ORIGINAL ARTICLE

Antimycotic activity of *Hemigraphis colorata* (Blume)

A. Tamilselvan[1], R. Bala Vikash[2]*, P. Dharaninath[1], SebastiSixtus Hilary[1], S. Vignesh[1]

[1]Department of Pharmacology, PSG College of Pharmacy, Coimbatore, Tamil Nadu, India, [2]School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, Punjab, India

Abstract

Aim: The aim of the study was to evaluate the antifungal activity of *Hemigraphis colorata* leaf extracts on various fungal strains. **Materials and Methods:** Leaves of *H. colorata* were extracted with the hydroethanolic and aqueous methods of extraction. A preliminary phytochemical analysis was done and confirmed by the high performance thin layer chromatography (HPTLC) analysis. *Candida albicans*, *Candida tropicalis*, *Aspergillus niger*, *Rhizopus*, and *Trichoderma* were the fungal organisms used for the study. Extractive values and qualitative analysis were found from the crude extracts. **Results and Discussion:** The flavonoids were confirmed by HPTLC analysis. Among the fungal organisms, tested aqueous extract had exhibited a maximum zone of inhibition for *C. albicans*, and *C. tropicalis* and the hydroethanolic extract exhibited the zone of inhibition for *C. tropicalis* alone. **Conclusion:** From the results obtained, it is interpreted that the presence of flavonoids was mainly responsible for antimycotic (antifungal) activity exhibited by the *H. colorata* leaves which were selective among the fungal strains. Further descriptive studies are needed to confirm the exact mechanism of action to know about how the extracts exhibit antimycotic activity and to elucidate the structure-activity relationships.

Key words: Antimycotic (antifungal) activity, aqueous extraction, caffeic acid, catechin, gallic acid, *Hemigraphis colorata*, high performance thin layer chromatography analysis, hydroethanolic, quercetin

INTRODUCTION

Natural products are a source of traditional herbal medicine. In some parts of the world, they are considered the only curative remedy rather than synthetic drug moieties.[1] There is a vast change in opinion about the ethnopharmacological applications in therapeutics which was observed in the past 10 years. Nearly 30–40% of drugs used to treat diseases are derived from natural products.[2] The advancements in synthetic chemistry have limited the research on natural products in the pharmaceutical sector. Recent data obtained from pharmaceutical industries show that the product produced naturally represents the most valuable source for the production of new secondary metabolites.[3] In the field of ethnopharmacology, the plants used in traditional medicine had been tested often in pharmacological models. The medicinal plants and secondary metabolites derived as natural extracts have been considered surrogate therapy against various diseases.[4]

Hemigraphis colorata is a purple-colored waffle plant and it had its origin in the family Acanthaceae. They are generally spreader kinds of plants and do not grow too much height above 15 cm. The extracts from the leaves of *H. colorata* had been proved to be showing wound healing activity.[5] A various number of phytochemicals such as carbohydrates, flavonoids, saponins, carboxylic acids, phenols, proteins, and alkaloids have been reported earlier. The fresh leaves of the plant are reported to have a variety of phytoconstituents present in the veins. Conventionally, it had been reported to treat gall stones and used as a diuretic. The extracts of this plant have been reported for antibacterial activity against various Gram-positive organisms and antidiabetic activity.[6]

Address for correspondence:
R. Bala Vikash, School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, Punjab, India.
Mobile: +91-8903438540.
E-mail: balavikash278@gmail.com

Received: 05-04-2022
Revised: 23-05-2022
Accepted: 08-06-2022

This study aimed to evaluate the antifungal activity of *H. colorata* (Blume) medicinal plant used in Ayurveda and traditional medicinal system for the treatment of manifestations caused by five different species of fungal organisms. Therefore, extracts of the plant were tested for their potential activity against fungal pathogens.[7]

MATERIALS AND METHODS

Collection of Plant Materials

Aerial parts and leaves of the plant *H. colorata* were freshly collected from Herbal Garden, Department of Pharmacognosy, PSG College of Pharmacy, Coimbatore, and the plant was identified and authenticated by Dr. C. Jayaraman, Director, Plant Anatomy and Research Centre, Chennai and the Reg. No of the Certificate: PARC/2019/3994. The leaves of *H. colorata* were washed with water and shade dried. After shade drying, the leaves were coarsely powdered in a cutter mill and the powder was subjected to various studies for which the materials and methods are presented below.

Extraction of Plant Materials

The extraction of successive solvent extraction by Soxhlet apparatus using the coarse powder and various solvents depending on their polarity from non-polar to polar.[8]

N-Hexane Extract

About 500 g of coarse powder was extracted with 2.5 l of n-Hexane (68°C) by continuous hot percolation using the Soxhlet apparatus. The extraction was continued for up to 24 h. After completion, the petroleum ether extract was filtered and the solvent was removed by distillation under reduced pressure. Then, the obtained residue was stored in a desiccator.

Hydroalcoholic (Ethanol 95% w/w) Extract

Marc obtained from the above extract was dried and extracted with a hydroalcoholic mixture of ethanol and water in the ratio of 70:30 (79–81°C) using the Soxhlet apparatus. The extraction was continued for up to 24 h. After completion of extraction, the alcoholic solvent was removed by a rotary evaporator. Then, it was stored in a desiccator, freeze-dried, and used for the study.

From the weight of each extractive residue, the extractive values were calculated in percentage. All the above extracts were used for the identification of constituents by preliminary phytochemical tests, hydroalcoholic extract was reported to have flavonoids as the result of preliminary phytochemical screening.[9] Hence, an attempt was made to determine the flavonoid content quantitatively using high performance thin layer chromatography (HPTLC). Quercetin is an essential chemical marker that was selected to perform the quantitative estimation. The data of extractive values are shown in [Table 1].

Preliminary Phytochemical Evaluation

Identification of phytoconstituents by chemical tests

The n-hexane and hydroalcoholic extracts of *H. colorata* were analyzed qualitatively for the phytochemicals by chemical tests.

Test for Alkaloids

A small portion of the solvent-free hydroalcoholic extract was stirred separately with a few drops of dilute hydrochloric acid and filtered. The filtrate was used to test the presence of alkaloids using reagents like Dragendorff's reagent which must indicate its presence by precipitating orange-brown precipitate. Cream-colored precipitate with Mayer's reagent indicates the presence of alkaloids in the extract.[10] The yellow-colored precipitate is observed in the presence of Hager's reagent. While with Wagner's reagent, a reddish-brown precipitate indicates the presence of alkaloids in the sample.

Test for Carbohydrates

A small quantity of the extracts was dissolved in 5 ml distilled water and filtered. This filtrate can be used for testing the presence of carbohydrates with many reagents/tests.

Molisch's test (General test)

To the filtrate of 2–3 ml taken in a test tube and a few drops of 1% alcoholic alpha naphthol were added, shaken well, and 2 ml of concentrated sulfuric acid was added along the

Table 1: Extraction of air-dried leaves of *Hemigraphis colorata* (Blume)

Method of Extraction	Solvents	Color	Consistency	Extractive values (%w/v)
Continuous Hot percolation by Soxhlet apparatus	Hydroethanollic (95%) Distilled water (70): Ethanol (30)	Dark brown	Liquid	3.5 g (17.5)
Cold Maceration	Distilled water with 1ml Chloroform	Dark brown	Liquid	0.95 g (4.75)

sides of the test tube. It can be understood that the presence of a violet ring at the junction of two liquids indicates the presence of carbohydrates.

Fehling's test (Test for reducing sugar)

To the filtrate 1 ml of Fehling's solution A and 1 ml of Fehling's solution B is mixed and heated in a boiling water bath. The presence of reddish-orange precipitate shows the presence of carbohydrates.

Test for Glycosides

The free sugar content in the extract is determined. The extract is made to undergo hydrolysis by treating with a mineral acid such as dilute H_2SO_4 and again the hydrolyzed extract is determined for sugar content. An increase in sugar content confirms the presence of glycosides in the extract.

Test for Cardiac Glycosides

Legal's test (Test for cardenolides)

To the extract which is hydroalcoholic 1 ml of pyridine and 1 ml of sodium nitroprusside solution was added. The appearance of pink to red color indicates the presence of cardiac glycosides.[11]

Test for Anthraquinone Glycosides

Borntrager's TEST

The extract was taken in a test tube and dilute H_2SO_4 was added and made to boil in a water bath. It was then filtered and the filtrate was cooled and an equal amount of benzene or chloroform was added and shaken well. The organic solvent was isolated from the mixture. Ammonia was added. If the ammonia layer turns pink in color, it indicates the presence of anthraquinone glycosides.

Test for fixed oils

The extract in small quantities was pressed separately between two filter papers. The appearance of a permanent oil stain on the filter paper indicates the presence of fixed oil.

Test for saponins (Foam test)

The extract was added with 20 ml of distilled water and it was agitated on a graduated cylinder for 15 min. The presence of saponins was indicated by the formation of 1 cm of foam.[12]

Test for tannins and phenolic compounds

Small quantities of the extract were taken in a test tube and 5% $FeCl_3$ was added in drops along the sides of the test tube. The appearance of deep blue-black color indicated the presence of tannins. The extract treated with gelatin solution if it forms a white precipitate, which indicates the presence of phenols. The extract treated with 10% lead acetate solution if white color precipitates indicate the presence of tannins.

Test for steroids

Salkowski reaction test

To some quantities of the extract were taken in a test tube and 2 ml chloroform and 2 ml concentrated H_2SO_4 were added and shaken well. If the chloroform layer turns into red color and the acid layer shows yellow-green fluorescence color, it indicates the presence of steroids.[13]

Test for proteins and free amino acids

Biuret test (general test)

To some quantity of extract biuret reagent which is a mixture of 1% copper sulfate and 4% NaOH was added and heated. If a violet pink color appears, it indicates the presence of proteins.

Ninhydrin reagent

The extract treated with Ninhydrin reagent, the appearance of purple color shows the presence of free amino acids.

Test for Flavonoids

Shinoda's test

To the extract taken in a test tube 5 ml of 95% ethanol was added and shaken well and a few drops of concentrated HCl were added along with 0.5 g of magnesium turnings. The appearance of orange, pink, and red to magenta colors shows the presence of flavonoids. The results are shown in [Table 2].

Table 2: Qualitative analysis of crude extracts of *Hemigraphis colorata* (Blume)

Name of the Test	Hydroalcoholic extract	Aqueous extract
Carbohydrates	+	+
Reducing sugar	+	-
Proteins	-	-
Amino acids	-	-
Cardiac glycosides	-	-
Anthraquinone glycosides	-	-
Saponins	+	-
Flavonoids	+	+
Alkaloids	+	+
Phenolics	+	+
Steroids	-	+
Enzymes	-	-

(+): Present, (-): Absent

HPTLC Analysis of Crude Extract

The hydroalcoholic extract which contains more phytoconstituents was chosen for the HPTLC. 1 g of freeze-dried hydroalcoholic extract powder was mixed with 10 ml of methanol and completely dissolved and filtered using Whatman filter paper Grade 1. The filtrate was used for the HPTLC analysis. 1 g gallic acid, caffeic acid, quercetin, and catechin powder were mixed with 10 ml of methanol and filtered using Whatman filter paper Grade 1. The filtrate was used for further studies. TLC was performed on precoated Silica gel 60F 254 TLC plates (E Merck, Germany) whose thickness is 0.2 mm. The mobile phase used is Toluene: Ethyl acetate: Methanol: Formic acid in the ratio of 5.5:3:1:0.5(v/v). The sample and standard were sprayed on plates at a distance of 0.8 cm as a narrow band by spray-on technique utilizing pressurized nitrogen gas (150 kg/cm^2) through CAMAG Linomat V fitted with a 100 µl syringe. The plates were kept in the twin-trough chamber which contains the mobile phase which had attained chamber saturation. Ascending development was performed in a twin–trough glass chamber (10 × 10 cm) at room temperature (25 ± 2°C) and relatively humidity (60 ± 5%) for a distance of 10 cm. The bands were visualized in CAMAG UV Cabinet at 254 nm and 366 nm and scanned through WINCATS 4 software.[14] The results of retention factor values of HPTLC analysis are given in [Table 3].

Antimycotic Activity

The samples were screened for antifungal activity against the following fungal strains using well diffusion method.[15] The sample (10 mg/ml) was prepared in a respective solvent. Agar well diffusion method was performed. Muller Hinton agar plates were prepared by adding the reagents and were inoculated with test organisms. The plates were evenly spread out. The wells were prepared by making holes in the agar. Each disk

Table 3: Quantitative analysis using retention factor (Rf) values of HPTLC peaks

Secondary Metabolites	Wavelength (nm)	Standard Rf	Hydroalcoholic extract Rf	Aqueous extract Rf
Gallic acid	254	0.17	-	0.168
Caffeic acid	254	0.21	-	0.214
Quercetin	254	0.23	-	0.231
Catechin	366	0.26	0.256	-

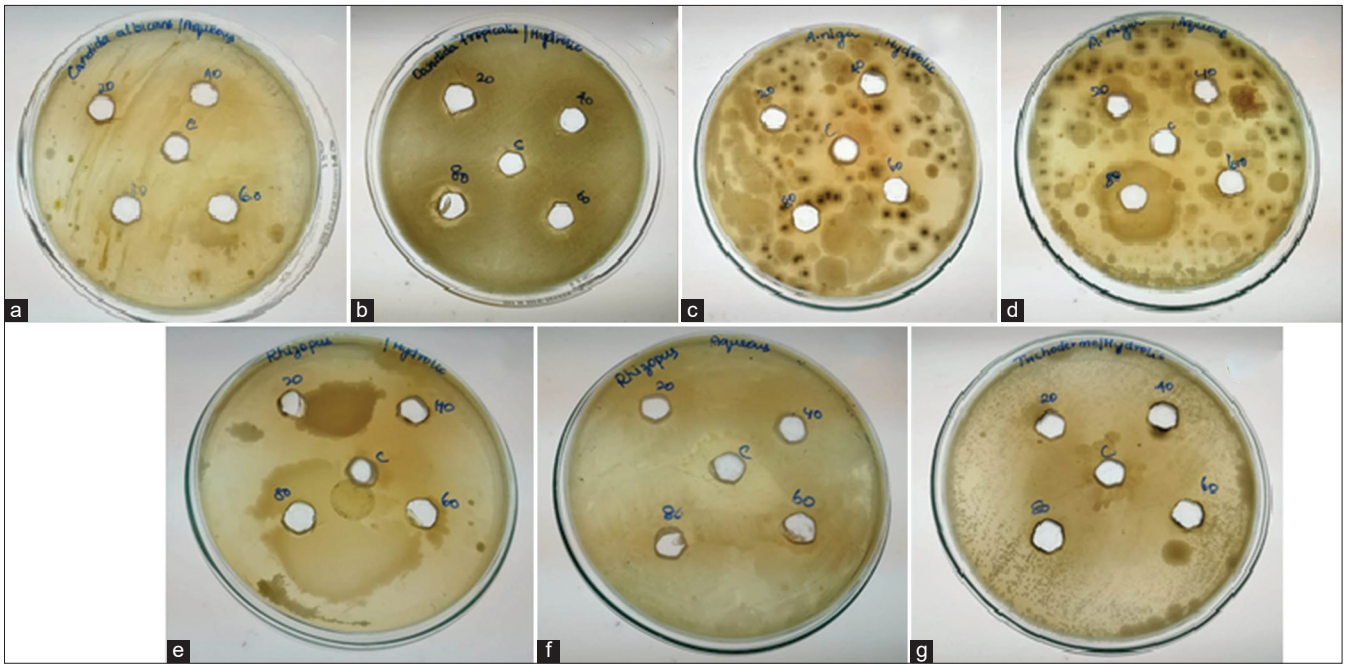

Figure 1: Zone of inhibition (a) aqueous extract on *Candida albicans,* (b) hydroalcoholic extract on *Candida tropicalis,* (c) hydroalcoholic extract on *Aspergillus niger,* (d) aqueous extract on *Aspergillus niger,* (e) hydroalcoholic extract on *Rhizopus,* (f) aqueous extract on *Rhizopus, and* (g) hydroalcoholic extract on *Trichoderma*

was loaded with 20, 40, 60, and 80 µg/ml of extract and fluconazole was used as a control for antifungal activity. The plates were incubated for 24 h at 37°C. The zone of inhibition around the well was measured as a value of the diameter of the circle and recorded. The results are presented in [Table 4 and Figure 1a-g].

RESULTS AND DISCUSSION

Extractive Values

It is the value obtained as a percentage amount of extract which was successfully extracted out of the crude coarse powder. The extracts were used for the identification of chemical constituents by phytochemical test and selective extracts which showed the presence of many phytoconstituents were analyzed by the instrumental analysis. The extractive value indicates the yield of the extract obtained from the air-dried plant powder by successive solvent extraction by Soxhlet extraction. Their percentage yield shows the solubility of the active principles in the organic solvents used based on the polarity nature. They are then identified and confirmed by the preliminary phytochemical evaluation.

HPTLC Analysis of *H. colorata* (Blume) Leaves

The fingerprint of the constituents present in the hydroalcoholic and aqueous extract was recorded using CAMAG and TLC visualizer and WinCats Software. Phytocompound isolated at various peaks is identified and presented in [Figures 2-9] and Rf values are tabulated in [Table 3].

The study was tested for the antifungal activity of crude extracts and their respective dilutions from a medicinal plant. The antifungal activity was determined by measuring the diameter of the zone of inhibition recorded as shown in Figure 1. The plant *H. colorata* leaves were extracted with hydroalcoholic and aqueous extraction methods. Both the extracts were used for antifungal activity in the concentrations of 20, 40, 60, and 80 mcg/ml. Standard drug used was Fluconozole 5 mcg/ml. The extracts were tested against *Candida albicans*, *Candida tropicalis*, *Aspergillus niger*, *Rhizophus*, and *Trichoderma* and the zone of inhibition was measured. The extract of hydroalcoholic mixture exhibited a maximum zone of inhibition against *C. tropicalis* 12 mm whereas the aqueous extract showed antifungal activity against *C. albicans* 1.9 mm and there was no specified activity against *A. niger*, *Rhizophus,* and *Trichoderma* with both the extracts.

Table 4: Antimycotic activity of hydroalcoholic and aqueous extract of *Hemigraphis colorata* leaves

Organism	Sample Concentration (mcg/ml)	Zone of inhibition (mm)	
		Sample	Control (Fluconazole)
Candida albicans (Hydroalcoholic)	20	0.5	10
	40	1.3	
	60	1.7	
	80	2.1	
Candida albicans (Aqueous)	20	0.2	9
	40	0.9	
	60	1.3	
	80	1.9	
Candida tropicalis (Hydroalcoholic)	20	-	6
	40	5	
	60	6	
	80	12	
Candida tropicalis (Aqueous)	20	-	5
	40	-	
	60	-	
	80	-	
Aspergillus niger (Hydroalcoholic)	20	-	7
	40	-	
	60	-	
	80	-	
Aspergillus niger (Aqueous)	20	-	4
	40	-	
	60	-	
	80	-	
Rhizopus (Hydroalcoholic)	20	-	6
	40	-	
	60	-	
	80	-	
Rhizopus (Aqueous)	20	-	5
	40	-	
	60	-	
	80	-	
Trichoderma (Hydroalcoholic)	20	-	4
	40	-	
	60	-	
	80	-	
Trichoderma (Aqueous)	20	-	4
	40	-	
	60	-	
	80	-	

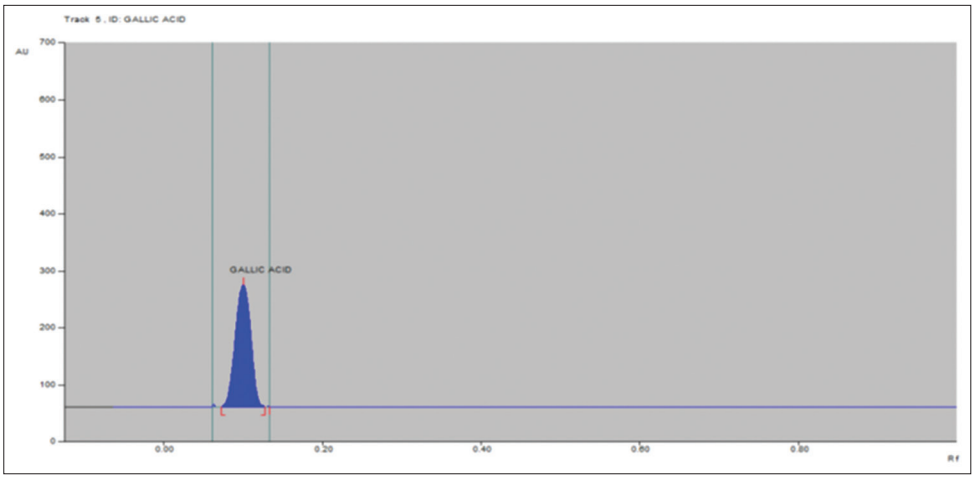

Figure 2: Standard peak of gallic acid

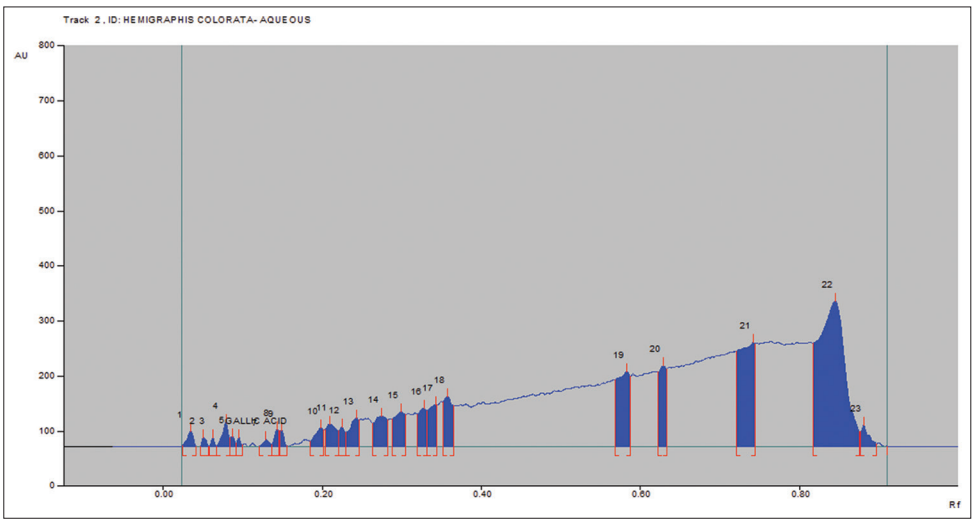

Figure 3: Gallic acid peak of aqueous extract of *Hemigraphis colorata*

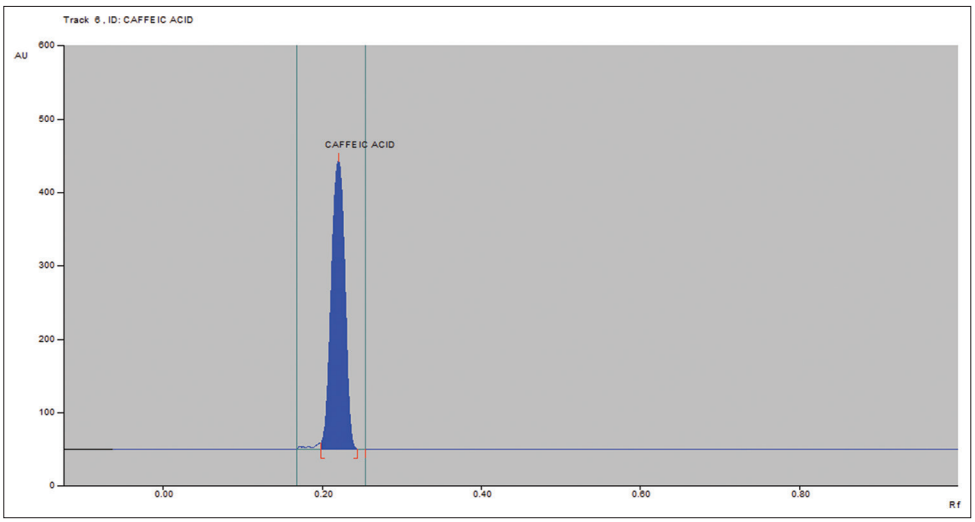

Figure 4: Standard peak of caffeic acid

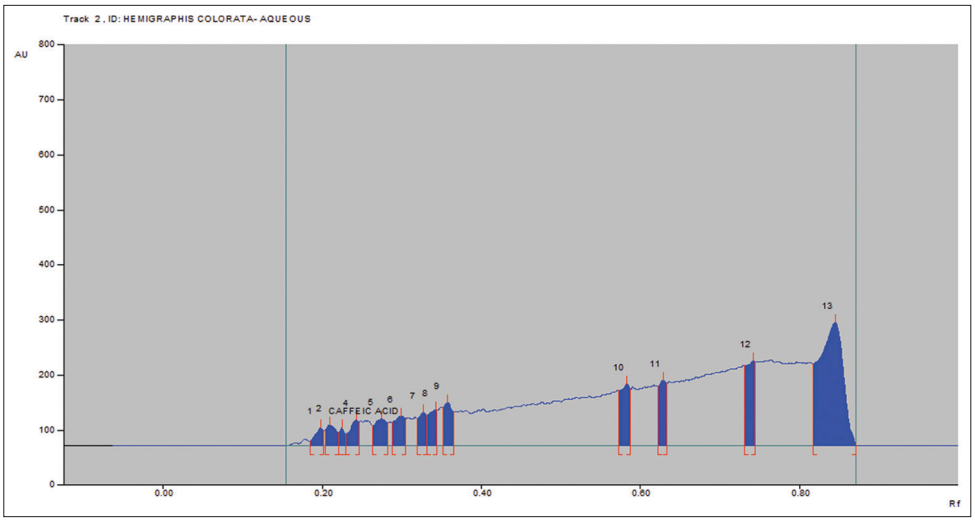

Figure 5: Caffeic acid peak of aqueous extract of *Hemigraphis colorata*

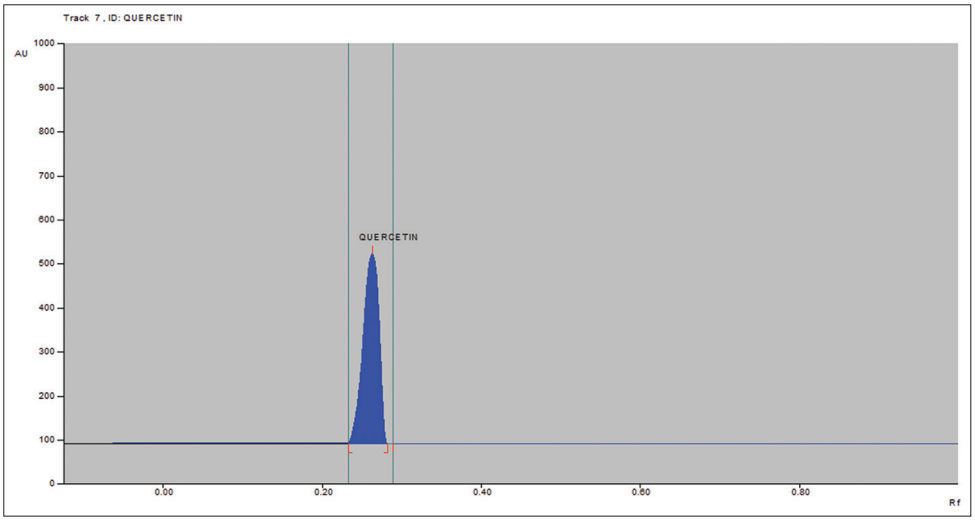

Figure 6: Standard peak of quercetin

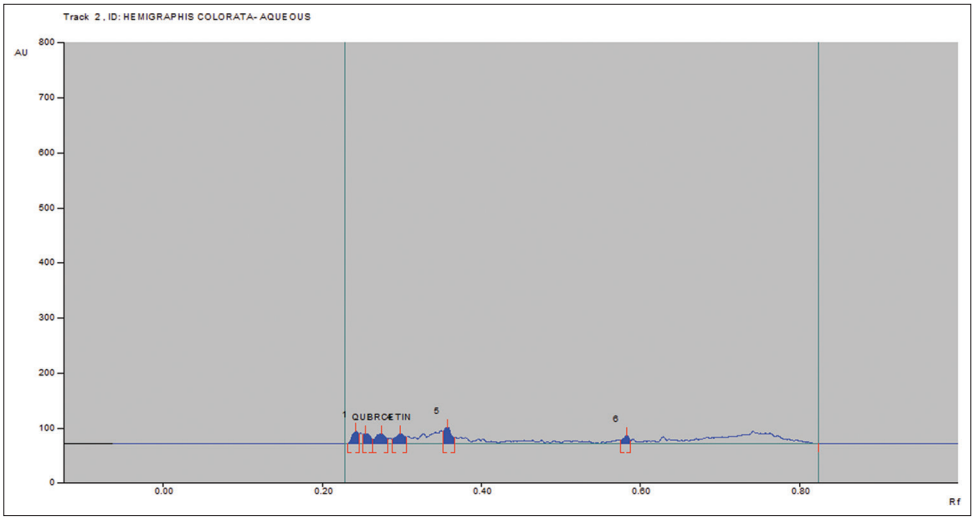

Figure 7: Quercetin peak of aqueous extract of *Hemigraphis colorata*

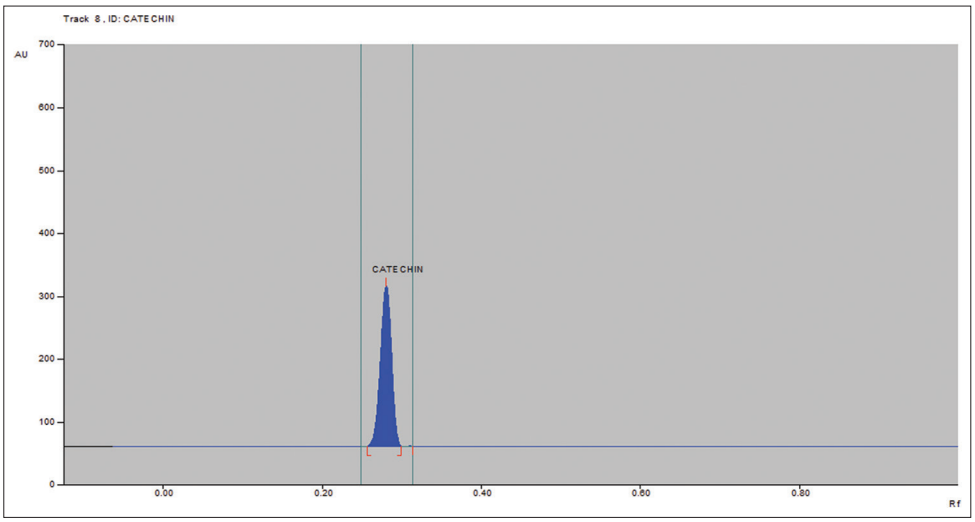

Figure 8: Standard peak of catechin

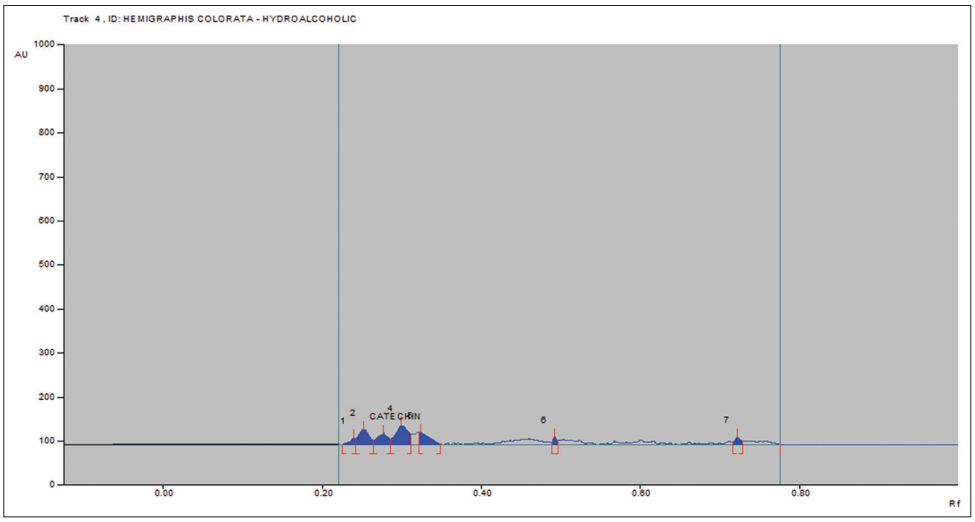

Figure 9: Catechin peak of hydroalcoholic extract of *Hemigraphis colorata*

CONCLUSION

The antimycotic activity was due to the secondary metabolites such as quercetin, caffeic acid, catechin, and gallic acid present in the leaves of *H. colorata* which was confirmed by the HPTLC technique. Further studies are needed to determine the mechanism of action, chemical identity, and structural activity elucidation of the observed antifungal activity. The natural plant-derived secondary metabolite fungicides may be a source of new alternative active compounds, in particular with antifungal activity.

REFERENCES

1. Gurgel LA, Sidrim JJ, Martins DT, Filho VC, Rao VS. *In vitro* antifungal activity of dragon's blood from *Croton urucurana* against dermatophytes. J Ethnopharmacol 2005;97:409-12.
2. Solórzano-Santos F, Miranda-Novales MG. Essential oils from aromatic herbs as antimicrobial agents. Curr Opin Biotechnol 2012;23:136-41.
3. Bobbarala V, Katikala PK, Chandrasekhar Naidu K, Penumajji S. Antifungal activity of selected plant extracts against phytopathogenic fungi *Aspergillus niger* F2723. Indian J Sci Technol 2009;2:87-90.
4. Wang X, Zhao X, Gu L, Zhang Y, Bi K, Chen X. Discrimination of aqueous and vinegary extracts of Shixiao San using metabolomics coupled with multivariate data analysis and evaluation of anti-hyperlipidemic effect. Asian J Pharm Sci 2014;9:17-26.
5. Anitha VT, Antonisamy JM, Jeeva S. Anti-bacterial studies on *Hemigraphis colorata* (Blume) H.G. Hallier and *Elephantopus scaber* L. Asian Pac J Trop Med 2012;5:52-7.
6. Ruddaraju LK, Pammi SV, Guntuku GS, Padavala VS, Kolapalli VR. A review on anti-bacterials to combat resistance: From ancient era of plants and metals to present

and future perspectives of green nano technological combinations. Asian J Pharm Sci 2020;15:42-59.
7. Cannas S, Usai D, Tardugno R, Benvenuti S, Pellati F, Zanetti S, *et al*. Chemical composition, cytotoxicity, antimicrobial and antifungal activity of several essential oils. Nat Prod Res 2016;30:332-9.
8. Kumar R, Patil MB, Patil SR, Paschapur MS. Evaluation of *Abelmoschus esculentus* mucilage as suspending agent in paracetamol suspension. Int J PharmTech Res 2009;1:658-65.
9. Gul R, Jan SU, Faridullah S, Sherani S, Jahan N. Preliminary phytochemical screening, quantitative analysis of alkaloids, and antioxidant activity of crude plant extracts from *Ephedra intermedia* indigenous to balochistan. Sci World J 2017;2017:5873648.
10. Angone SA, Mewono L, Mounanga MB, Medzegue S, Mendene HF, Mba Ndong JG, *et al*. Phytochemical screening and cytotoxicity studies of *Chrysophyllum pruniforme* Pierre ex Engl. barks. Pharmacogn Res 2013;5:195-9.
11. Petschenka G, Fei CS, Araya JJ, Schröder S, Timmermann BN, Agrawal AA. Relative selectivity of plant cardenolides for Na+/K+-ATPases from the monarch butterfly and non-resistant insects. Front Plant Sci 2018;9:1-13.
12. Auwal MS, Saka S, Mairiga IA, Sanda KA, Shuaibu A, Ibrahim A. Preliminary phytochemical and elemental analysis of aqueous and fractionated pod extracts of *Acacia nilotica* (Thorn mimosa). Vet Res Forum 2014;5:95-100.
13. Hossain MA, AL-Raqmi KA, AL-Mijizy ZH, Weli AM, Al-Riyami Q. Study of total phenol, flavonoids contents and phytochemical screening of various leaves crude extracts of locally grown *Thymus vulgaris*. Asian Pac J Trop Biomed 2013;3:705-10.
14. Nile SH, Park SW. HPTLC analysis, antioxidant and antigout activity of indian plants. Iran J Pharm Res 2014;13:531-9.
15. Bhalodia NR, Nariya PB, Shukla VJ. Antibacterial and antifungal activity from flower extracts of *Cassia fistula* L.: An ethnomedicinal plant. Int J PharmTech Res 2011;3:160-8.

Source of Support: Nil. **Conflicts of Interest:** None declared.