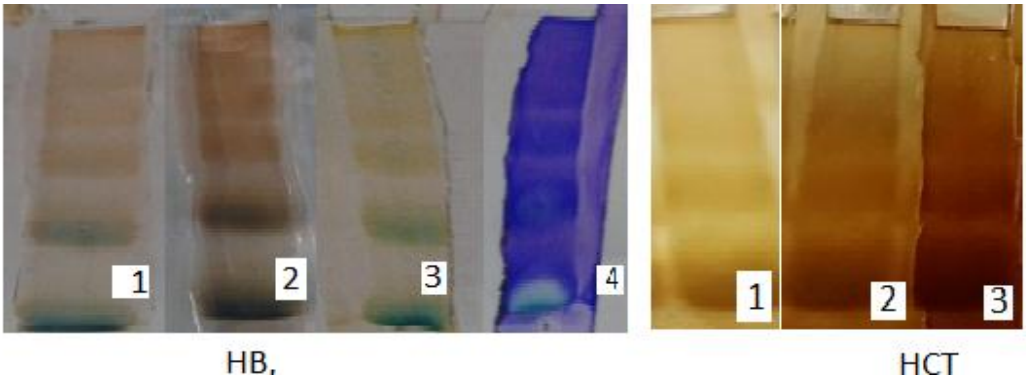

Fig 2: Efficiency of Natural stains' (Henna Beetroot (HB) and Henna, Coffee and Tea(HCT)) staining time and Protein band expression. Serum was separated using PAGE and stained with staining solutions for 30 minutes (1), 60 minutes (2), and 90 minutes (3).

Staining protocol for both phytal solutions was standardized. Best results obtained when the PAGE gels were stained at 37°C for 1 hour followed by destaining with 6% Acetic acid for 1 hour. Protein bands of BSA and blood serum were observed after staining the gel with aqueous extract of beetroot and henna (Fig.3). Similarly, separated protein bands of blood serum and BSA samples were observed after staining of gel with phytal extract of HCT (Fig.4).

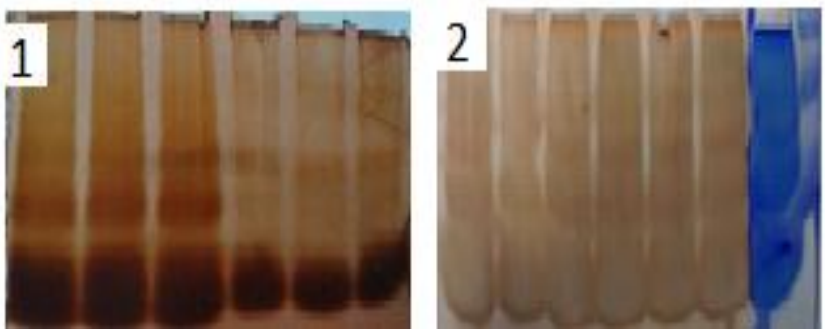

Fig. 3: Gels with BSA(1) / serum (2)sample stained by Henna and beetroot (HB) phytal extract.

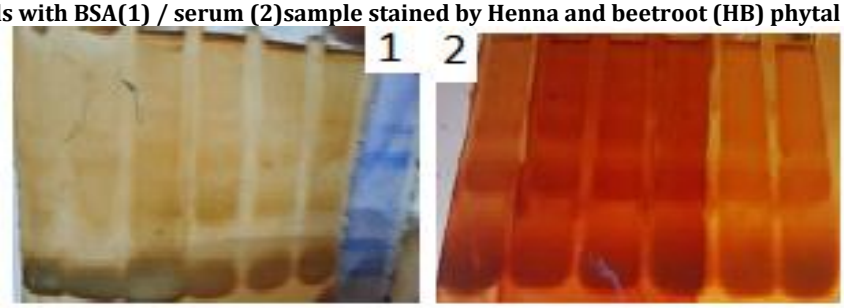

Fig. 4: Gels with Serum(1) / BSA sample (2) stained with henna, coffee and tea phytal stain (1:stains used 1-6, 7- CBB and, 2 : 1-4 fresh stain; 5,6 - 1 month old stain).

MyImage Analysis software of Thermo Scientific was used to assess expression of the intensity levels and binding characterization of protein bands by natural stains and the standard CBB. The photographs of gels stained with both stains were used for analysis. The Rf value, relative quantity and percentage purity of each band of protein was assessed using this computer software. (Fig.5 and 6)

It was observed that percentage purity of the protein band stained with standard CBB stain and test stain i.e. phytal extract of beetroot and henna; and CBB stain and test stain i.e. phytal extract of henna, coffee and tea showed almost similar results (Table-2). Similar trends were observed for relative quantity of each protein band as well. However, relative quantity and percentage purity of protein bands visualized by HCT stain were relatively higher compared to that of henna and beetroot stained protein bands indicating the efficiency of HCT stain better than that of beetroot and henna stain.

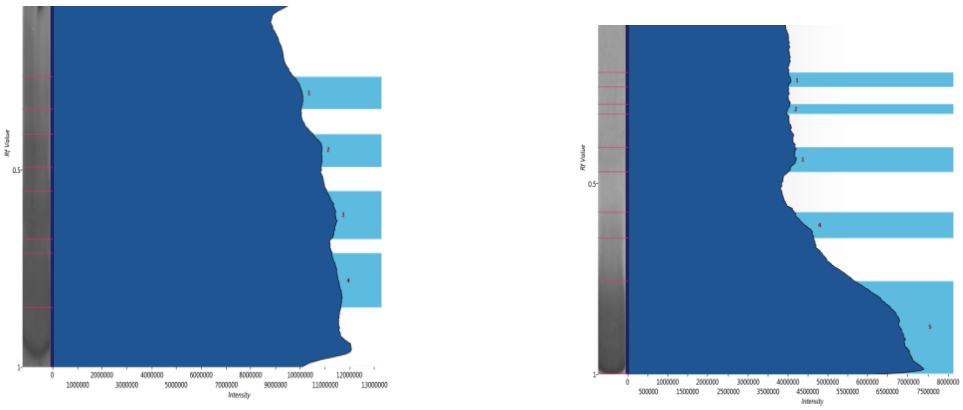

Fig.5. Rf value of bands of BSA and serum samples stained with HB stain.

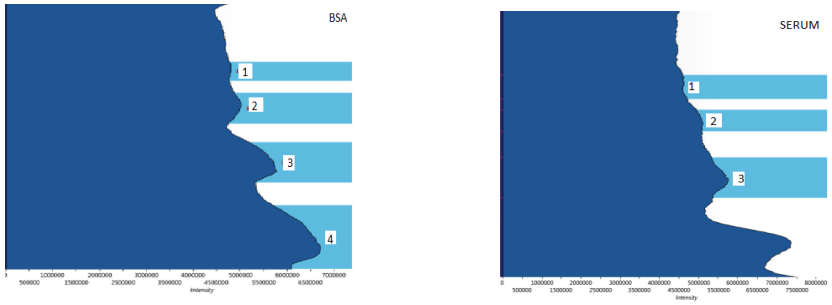

Fig.6: Rf value of bands of BSA and serum samples stained with HCT stain

Table 2: Percentage purity of each protein band was analysed. Proteins were stained with CBB, Henna + beetroot, and Tea+ coffee.

Stains	Sample	Percentage purity			
		Band 1	Band 2	Band 3	Band 4
Henna+ Beetroot	BSA	7.82±0.50	8.64±1.00	13.15±0.99	28.39±11.56
CBB	BSA	8.01	8.19	13.37	14.77
Henna + Beet root	Blood Serum	3.48±0.06	2.48±0.40	6.33±0.36	6.48±0.41
CBB	Blood Serum	3.93	1.98	8.82	5.66
Henna+ Tea + Coffee	BSA	6.4±0.14	10.33±0.91	15.25±0.71	23.14±9.06
Henna + Tea + Coffee	Blood Serum	6.66±1.33	7.79±1.09	11.77±2.79	
CBB	Blood Serum	6.21	8.25	13	

Statistical analysis of the results of two phytal stains and CBB staining techniques with respect to the human serum sample and Bovine albumin serum samples separated by Polyacrylamide gel electrophoresis was performed. A positive correlation between Rf values of bands, relative quantity and purity of the bands was observed. The significant differences with respect to RF values, purity of the band observed and relative quantity expressed after staining were assessed with student T test. The test results between HB staining, HTC staining and standard CBB have not shown any significant differences with respect to different parameters. These results were observed irrespective of the protein sample such as serum and BSA. (t_{cal} values <2.42 at 0.05 significance)

Discussion

1DE is the most sought after procedure due to its high resolving power, large sample loading capacity and display of several proteins on a single gel. This gives an ample scope of producing a direct and global view of a sample proteome at a given time. Several dyes are available to label proteins, either before or after electrophoresis. These include quite expensive (Sypro ruby) or economical (colloidal Coomassie blue, silver nitrate) and affordable stains, which may not cause any obstacles in protein identification by mass spectrometry (CBB). Despite a moderate amount of success in detecting and quantifying proteins in-gel, each staining procedure has its own limitations. All the traditional staining solutions contain methanol, acetic acid, or phosphoric acid which not only produce unpleasant smell but also cause environmental pollution.

The two staining solutions developed along with their staining procedures largely depend on water and no acids are needed for the staining solution preparation. The phytal extracts used for staining process have promising future in terms of staining the protein as efficiently as that of widely used CBB. The current staining procedure does not require washing and fixing steps but requires only the staining step. However, the protocol requires 6% acetic acid for destaining.

Conclusion

Post-electrophoretic protein staining procedures consider most widely used non covalent dye Coomassie blue. The other classical protein stains include silver nitrate, different fluorescent dyes such as Sypro Ruby, and Ruthenium red-based dyes that have limited usage probably due to their cost and/or technical difficulties[16]. In comparison, the current aqueous based phytal stains have advantages such as simplicity, low cost, environmental friendliness; do not require many reagents (except Vinegar or 6% acetic acid). Unlike the other chemical based extractions of henna, the current procedure involves extraction of stain from the leaves of henna and in combination of beetroot or tea and coffees using only the distilled water as extraction source. The protocol includes only staining and quick destaining steps. Stains are reusable up to several times, provided properly refrigerated but indicate a minimal intensity differences in the gel[13].

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Competing Interests

None declared.

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