Research Article

Phytochemical Study & Chromatographic Separation of Microbial Pigments

Sri Vani Aparna. Sriram M.Sc, M.Tech, PGDCAQM[1], Dhurjeti Sarvamangala M.Sc, PhD, PGDBI[2]
[1] Research Scholar, Department of Biotechnology, Gitam Institute of Science,
[2] Associate Professor, Department of Biotechnology, Gitam Institute of Technology,
Gitam (Deemed to be University), Visakhapatnam, India.
***Corresponding author's E-mail:** aparnasriram198@gmail.com

Received: 10-04-2019; **Revised:** 24-05-2019; **Accepted:** 03-06-2019.

ABSTRACT

The current study aimed to screen the phytochemical constituents and chromatographic separation of pigments from various carbon sources using *Penicillium purpurogenum*. The microbial extracts were subjected to various phytochemical tests. Phytochemical analysis of microbial pigments shows the presence of alkaloids, tannins, flavonoids, steroids, saponins, phenolic compounds. The Rf values of all the microbial extracts were determined using paper and thin layer chromatography techniques.

Keywords: Phytochemicals, alkaloids, phenolic compounds.

INTRODUCTION

The bioactive compounds which can be produced by plants are known as Phytochemicals. They include Alkaloids, flavonoids, phenols, saponins, etc. These are plant derived secondary metabolites protects plants from stress, insects, microbes and climatic changes. Therefore, these are also protecting humans against diseases. The leaves of *Moringa.oleifera*[1] is a tropical plant that grows rapidly with well-established leaves and well documented nutritional, economic and medicinal benefits. Fenugreek, vernacular name for *Trigonella foenum graecum*[2] has been referred to as medicinal herb in both Indian Ayurvedic and traditional Chinese medicines. *Spinacia oleracea*[3] also called as palak, edible plant having curative activity against several human diseases by presence of bioplogical tannins. *Hibiscus rosa sinensis*[4] grows as an evergreen herbaceous plant in tropical regions. *Ixora coccinea* [5] are found growing profusely in dry lands. *Gomphrena globosa*[6] is used in folk medicine in the treatment of high blood pressure and other diseases. *Tagetes patula*[7] belongs to family Asteraceae. It is very popular as a garden plant and yields aromatic oil. *Nerium.oleander*[8] is an evergreen shrub, widely grown for its showy and fragrant flowers having ornamental and religious significance. *Citrus reticulata, and Citrus limon*[9] are the two species from citrus family to their diversified uses and largest growing fruits. *Carica.papaya*[10] with medicinal significance and is seen in the tropical regions. *Luffa.acutangula*[11], and *Solanum.tuberosum*[12] are the two vegetables known to all the important phytochemicals.

In paper chromatography support material consists of a layer of cellulose highly saturated with water. In this method, a thick filter paper comprised the support, and water drops settled in its pores made up the stationary "liquid phase." Mobile phase consists of an appropriate fluid placed in a developing tank. Paper chromatography is a "liquid-liquid" chromatography[13].

Thin-layer chromatography is a "solid-liquid adsorption" chromatography. In this method stationary phase is a solid adsorbent substance coated on glass plates. As adsorbent material all solid substances used. in column chromatography (alumina, silica gel, cellulose) can be utilized. In this method, the mobile phase travels upward through the stationary phase. The solvent travels up the thin plate soaked with the solvent by means of capillary action. During this procedure, it also drives the mixture priorly dropped on the lower parts of the plate with a pipette upwards with different flow rates. Thus the separation of analytes is achieved. This upward travelling rate depends on the polarity of the material, solid phase, and of the solvent[14].

In cases where molecules of the sample are colorless, florescence, radioactivity or a specific chemical substance can be used to produce a visible coloured reactive product so as to identify their positions on the chromatogram. Formation of a visible colour can be observed under room light or UV light. The position of each molecule in the mixture can be measured by calculating the ratio between the the distances travelled by the molecule and the solvent. This measurement value is called relative mobility, and expressed with a symbol R_f. R_f. value is used for qualitative description of the molecules[15].

MATERIALS AND METHODS

Collection of raw materials

The flowers (*Hibiscus rosa-sinensis, Gomphrena globosa, Tagetes patula, Ixora coccinea, Nerium oleander*), were collected from Gandhi Nagar park, Kakinada and the leaves (*Moringa oleifera, Trigonella foenum graecum, Spinacia oleracea*), fruit pulp and peels (*Carica papaya, Citrus*

reticulata, Citrus limon species), and vegetables (*Luffa acutangula, Solanum tuberosum*) were collected from the local market in Kakinada. All these materials are weighed followed by rinsing with distilled water and further reduced to paste using an electric blender. The paste is transferred into closed containers.

Pigment production, extraction, and purification

A loopful of well sporulated culture of the *Penicillium purpurogenum* was inoculated into a corresponding 250 mL Erlenmeyer flask containing 100 mL of production medium composed of PDB (2%), $MgSO_4$ (1%), $MnSO_4$(1%), K_2HPO_4 (1%) and KH_2PO_4 (1%) and Urea (0.5%) with pH 5.5. The inoculated flask was incubated on a rotary shaker (200 rpm) at 25ºC for 7 days. First, 250gms of all the fresh leaves, flowers, fruit pulp, and peel, vegetables was taken which is rich in sugar content and was ground for the extract. The sample extracts were diluted with an equal amount (250ml) of distilled water and stirred properly. It was added with 10ml of micro and macro nutrients KH_2PO_4-0.5gms, K_2HPO_4-0.5gms, $MgSO_4$-0.1gms, and $CO(NH_2)_2$-0.2gms for the growth of organism. 20ml of inoculum was added in the substrate. The solution is incubated for 1week at 37°C. After incubation, the broth obtained was taken and heated on a heating mantle at 70 degrees Celsius for 2hours. After heating, the broth was filtered, separating the biomass and the filtrate. The pH of the filtrate was checked. The solution obtained was evaporated and concentrated at 70 degrees Celsius. The water molecules were slowly removed on evaporation leaving the solid concentrate. The concentrate was cooled immediately. The crude extract obtained was allowed for crystallization to form crystals of colored pigment. The colored pigment obtained was purified and weighed.

Phytochemical Screening of microbial pigments:

The evaluation of phytochemicals from the microbial pigments was carried out according to the methodology of Sofowara (1994) [16], Harborne (1998)[17] and Kokate (2001)[18] and tabulated in table 1.

- Test for alkaloids: 2 ml filtrate was mixed with 1% HCl and about 6 drops of Mayor's reagents. A Creamish or pale yellow precipitate indicated the presence of respective alkaloids.
- Test for flavonoids: 2 ml filtrate was added to conc. HCl and magnesium ribbon. Pink-tomato red color indicated the presence of flavonoids.
- Test for amino acids: 1 ml of the extract was treated with few drops of Ninhydrin reagent. The appearance of purple color shows the presence of amino acids.
- Test for tannins: 1 ml of the extract was treated with few drops of 0.1% ferric chloride and observed for brownish green or a blue-black coloration.
- Test for phlobatannins: Deposition of a red precipitate when an aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatanins.
- Test for anthraquinones (Borntrager's test): To 1ml of the test was hydrolyzed 3ml of chloroform is added and layer obtained is removed. 10% ammonia was added to it. Rose pink tint demonstrated the closeness of anthraquinones.
- Test for saponins: Froth test for saponins was used. 1g of the sample was weighed into a conical flask in which 10ml of sterile distilled water was added and boiled for 5 min. The mixture was filtered and 2.5ml of the filtrate was added to 10ml of sterile distilled water in a test tube. The test tube was stopped and shaken vigorously for about 30 seconds. It was then allowed to stand for half an hour. Honeycomb froth indicated the presence of saponins.
- Test for steroids: To 1ml of the test, including 2 ml of chloroform was added to 2 ml of conc. H_2SO_4. The shading changed red demonstrating the proximity of steroids.
- Test for phytosterol: The extract was refluxed with a solution of alcoholic potassium hydroxide till complete saponification takes place. The mixture was diluted and extracted with ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol. The residue was dissolved in few drops of diluted acetic acid; 3 ml of acetic anhydride was added followed by few drops of Conc. Sulphuric acid. The appearance of bluish-green color showed the presence of phytosterol.
- Test for reducing sugars: The residue was re-dissolved in the water on the water bath. To 2ml of the solution, in the test tube was added, 1ml each of Fehling's solutions A and B. The mixture was shaken and heated in a water bath for 10min. The color obtained was recorded. A brick-red precipitate indicates reducing sugar.
- Detection of Terpenoids: Salkowski s Test: Five ml of the extract of the peel, flesh, and seeds were mixed with two ml of chloroform and then added carefully the 3 ml of concentrated H_2SO_4 to form a layer. An appearance of reddish brown color in the inner face indicates the presence of terpenoids.
- Detection of Phenols: Ferric chloride test: 10ml of the extract was treated with few drops of ferric chloride solution. Formation of a bluish black color indicates the presence of phenol. Lead acetate test: 10 ml of the extract was treated with a few drops of lead acetate solution. The formation of yellow color precipitate indicates the presence of phenol.
- Detection of Oils and Resins: The extract was applied to the filter paper. It develops a transparent appearance on the filter paper. It indicates the presence of oils and Resins.

- Test for cardiac glycosides (Keller-Killani test): 5 ml of each extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides.

Paper Chromatography:

A strip of Whatmann No. Filter paper, about 25-30 cm long, and 1.5 cm wide was marked lightly with a pencil line about 5cm from one end. The extract was spotted from a capillary pipette on to a spot marked in the middle of a pencil line. The solvent was allowed to evaporate. The paper is allowed to hang. The solvent Isopropyl alcohol: water (9:1) was introduced to saturate in a glass chamber. The solvent moves by capillary action into the paper. After the solvent moves lower to the upper edge of the paper, the paper is removed and the solvent front is marked. The R_f value was calculated and tabulated in table 2 and figure 1.

Thin Layer Chromatography:

TLC plates (7.5 x 2.5 cm glass plates) were coated with silica gel - G (200 mesh, Hi-media) to 1 mm thickness using a spreader. After air-drying, the plates were activated by exposure at 90°C for 15 min in a hot air oven. A quantity of 10 μL of the crude pigment was spotted. The mobile phase was Hexane: Ethyl acetate: Methanol: distilled water (6:4:7:3) solvent system. Individual colour spots on the TLC plates were marked and R_f values were calculated and tabulated in table 2 and figure 2.

Rf = Distance travelled by the solute/ Distance travelled by the solvent

RESULTS AND DISCUSSION

In the present investigation table 1 shows the presence of phytochemicals in microbial extract of flowers, leaves, fruit extracts and vegetables. The phytochemical analysis showed the presence of phenols, alkaloids, glycosides, saponins, and terpenoids, steroids, reducing sugars etc.

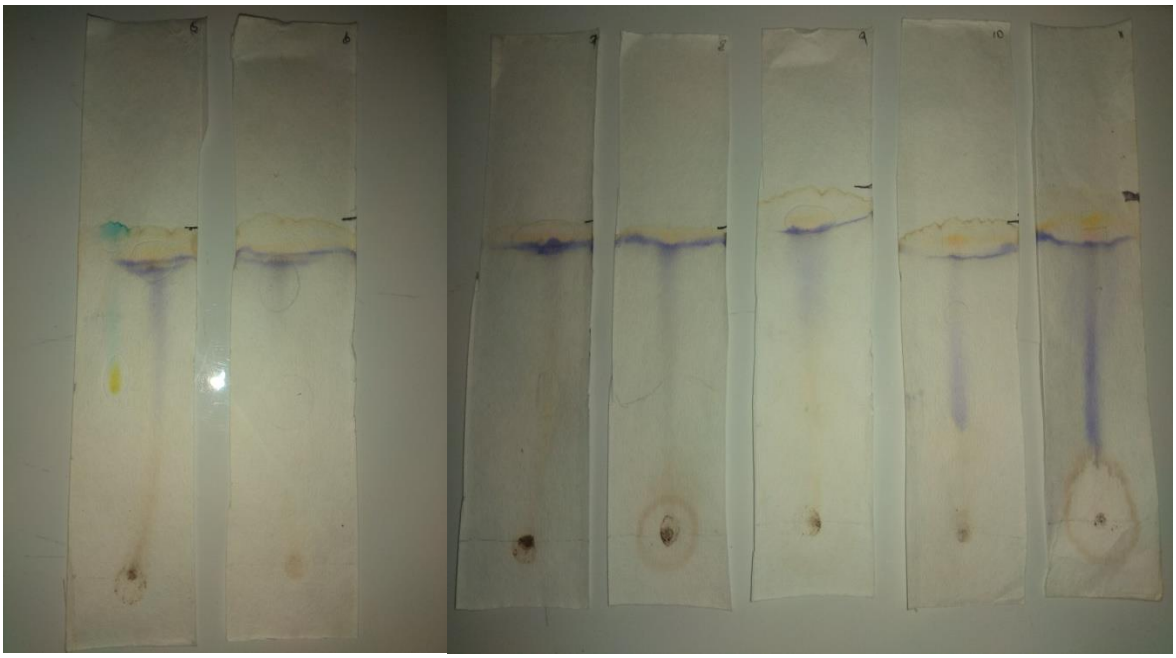
Table 1: Preliminary Phytochemical Screening of microbial pigments

Scientific name	Alkaloids	Flavonoids	Amino acids	Tannins	Phlobatannis	Anthraquinones	Saponins	Steroids	Phytosterols	Reducing sugars	Terpenoids	Phenols	Oil & resins	Cardiac glycosides/ Coumarins
M.oleifera	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve
T.foenum	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve
H.rosa sinensis	+ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve
I.coccinea	+ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve
G.globosa	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve
T.patula	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
N.oleander	-ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
T.patula + C.reticulata	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
C.reticulata + C.papaya	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
T.patula+ C.papaya	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve
S.oleracea + T.patula	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
C.limon + C. Papaya	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
L. acutangula	-ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve
S. tuberosum	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve

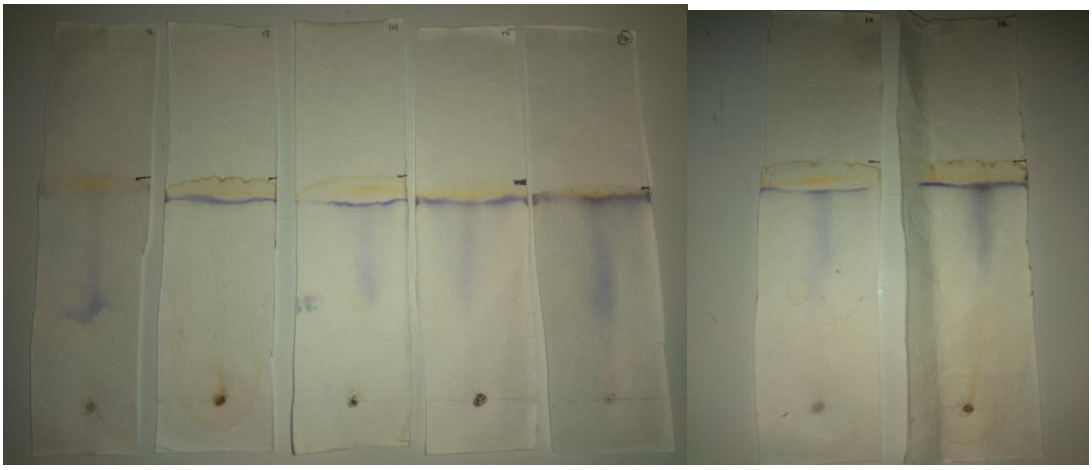
Table 2: Rf values of Paper and Thin Layer Chromatography of microbial pigments

Scientific name	Rf values of paper chromatography	Rf values of Thin Layer Chromatography
M.oleifera	0.925	0.75
T.foenum	0.898	0.66
H.rosa sinensis	0.948	0.933
I.coccinea	0.973	0.866
G.globosa	0.891	0.826
T.patula	0.933	0.970
N.oleander	0.948, 0.896	0.985
T.patula + C.reticulata	0.973	0.955
C.reticulata + C.papaya	0.931	0.692
T.patula+ C.papaya	0.945,0.918,0.405,0.457	0.70
S.oleracea + T.patula	0.944	0.833
C.limon + C. Papaya	0.971	0.971
L. acutangula	0.921,0.894	0.985
S. tuberosum	0.947	0.828

Figure 1: Paper Chromatogram of Pigments



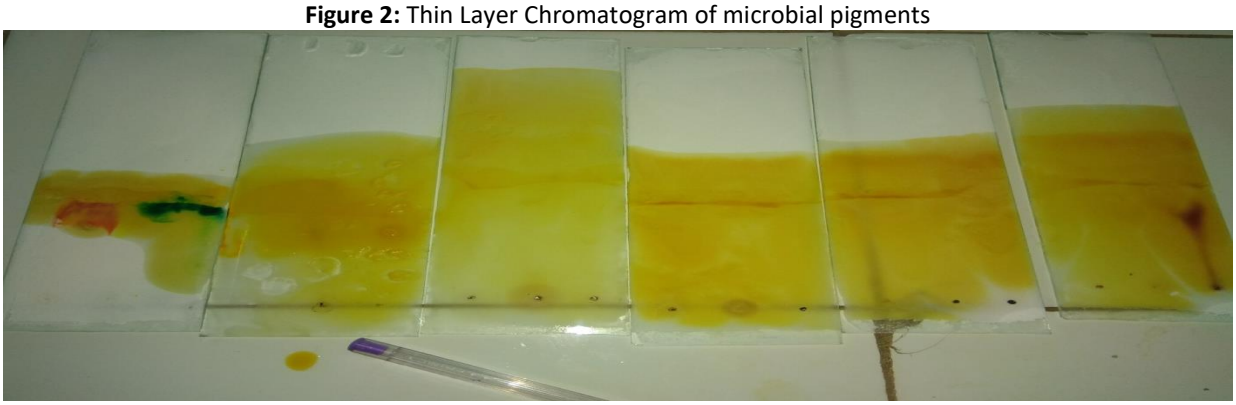
Moringa Trigonella Hibiscus, Ixora, Gomphrena, Tagetes, Nerium



1 2 3 4 5 6 7

1) T.patula + C.reticulata; 2) C.reticulata + C.papaya; 3) T.patula + C.papaya; 4) S.oleracea + T.patula 5) C.limon + C.papaya; 6) L.acutangula; 7) S.tuberosum

Figure 2: Thin Layer Chromatogram of microbial pigments



From left to right, every plate has 3 samples

Plate no 1: Standard red, blue, green food colors

Plate no 2: Standard yellow color, *Moringa, Trigonella*

Plate no 3: *Hibiscus, Ixora, Gomphrena*

Plate no 4: *Tagetes, Nerium, T.patula + C.reticulata*

Plate no 5: *C.reticulata + C.papaya , T.patula + C.papaya, S.oleracea + T.patula*

Plate no 6: *C.limon + C.papaya, L.acutangula, S.tuberosum*

CONCLUSION

The preliminary qualitative phytochemical screening of all the microbial extracts of leaves, flowers, fruit pulp and peels, vegetables were done to assess the presence of secondary metabolites. The paper and thin layer chromatography revealed that the microbial extracts having carotenoids, anthocyanins, terpenoids, flavonoids and phenolic compounds.

REFERENCES

1. Cáceres A, Saravia A, Rizzo S, Zabala L, De Leon E, Nave F. Pharmacologic properties of Moringa oleifera. 2: Screening for antispasmodic, antiinflammatory and diuretic activity. J Ethnopharmacol., 36(3), 1992 Jun, 233-7.

2. Anuradha CV; Ravikumar P. Indian J Physiol Pharmacol., 45, 2001, 408–420.

3.Vasthi kennedi Evenjelene. Evaluation of free radical scavenging activity and biological properties of Spinacia oleracea L. International Journal of Engineering science and Technology, 3, 2011, 25-29.

4. Mahadevan N, Shivali, P. Kamboj. Hibiscus sabdariffa Linn.– an overview. Natural Product Radiance, 8, 2009, 77-83.

5. Baliga MS, Kurian PJ "Ixora coccinea Linn.: traditional uses, phytochemistry, and pharmacology", Chin J Integr Med., 18(1), 2012 Jan, 72-9.

6. Lans CA. Ethnomedicines used in Trinidad and Tobago for urinary problems and diabetes mellitus. Journal of Ethnobiological and Ethnomedical., 2, 2006, 45.

7. Kaplan L., "Historical and Ethnobotanical aspects of domestication in tagetes," Economic Botany, vol. 14, no. 3, pp. 1960, 200–202.

8. Goetz, Rebecca J. "Oleander". *Indiana Plants Poisonous to Livestock and Pets*. Cooperative Extension Service, Purdue University (1998). Retrieved 2009-07-27.

9. Manthey. A and Grohmann. K. (2001): Phenols in citrus peel byproducts: concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses, J. Agric. Food Chem. 49 pp. 3268.

10. Lydia E., Riyazudin M., John S., Thiyagarajan S. Investigation on the phytochemicals present in the fruit peel of Carica papaya and evaluation of its antioxidant properties. Int J Health Allied Sci, 5, 2016, 247-52.

11. Anitha J and Miruthula S, Traditional medicinal uses, phytochemical profile and pharmacological activities of Luffa acutangular Linn, Int J Pharmacogology, 1(3), 2014, 174-83.

12. Al-Saikhan, Howard M. S., L. R., & Miller, J. C., Jr. (1995). Antioxidant activity and total phenolics in different genotypes of potato (Solanum tuberosum L.). Journal of Food Science, 60, 341–343.

13. Stoddard JM, Nguyen L, Mata-Chavez H, Nguyen K. TLC plates as a convenient platform for solvent-free reactions. Chem Commun (Camb), 12, 2007, 1240–1. [PubMed]

14.Sherman J, Fried B, Dekker M. New York, NY: Handbook of Thin-Layer Chromatography; 1991.

15.Donald PL, Lampman GM, Kritz GS, Randall G. Engel introduction to organic laboratory techniques. 4th ed. Thomson Brooks/Cole; 2006. pp. 797–817.

16. Sofowora A. J. Altern. Complement. Med., 2(3), 1996, 365-372.

17. Harborne JB. Phytochemical method: A Guide to Modern technique of Plant Analysis. 3rd Edition, Chapman and Hall: New York; 1998.

18.Kokate CK. A text book of Practical pharmacognosy. 5th Edition, Vallabh Prakashan: New Delhi 2005; 107- 111.

Source of Support: Nil, **Conflict of Interest:** None.