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EXTRACTION AND PURIFICATION OF C-PHYCOCYANIN FROM ARTHROSPIRA SPECIES AND ITS APPLICATION IN LIP-BALM FORMULATION

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Abstract: C-Phycocyanin, a natural dye is used in food, cosmetics and pharmaceutical industries due to its potent antioxidant and anti-inflammatory properties. In lip balm formulation, the use of natural color dyes from different natural sources are under research to avoid the adverse side effects caused by synthetic colorants. The main objective of this study is to isolate and purify phycocyanin pigment from *Arthrospira* species for its formulation in lip balm as a natural colorant. The pigment was extracted by enzymatic and ultrasonic methods followed by ammonium sulphate precipitation, dialysis and HPLC. The formulated lip balm was analyzed for different parameters like pH and skin irritation. From HPLC analysis, it was observed that the maximum yield of 18.1 mg/ml of phycocyanin was obtained through ultrasonic methods when compared with enzymatic yield of 2.59 mg/ml. The extract also exhibited antimicrobial effect against *Staphylococcus*, *E. coli*, *Pseudomonas* and *Klebsiella* species, 37.7% of antioxidant activity and *in vitro* anticoagulation efficiency of 30 minutes. The lip balm didn't induce any rashes or redness on the skin after the application and the pH was found to be 6.8. Thus, the extracted C – Phycocyanin can be used in lip balm formulation as a bio-colorant as it is non-toxic.

Keywords: Phycocyanin, *Arthrospira* sp, Antioxidant, Cosmetics, Lipbalm

I. INTRODUCTION

Lip-balm is a cosmetic product containing pigments, oils, waxes and moisturizers that apply color, texture and provides protection to the lip (Azmin et al., 2020). It is made up of fatty acids like oil, wax, paraffin that act as emollients and additives such as colouring agents, antioxidants, and preservatives (Fernandes et al., 2013). The artificial colour additives tends to impart undesirable taste, negative health issues like allergy, carcinogenic effects, hyper-activity and intolerance reaction in kids (Sen et al., 2019). Hence alternatives to artificial color like bio - pigments for addition into cosmetics is the need of the hour as it is biocompatible and safe for human intake and also the demand for herbal cosmetics is increasing in the market (Gorgich et al., 2020; Panesar et al., 2015). The pigments that are used for natural colouring purpose are called as the bio – pigments. They are obtained from biological sources like plants, animals and microbes. The microbial pigments extracted from bacteria, fungi and algae are promising source of food colorants (Tuli et al., 2015). Microbial production has various benefits as their production is independent to weather condition, easy and fast with array of pigments in a very less time with low cost (Panesar et al., 2015). In addition, production of natural colorants will also be an advantage for the preservation of biodiversity as this can prevent the release of harmful chemicals into the environment (Abdulkadir, 2017). Currently, there are many microbial pigments like Zeaxanthin from *Flavobacterium* sp. Phycoerythrin from Porphyridium, astaxanthin from Haemotococcus pluvialis, Chlorella in skin cream formulation, (Dufossé, 2018; Gupta et al., 2019; Stoyneva-Gärtner et al., 2020) are used in cosmetic and food industries.

Arthrospira sp is the most popular algal source of Phycocyanin, the blue pigment. Phycocyanin is a water-soluble blue pigment that gives *Arthrospira* sp its bluish tint (Viskari & Colyer, 2002). Apart from natural dye application, the phycocyanin have shown various health benefits and wide range of pharmaceuticals application (Amara & Steinbüchel, 2013). *Arthrospira* sp is believed to stimulate the immune defense system and possess antioxidant, anti-inflammatory, anti-viral, anti-cancer, and cholesterol-lowering effects because of their high contents of phycocyanin and other biologically active molecules (Jiang et al., 2017; Kissoudi et al., 2018). Phycocyanin can make up more than 15% of the biomass in *Arthrospira* sp. This cyanobacterium tolerates pH values up to pH 10.5 and is grown photoautotrophically in outdoor, open ponds or raceways in tropical and subtropical regions (Sujatha Elumalai, 2010). Purified phycocyanin is quite a novel food ingredient in most parts of the world. Phycocyanin extracted from Porphyridium purpureum is added as coloruing agent in eye shadow formulation (Stoyneva-Gärtner et al., 2020).

The present study focuses on the extraction, purification of phycocyanin from a blue green algae *Arthrospira* and its evaluation in lip balm formulation as a bio - pigments using all the natural ingredients.

II. MATERIALS AND METHODS

Sample collection

The water sample was collected in a clean plastic 1 l white bottle from DGVC Pond water and Avadi lake and brought to laboratory.

Isolation, Purification and Culturing of Microalgae

To the two sets of 100 ml modified Zarrouk's medium, 1ml of water sample collected was added and incubated at 250 ºC under 16 h of light (100 lE/m2/s) alternating with 8 h of darkness for photoautotrophic cultivation. After 2 weeks, the samples were serially diluted and plated on sterile Bennett's agar plates under 16 h of light (100 lE/m2/s) alternating with 8 h of darkness. Algal species were examined under compound microscope 10X, 45X objective. The isolated colonies of Microalgae grown were then transferred to 4 ml media followed by scaling up of media to 1000 ml medium.

Optimization of pH

The Zarrouk's media and the pH of the medium in the range of 5.8 to 7.8 was optimized with slight modifications. All the experiments were done in 250 ml flasks containing 100 ml of media by inoculating 500 µl of cyanobacterial samples. The growth rate was analyzed photometrically at 615 nm.

Mass Cultivation and harvesting of of *Arthrospira* sp

Mass cultivation of *Arthrospira* sp was carried out in 1l bottle containing 600ml of the modified Zarrouk's medium, incubated at 250 ºC under 16 h of light (100 lE/m2/s) alternating with 8 h of darkness. The cultivation time depends on the optimum growth rate till reaching stationary phase between 15-20 days. The microalgal cells of *Arthrospira* sp were filtered through muslin cloth and wet weight (g) was calculated. The harvested biomass was dried in shade for sufficient time. After drying, the dried biomass was crushed to powder using piston-motor and dry weight (g) was measured.

Extraction of C-Phycocyanin by ultrasonic method

The dried *Arthrospira* sp was dissolved in distilled water at a ratio of 1:25 (w/v) for 24 h at 4 °C. Then the solution was irradiated at 40 kHz for 1 h and centrifuged at 10000 rpm at 4 °C for 15 min. The sediment was discarded and the supernatant containing the crude extract was collected, stored at 4 ºC and labelled as C – Phycocyanin (C – PC).

Extraction of C-Phycocyanin by physical method

The harvested microalgae were subjected to centrifugation at 3000 x g for 5 min at 20 ºC and the pellets were washed with potassium phosphate buffer. To one volume of washed cell mass five volumes of buffer was re-suspended and subjected for freezing at 20 ºC for 4 h and thawing process at 25 ºC in four cycles for the extraction of phycocyanin. In all the treatments the cell debris were removed by centrifugation at 10000 g and the supernatant was pooled and stored at 4 ºC. The absorbance was read at 620 nm and 652 nm. The evaluation of C-PC extraction based on the concentration of C-PC was calculated by the following equation according to (Bennett & Bogobad, 1973).

The evaluation of C-Phycocyanin extraction based on the concentration of C- Phycocyanin was set by

equation 1 deduced by Bennett and Beograd (1973).

$$PC = \frac{A620 - 0.474\ (A652)}{5.34} \qquad \text{Eq (1)}$$

Where, PC = Concentration of C-Phycocyanin mg/ml, A620 = Absorbance of the sample at 620nm, A652 = Absorbance of the sample at 652nm.

The extraction yield was calculated by using the equation,

$$Yield = \frac{(PC)V}{DB} \qquad \text{Eq (2)}$$

Where, PC is the concentration of C- Phycocyanin in terms of mg/dry biomass (g), V is the solvent volume (ml) and DB is the dry biomass (g).

Purification of C – Phycocyanin

The crude extract was partially purified by ammonium sulfate - $(NH_4)_2SO_4$ precipitation method. Briefly, $(NH_4)_2SO_4$ was gradually added to the crude extract to achieve 50% saturation under continuous stirring for 1 h and left overnight at 4 ℃ under dark condition. Then this solution was centrifuged at 10000 rpm for 30 min at 4 ℃ to collect the blue sediment containing phycocyanin and further dissolved in small volume of sodium phosphate buffer (pH -7) and dialyzed for 24 h at 4 ℃ against the phosphate buffer. In order to separate the existing bioactive compounds, the extracted C - PC was exposed to Thin Layer Chromatography (TLC).

High performance liquid chromatography (HPLC)

The extracted C – PC was identified by HPLC method using C18 column. Methanol and ammonium acetate (3%) in the ratio of 7:3 (v/v) was the mobile phase and temperature was set at 25 ℃ and wavelength at 615 nm. The phycocyanin obtained by physical method was injected into HPLC.

Determination of antibacterial activity by disc diffusion method

Antibacterial activity of the purified C - PC extracts was evaluated against different bacterial cultures - *E.coli, S. typhi, K. Pneumonia and P. aeruginosa.* The experiment was performed by following Kirby-Bauer disc diffusion method. Sterilized discs were made from Whatmann no. 1 filter paper (5 mm diameter) and 50 µl of C – PC extracts were loaded to the discs and dried completely in sterile condition. All the bacterial species were spread on sterile muller hinton agar plates and the crude extract loaded discs were placed on the seeded plates by using a sterile forceps. The plates were then incubated for 24 - 48 h and observed for clear zone of inhibition. The inhibition zone was measured in mm.

Antioxidant activity of the extracted crude extract

To different concentrations of C -PC solution (20 µl), 180 µl of DPPH (0.06 mM) solution was added and incubated for 30 min at room temperature in dark. An equal amount of phosphate buffer saline and DPPH served as the control. After incubation, the absorbance was recorded at 517 nm. The scavenging activity was determined and ascorbic acid was used as the control sample.

%RSA IC $_{50}$ = (1- Absorbance of sample /Absorbance of control) x 100

Determination of anticoagulation activity

The anticoagulation activity of purified C- Phycocyanin was investigated using the method of USA pharmacopeia. To 0.8 ml of 1% extract solution, 0.8 ml of standard heparin sodium solution, 1 ml plasma and 0.2 ml of 1% calcium chloride solution were added and the contents of the tubes were mixed by inverting the tubes thrice. The time required for clotting was recorded to determine the anticoagulation activity of the extract.

Formulation of lip balm

The lip balm ingredients used along with their formulation is described below in Table1 as described by *Anju et al.* (Varghese et al., 2017). Lip balm is composed of waxes, oils, pigments, and emollients which are adjusted to desired melting point and viscosity. Various agents used in the present study lip balm formulation are presented in Table 1.

Ingredients	Quantity	Function
Solid waxes (bees wax, candelilla wax)	10 g	Provides hardness and creaminess
Softening agents (cocoa butter)	15 g	Lubricates lip balm after application
Oil (castor oil, coconut oil, liquid paraffin)	10 ml	Dispensing the pigment and give high gloss to the lip balm
Colouring agents/Pigments	10 ml	Phycocyanin pigment
Perfumes	Adequate	Give aroma
Strawberry essence	2ml	Flavoring agent

Table 1: Components of lip stick and function

Characterization of formulated lip balm

Determination of pH:

The pH of formulated herbal lip balm was determined using pH meter.

Skin irritation test:

The surface of the area to be tested was cleaned and wiped. The product was applied over the skin and undisturbed for 10 min. After 10 min, the skin was observed for the absence of rashes, redness and irritation.

III. RESULTS AND DISCUSSION

Isolation, identification and culturing of microalgae

The algal medium inoculated with water sample collected from DGVC pond water showed filamentous growth, whereas the water sample from Avadi lake did not show any growth as shown in figure 1a&b. Further, the sample was serially diluted and plated on the Bennett's agar plate. The microscopical view revealed the free-floating filamentous green algae, which is characterized by cylindrical, multicellular trichomes in an open left-hand helix along the entire length. They show easily – visible transverse cross-walls. Then the filaments are solitary and free floating and display gliding motility and it belongs to the Genus *Arthrospira* species which is observed in figure 1c. The Zarrouk's media and pH were optimized, then the pH 7.8, were concluded to be the best when compared to other pH respectively. The growth rate analysis of cyanobacteria showed to be reaching high optical density in photometric analysis. Thus, considered to yield high biomass.

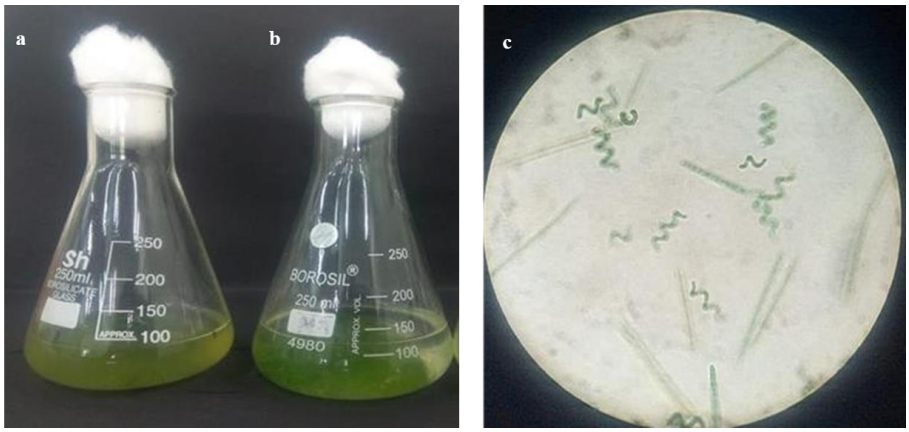

Figure 1 represents the isolation and identification of microalgae

Mass cultivation and harvesting of the isolated Arthropsira sp

Algal culture maintained in Zarrouk's medium was made to adapt to the new environment and growth progressed slowly for mass production of the pigment. The growth rate was maintained by controlled conditions of aeration, temperature and light as mentioned in figure 2a. The algae cells were harvested after 25 days and the wet weight of algae before dewatering was 54.7 g and it was dried in hot air oven at 60℃ for sufficient time and dry weight was 20.5 g which is represented in figure 2b.

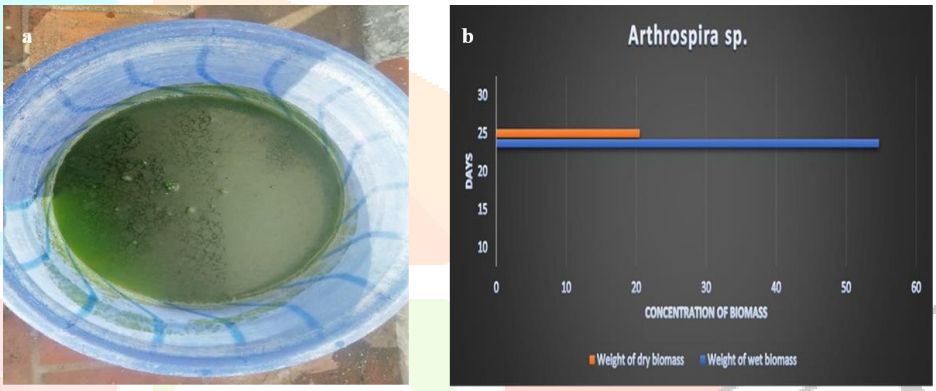

Figure 2a shows the mass cultivation of isolated *Arthrospira* species and 2b represents the yield of biomass bio-mass recovered after 25 days of cultivation

Extraction and Purification of C - Phycocyanin

The extraction yield and purity of phycocyanin concentration depends on how the destruction of the cell coverage happens. In this study we have used two methods to extract phycocyanin. In the first method, sodium phosphate buffer was used that would destabilize the cell membrane which is a physical means of extraction (figure 3a). Briefly, the freeze thawed biomass treated with sodium phosphate buffer of two different pH – 5.8 and 7.8 was centrifuged at 3000 rpm for 5 mins at 20 ℃ and the supernatant containing pigment was collected. In the second technique, ultrasonication process (figure 3b) was used to destroy the cell membrane of algae for extracting the pigment. The dry powder of *Arthrospira sp* was subjected to sonication method with distilled water and further centrifuged. The highest presence of C- Phycocyanin was observed in the supernatant and its concentration and purity was assessed.

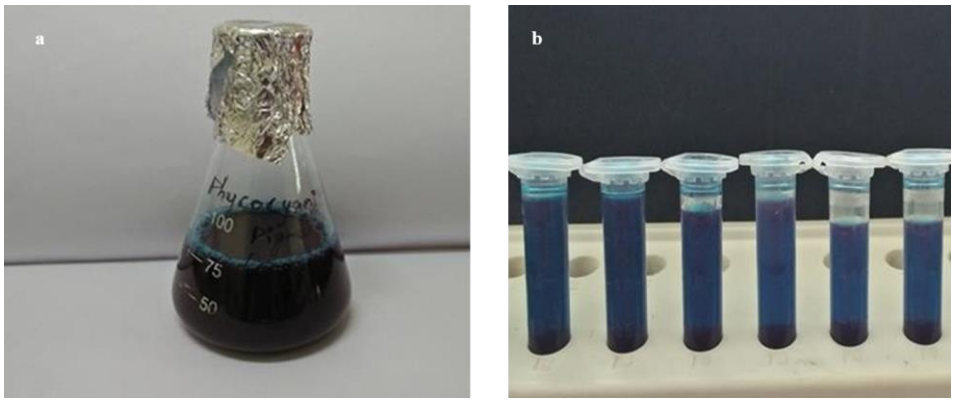

Figure 3a and 3b illustrates the extraction of C – Phycocyanin by Ultrasonic and Physical method respectively

The C- Phycocyanin concentration (CPC) in mg/ml was calculated from the optical densities at A620 and A652 nm and the ratio of A620 and A652 nm was used to determine the purity of the extracted pigment. Maximum yield of 18.1 mg/ml of C- Phycocyanin was extracted through ultrasonication with a purity of 0.57 (Table 2). The physical method at pH - 5.8 yielded 0.84 mg/ml and pH -7.8 yielded 0.97 mg/ml of C- Phycocyanin with the purity of 1.01 and 1.29 respectively (Table 3).

Extraction method	C- Phycocyanin (mg/ml)	Purity ratio (A620/652)
Sonication (Distilled water)	18.1	0.57

Table 2: Phycocyanin yield obtained from *Arthrospira* sp by physical method.

Sample	pH	Phycocyanin	C-Phycocyanin concentration (mg/ml)	Purity Ratio(A620/A652)
1.	5.8	Crude Phycocyanin	0.84	1.01
2.	7.8	Crude Phycocyanin	0.97	1.29

Table 3: Phycocyanin yield obtained from *Arthrospira* sp by ultrasonication method.

Phycocyanin was purified in several steps. First the PC was salted out with 50 % ammonium sulphate which neutralizes the pigment surface and precipitate outside. It also prevents the phycocyanin denaturation. Then the crude was dialyzed against phosphate buffer to remove small contaminants and the concentration of the phycocyanin measured by the UV absorption showed maximum absorbance at 652 and 615nm. The purified phycocyanin had the highest absorption at 652 nm which represents a covalent bond that binds to prosthetic groups as shown in figure 4.

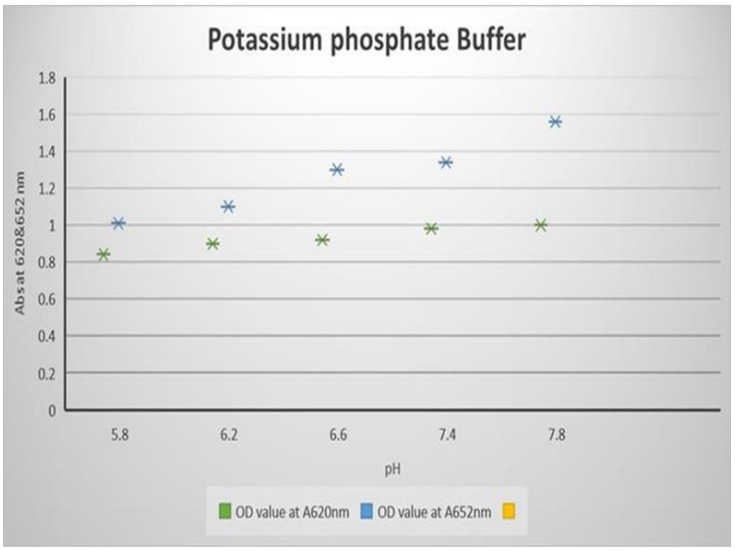

Figure 4 represents the determination of C – Phycocyanin purity by Ultraviolet Spectrophotometric analysis

Thin layer chromatography (TLC) was used for further characterization of phycocyanin. Silica gel was used as adsorbents to separate the more polar substrates. The plate developed in methanol 100% showed spots of phycocyanin chromophore. The Rf values of sample was equal to 0.6 (figure 5).

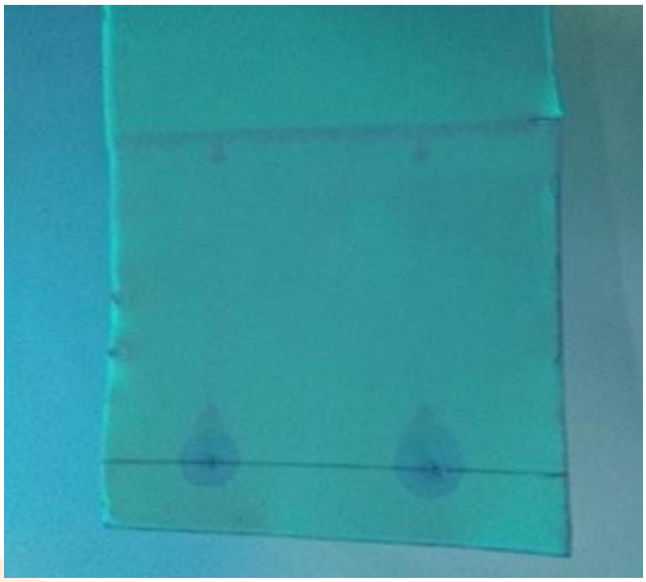

Figure 5 shows the qualitative analysis of pigment determined using Thin layer chromatography sheets

HPLC was used to view the major peak of phycocyanin and calculated the concentrations of phycocyanin (figure 6a). The concentration obtained for the enzymatic treatment was 2.591mg/ml. According to concentrations obtained by the ultrasound method seems more efficient at extracting phycocyanin, and the procedure is far more economically affordable. (124) **(Prabakaran. P, et al., 2013)** The crude form of C – Phycocyanin was purified by ammonium sulfate precipitation and dialysis method. The purity of phycocyanin increased after every stage of fraction. The C - PC fraction was then salted out with 50% ammonium sulphate concentration and dissolved in sodium phosphate buffer, eliminating other basic proteins to a remarkable degree with a purity, which results by fractionation better than those obtained by direct precipitation at 50% saturation.

The purification technique, ammonium sulfate precipitation is of great value, since it can be applied on a large scale and requires simple equipment and is simple and cheap. Moreover, for recovering C – PC biological activity, it is usually excellent after precipitation and its dissolution in easy. The partial purification of phycocyanin concentration was observed in dialysis method in the range of 1.96 mg/ml (figure 6b).

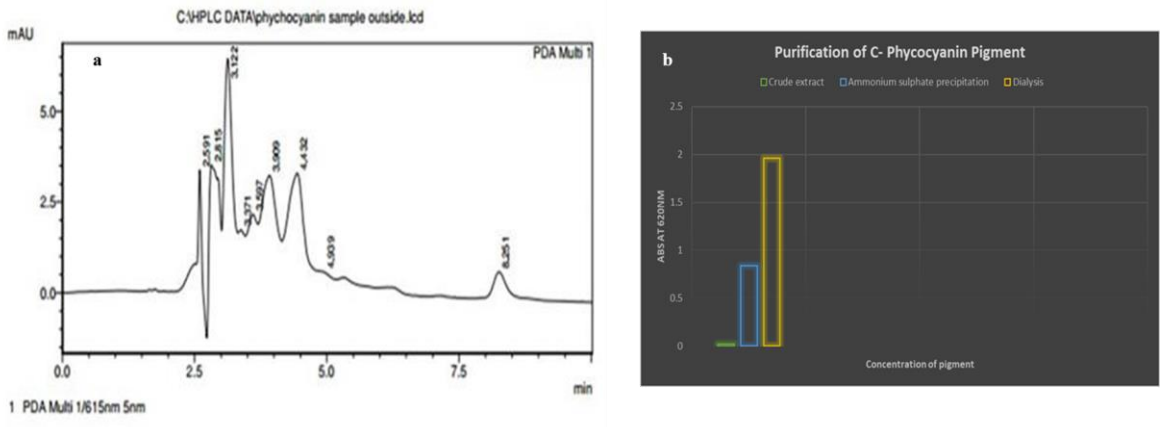

Figure 6 represents the absorption spectra of α and β subunits of C – Phycocyanin by the High-Performance Liquid Chromatography

Efficacy of extracted C - Phycocyanin

Anti-bacterial activity

The results of the study showed that the extraction of C – PC produced zone of inhibition against the representative bacterial strains in figure 7. The clear zone with maximum antibacterial activity was observed against *E.coli* and *S. aureus* with a zone of inhibition of 5 mm and 4 mm. The minimum antibacterial activity was observed against *K. Pneumonia* and *P. a*eruginosa with a zone of inhibition of 2 mm and 1 mm when treated with
the extraction of C – PC.

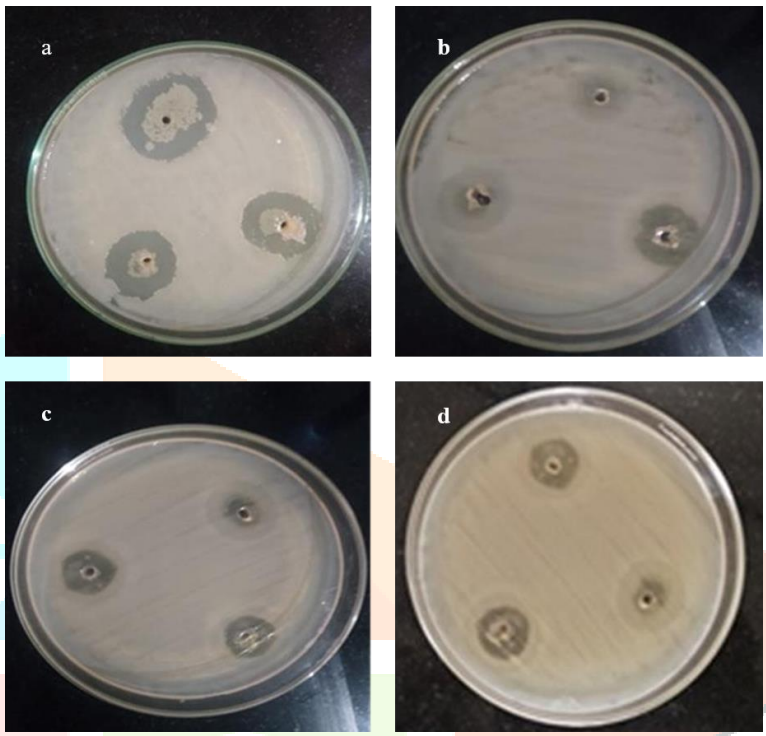

Figure 7 displays the anti-bacterial activity of C – Phycocyanin *against* (a) *E.coli* (b) *S. typhi* (c) *K. Pneumonia and* (d) *P. aeruginosa*

Determination of anti-oxidant activity

DPPH is a stable purple colour radical that turns to blue when it reacts with antioxidant and the degree of dis-colouration indicates the scavenging potential of the extracted pigment. The antioxidant activity of C - PC was compared with the standard ascorbic acid and the blue pigment showed radical scavenging activity of 37.7% (figure 8). Therefore, it could be concluded that the microbial pigment is a potent free radical scavenger.

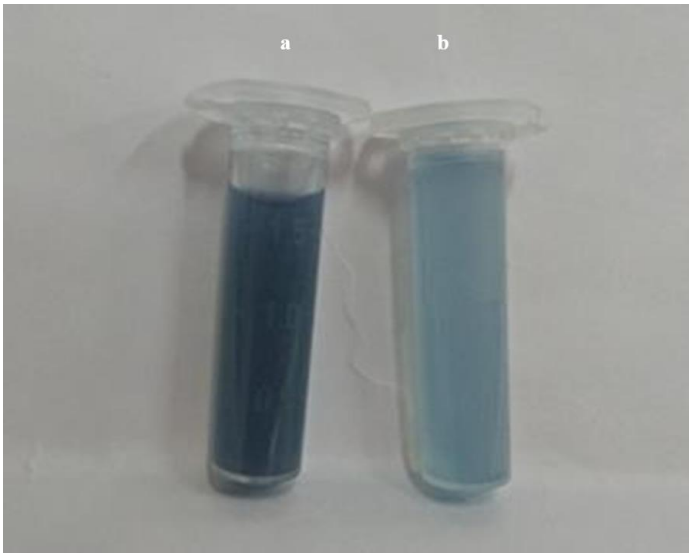

Figure 8. Estimation of anti-oxidant capacity of C – Phycocyanin by DPPH assay

Determination of anticoagulation activity

The obtained result for anticoagulation activity of extracted C – PC showed that it possesses great anticoagulating efficiency (expressed by clotting time assay) compared with that of the standard anticoagulant heparin (sulfate glucuronic acid) was found to be 30 and 35 min respectively as shown in figure 9.

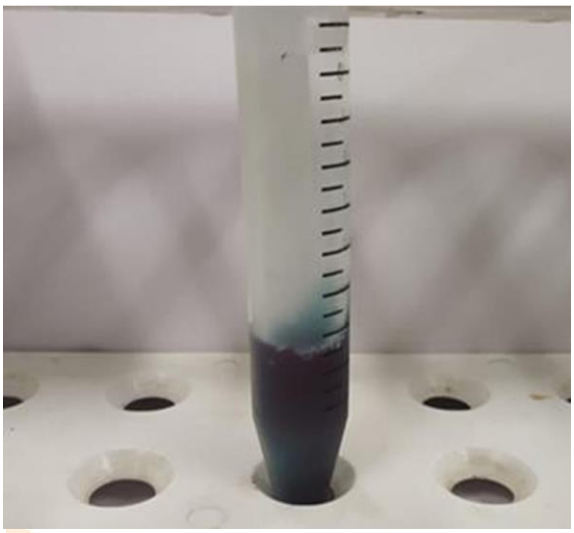

Figure 9 Determination of clotting time activity of C – Phycocyanin

Formulation of Lipbalm

The extracted Phycocyanin was used as coloring agent using natural ingredients and lip balm was formulated (figure 10). It was blue in colour with a pH of 6.8 and it didn't induce any skin irritation as tabulated in table 4. Thus, it was non-toxic and compatible to the human usage.

S. No	Parameters	Inference
1	Colour	Blue
2	pH	6.8
3	Skin irritation test	No

Table 4: Characterization of formulated lipbalm

Figure 10 shows the formulated lip – balm using C – Phycocyanin as the colouring agent

IV. CONCLUSION

There is a high demand for natural cosmetics in the recent times due to its safety. Lip balms or lip sticks are the cosmetic products that enhance the beauty of lips. The formulation of lip balms includes certain synthetic coloring agents that have been found to produce carcinogenic effects and that causes harm to the lips. Thus, we can move towards the use of natural colorants to prepare lip balm. Hence the use of natural colouring agents is a step towards healthy cosmetics and which can be widely utilized by the women with great pleasure. In conclusion, the present work describes an efficient method for extraction and purification of phycocyanin from *Arthrospira* sp. The awareness of natural pigment among people and their therapeutic uses are increasing because of their safety and cost. On the other hand, synthetic pigments are based on toxic raw materials. Hence, the natural products produced by microorganisms as pigments are safer and better than the use of synthetic products.

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