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ASSESSMENT OF ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF WHEY PROTEIN AND QUANTIFICATION BY REVERSE PHASE HPLC

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ABSTRACT: Dairy and milk consumption are habitually included as important essentials in a healthy and balanced diet. It is the principal food for mammals and provides all the essential energy and nutrients to guarantee proper growth and development. Whey protein present in the milk is quickly digested and therefore amino acids are delivered quickly to muscles to help start the muscle-building process. The major components of milk whey of nutraceutical potential include beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin (BSA), lactoferrin (Lf), immunoglobulin's (Igs), lactoperoxidase (Lp) enzymes, glycomacropptides (GMP), lactose and minerals. The main aim of this study is to determine the antimicrobial and antioxidant activity of whey protein concentration from cow's milk. The result of the present study reveals the fact that the whey milk exhibited good antimicrobial activity. Whey protein α - lacto albumin and β - globulin were confirmed by two peaks with the retention time of 3.96 and 9.45. Thus, HPLC confirmed the presence of protein in the whey milk sample.

INTRODUCTION: Whey protein, with its high protein quality score and a high percentage of BCAAs (branched-chain amino acids), has long been popular in the exercise industry as a muscle-building supplement^{1, 2}. However, research suggests it may have far wider applications as a functional food in the management of conditions such as cancer, hepatitis B, HIV, cardiovascular disease, osteoporosis and even chronic stress^{3, 4}. About 20% of the protein in milk is whey. Whey proteins are quickly and easily digested, and they have essential amino acids-including the three branched-chain amino acids and other micro fractions⁵.

Despite the prevalence of high protein diets in athletic and sedentary populations, information available concerning the type of protein (*e.g.*, animal or vegetable) to consume is limited^{6, 7}. The purpose of this paper is to examine and analyze key factors responsible for making appropriate choices on the type of protein to consume in both athletic and general populations^{8, 9}.

Cow's milk contains approximately 3.5% protein, about 18% of which are whey proteins, mainly lactalbumin (about 12% of the whey protein) and lactoglobulin (about 50% of the whey protein), and about 82% are caseins^{10, 11}. During cheese making, the casein proteins precipitate out in the acidic environment and/or are coagulated by rennet to form the cheese while the whey proteins stay in solution^{12, 13}. This liquid whey contains proteins, peptides, lactose, milk fat, and other lipids; mineral salts and ions, such as sodium, potassium and calcium; vitamins; and water^{14, 15}. Manufacturers remove the water and concentrate the whey

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proteins, ending up with a white to a cream-colored product containing 90% or more protein called whey protein isolate (WPI) ^{16, 17}.

Bioactive peptides present in milk whey are one of the most studied compounds in different dairy products ^{18, 19}. Bioactive peptides from dairy sources are majorly classified on the basis of their biological roles as anti-hypertensive, anti-oxidative, immuno-modulant, anti-mutagenic, anti-microbial, anti-thrombotic, anti-obesity and mineral-binding agents ^{20, 21}. These bioactive peptides are produced by enzymatic hydrolysis during fermentation and gastrointestinal digestion ²². Thus, fermented dairy products like yogurt, cheese, and buttermilk are gaining popularity worldwide and are considered as an excellent source of dairy peptides. Furthermore, these dairy products are also associated with lower risks of hypertension, coagulopathy, stroke and cancer insurgences.

MATERIALS AND METHOD:

Collection of Milk: Fresh cow's milk was collected from a milk farm at Woraiyur, Tiruchirappalli district, Tamil Nadu in the month of December 2017 and was brought to the laboratory for further analysis.

Separation of Whey Protein from Cow's Milk: 250 ml of Fresh milk was boiled at 100 °C. Lemon was cut into two pieces. One half of the lemon was squeezed into the boiling milk. The milk is then allowed for cooling. After cooling, the content was filtered to separate the Whey milk. Finally, 120 ml of Whey milk was collected and stored in the refrigerator. The sample is then used for further examinations.

Estimation of Protein:

Lowry's Method: It is the most commonly used method for the determination of protein in cell-free extracts. The CO-OH (Peptide Bond) in the polypeptide chain reacts with copper sulphate in an alkaline sulphate medium to give a blue colored complex. In addition, tyrosine and tryptophan residues of protein complex cause reduction of the phosphate bond. Phosphomolybdate and phosphotungstate components of Folin ciocalteu reagents give bluish products that contribute towards enhancing the sensitivity of this method.

It is, however, important to remember that several compounds like EDTA, NH₄⁺, K⁺, Mg⁺ ions thiol reagent phenol *etc.* Interface with the color development and it should be ensured that substances are not present in sample preparation.

Antimicrobial Activity:

Test Organism: The test organism used in this study include gram-positive bacteria - *Bacillus* sp, and two gram-negative bacteria *E. coli* sp, *Klebsiella* sp. The fungal species like *Aspergillus* sp was used. All cultures were obtained from the Microbiology lab of our institute and Srimad Andavan Arts and Science College. All bacterial species were maintained in Nutrient agar medium and fungal species in Rose Bengal agar medium.

To carry out an antimicrobial activity, the agar diffusion method was used and the diameter of growth inhibition zone surrounding the antibiotic disc was measured to determine it. At first, the sterilized Muller –Hinton Agar medium 20 ml was poured into a sterile Petri plate. Then the sterile cotton swap was dipped into the culture suspension of bacteria.

The agar surface of each plate was inoculated by using the swab and ensuring the even distribution of the organism over the agar surface. The agar surface was allowed to dry for ten minutes. A sterile disc was picked up by the outer edge with sterile forceps and dip the disc in a prepared solution of the extract with different concentration (25 µl, 50 µl, 75 µl, 100 µl). The disc was placed near the edge of the agar surface of the inoculated plates. All plates were incubated at 37 °C for 24 h. The diameters of the zones of inhibition appearing around the discs were measured to the nearest millimeter (mm) and recorded.

Antioxidant Activity: 300 µl of each sample solution or ascorbic acid is added to the following: (100 µg/ml was combined with 3 ml reagent (0.6 M sulphuric acid, 2.8mM sodium phosphate, and 4mM ammonium molybdate). A blank solution containing 3 ml of reagent solution an appropriate volume of the same solvent was used for the sample. All tubes were capped and incubated in a boiling water bath at 95 °C for 9 min. After the samples were cooled to room temperature, the absorbance of the solution of each sample was

measured at 695 nm against the blank using a UV/VIS spectrophotometer. The experiment was performed in triplicates. The antioxidant activity was expressed as the number of the equivalent of ascorbic acid (Abdel-Hameed et al., 2009).

Total antioxidant capacity (% of inhibition) = $\frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$

Paper Chromatography: Amino acids in a given mixture of sample aliquots are separated on the basis of difference in their solubility. The separated amino acids are detected by spraying the air-dried chromatogram with ninhydrin agent. All amino acids give purple or bluish color reaction with ninhydrin except proline and hydroxyl proline which give a yellow-colored product.

HPLC: A validated sensitive and selective method of high-performance liquid chromatography (Shimadzu LC 8A with variable wavelength UV detector) was used for the determination of whey protein. A reverse-phase C 18 analytical columns (4.6 × 250 mm, particle size 5 μm), with a mobile phase that consists of solvent A acetonitrile and solvent B water is used for this experiment.

pH is maintained at 4.5. The ratio of acetonitrile is ranging from 95% to 55% with the flow rate of 1 ml/min at 45 ± 2 °C was maintained for 26 min.

The elute was monitored at 205 nm. The recorded absorbance at 205 nm was considered as a reference concentration. Thus, calibrated HPLC was used for the determination of Whey protein. HPLC analysis was performed at Cauvery College for women, Trichy.

RESULTS:

Protein Analysis (Lowrey’s Method): The results of protein analysis of lowrey’s method were presented as graphical representation Fig. 1.

Concentration of Test Sample = $\frac{\text{Test O.D.} \times \text{Con. Std} \times 100}{\text{Std O.D.}}$

$$\frac{0.20 \times 50 \times 100}{0.20}$$

Protein in 0.1 ml of sample = 5mg

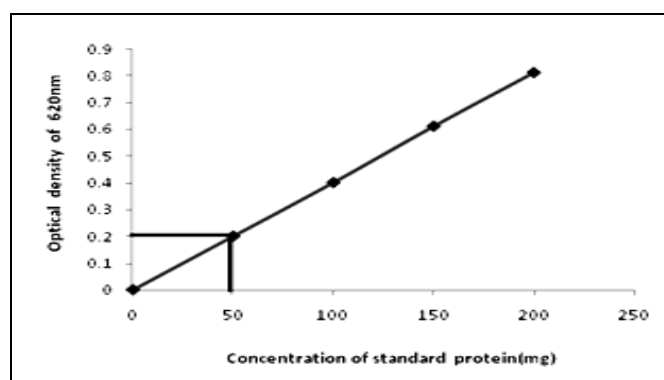


FIG. 1: STANDARD GRAPH OF PROTEIN ESTIMATION BY LOWREY’S METHOD



E. coli



Bacillus

Klebsiella

FIG. 2: ANTIBACTERIAL ACTIVITY OF WHEY PROTEIN

Antibacterial Activity: This antibacterial potency was accessed by the zone of inhibition (mm). Whey milk showed the best antimicrobial activity against gram-negative bacterial isolates and gram-positive bacterial isolate which showed resistance against it. The result of the present study reveals the fact that whey milk exhibited the greatest antimicrobial principles.

The present study justifies the claimed uses of whey milk in the traditional system of medium to treat various infectious diseases caused the micro-organisms. Some isolates showed various antimicrobial activities pathogen bacteria such as *E. coli*, *Bacillus* and *Klebsiella* **Fig. 2 & Fig. 3**. The results show that every isolate has different characteristics in generating antimicrobial activity. Whey milk sample isolates give the best antimicrobial activity with 24 h clear zone diameter in *E. coli*, *Bacillus* and *Klebsiella*.

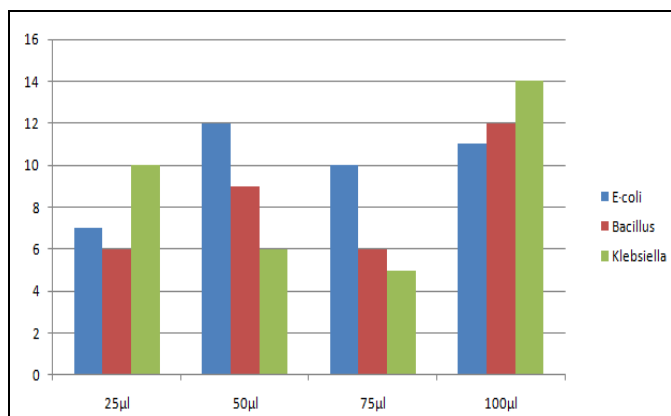


FIG. 3: COMPARISON OF ANTIBACTERIAL ACTIVITY OF WHEY PROTEIN

Antifungal Activity: Whey milk has notable antifungal activity against fungal species tested. The growth of *Aspergillus* sp was found to be decreased with increasing concentration of whey milk **Fig. 4 & Fig. 5**.



FIG. 4: ANTIFUNGAL ACTIVITY OF WHEY PROTEIN

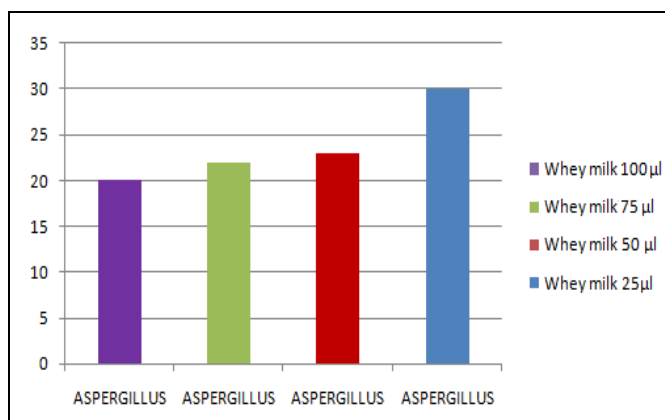


FIG. 5: DIFFERENT CONCENTRATION OF WHEY MILK SHOWING ANTIFUNGAL ACTIVITY

Antioxidant Activity: The absorbance of the sample was measured at 695 nm. The antioxidant activity of whey milk was 3.645 mg ascorbic acid equivalent/gram extract **Fig. 6**. The inhibition concentration of whey milk was 89. This antioxidant property may present of phenolic compounds such as flavonoids, phenolic acids and tannins.

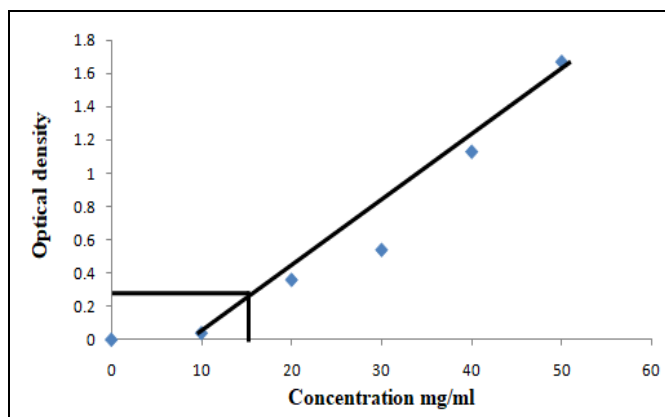


FIG. 6: STANDARD GRAPH OF ANTIOXIDANT ACTIVITY OF WHEY PROTEIN

Paper Chromatography: The tyrosine ($R_f = 0.076$) amino acid is identified in the whey protein sample by paper chromatography method **Fig. 7**.

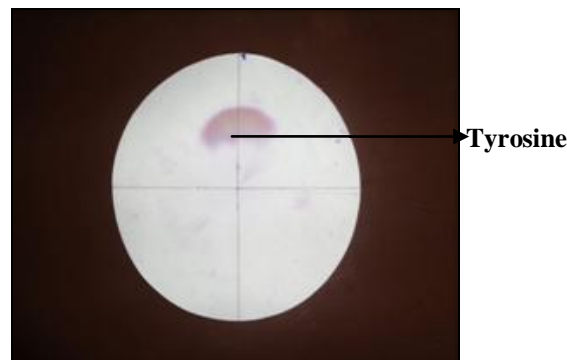


FIG. 7: PAPER CHROMATOGRAPHY

HPLC: Whey protein α – lacto albumin and β – globulin was confirmed by two peaks with the retention time of 3.96 and 9.45 **Fig. 8**. Thus, HPLC

confirmed the presence of protein in the whey milk sample.

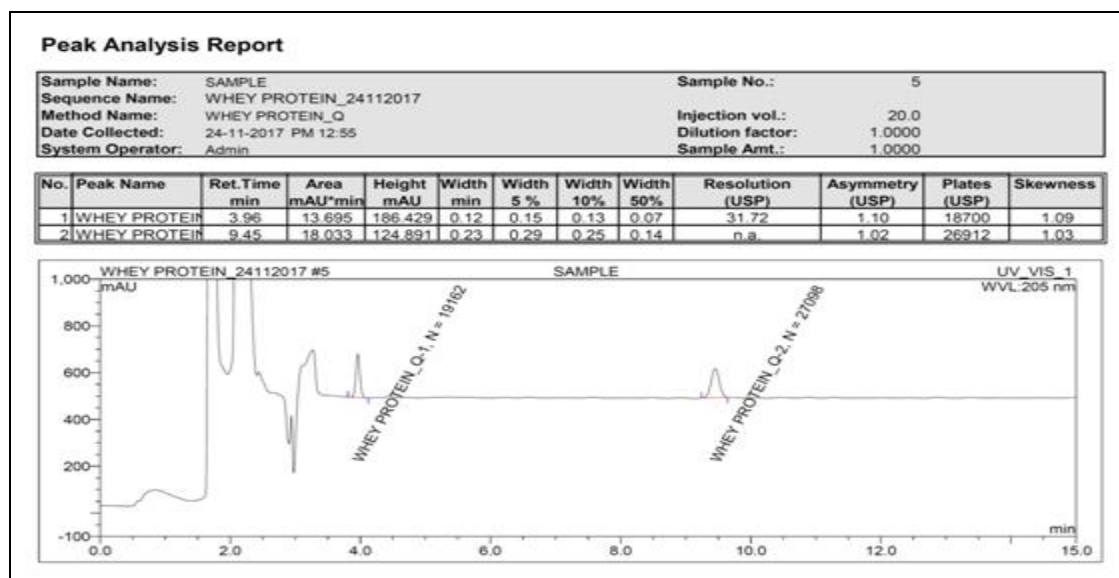


FIG. 8: HPLC

CONCLUSION: The amount of protein present in the whey milk is estimated by Lowery's method and it is shown in the graphical representation. The amino acid test showed a positive result for the Biuret test, Xanthoprotein test, Ninhydrin test, Sulphur test, Pauli's test, and a negative result for Million's test, Ehrlich's test. Antimicrobial activity was analyzed by three different methods. They are agar well diffusion method, disc diffusion method and minimum inhibitory concentration method. The result of the present study reveals the fact that whey milk exhibited the greatest antimicrobial activity. Antioxidant was analyzed by phosphomolibdenum method where we used ascorbic acid as standard and at the same time antioxidant in the number of equivalent ascorbic acid. The tyrosine amino acid is identified in the whey protein sample by paper chromatography method. Whey protein α – lacto albumin and β – globulin was confirmed by two peaks with the retention time of 3.96 and 9.45. Thus, HPLC confirmed the presence of protein in the whey milk sample.

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