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IN VITRO STUDY OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF THE 2-(3,4-DIHYDROXYPHENYL)-3,5,7-TRIHYDROXY-4H-CHROMEN-4-ONE ISOLATED FROM THE METHANOLIC EXTRACT OF *ANDROGRAPHIS ECHIOIDES* LEAVES

S. Gurupriya*[1], L. Cathrine[2], J. Ramesh[3] and P. Pratheema[4]

[1,3]Research Scholar, P.G. and Research Department of Chemistry, Holy Cross College, Tiruchirappalli 620002, TamilNadu, India.

[2]Research Supervisor, P.G. and Research Department of Chemistry, Holy Cross College, Tiruchirappalli 620002, TamilNadu, India.

[4]Assistant Professor, P.G. and Research Department of Chemistry, Holy Cross College, Tiruchirappalli 620002, TamilNadu, India.

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***Corresponding Author**
S. Gurupriya
Research Scholar,P.G. and Research Department of Chemistry, Holy Cross College, Tiruchirappalli 620002, TamilNadu, India.

ABSTRACT

Objective: The present study was undertaken to determine antimicrobial and antioxidant activities of the 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one isolated from the methanolic extract of leaves of *Andrographis echioides*. **Materials and methods:** Antimicrobial activity was tested against *Escherichia coli* (MTCC 25922), *Enterococcus aerogenes* (MTCC 29212). *Pseudomonas aeruginosa* (MTCC 27853), *Staphylococcus aureus* (MTCC 25923) and *Proteus vulgaris* (MTCC 7299) by disc diffusion assay method. Antioxidant activity was determined by DPPH free radical scavenging assay. The isolation was done using column chromatography using gradient elution with different mobile phase. Structural elucidation was carried out on basis of spectral analysis. **Results:** The 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one exhibited significant antioxidant inhibitory activities with an IC50 value 43.81 and 47.4% respectively and well compared with standard ascorbic acid drug. As the concentration of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one increased from 20-100 µg/ml, the inhibitory actions of the

isolated compound increased towards all the strains used in this study. At concentration 100 µg/ml, the 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one exhibited the antimicrobial activity all the five bacteria and five fungal pahogens, but was more susceptible against *Staphylococcus aureus* (11 mm), *Proteus vulgaris* (10 mm), *Candida albicans* (10 mm zone of inhibition) at 100 µg/ml, followed by the highest activity against *Aspergillus flavus* and *Candida tropicalis* (9 mm zone of inhibition). **Conclusion:** The result confirms that the isolated compounds 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one from the methanolic extract of leaves of *Andrographis echioides* exhibited antibacterial and antifungal activity against the tested strains.

KEYWORDS: *Andrographis echioides*, leaves, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one, antioxidant, antimicrobial.

INTRODUCTION

Medicinal plants are the backbone of traditional medicine and the antibacterial activity of plant extract is due to different chemical agent in the extract, which was classified as active antimicrobial compound.[1-4] In recent years, pharmaceutical companies have been doing phytochemical research and investing billions of dollars in developing natural remedies to produce drugs in affordable price to general population.[5] The rising incidence in multidrug resistance amongst pathogenic microbes has further necessitated the need to search for fewer antibiotic sources from plants.[6]

Andrographis echioides belongs to *Acanthaceae* family and contains plenty of phytochemical constituents such as flavonoids, flavones, steroids, tannins, carbohydrate, glycosides and alkaloids.[7-8] Genus of *Andrographis* family plants are used to cure various diseases like goiter, liver diseases, fertility problems, bacterial, malarial and fungal disorder.[9] From the leaves extract of *Andrographis echioides* various chemical constituents were isolated dihydro echioidinin, skullcap avone 1 2'-methyl ether, echioidinin, echioidin, skullcap avone 1 and 2'-O-bD-glucopyranoside.[10,11] Some of the other chemical constituents present in the *A. echioides* are more than 17 compounds such as borneol, cyclohexanol 2,4 dimethyl phenol, 3,4 altroson, ndeconoicacid, Squalene, vitamin E, Methoprene, 2-nonenlol Oxirane,octyl-, 2, 2-cyclopentene-1-undecanoic acid, ketone, 1,5-methylbicyclo [2.1.0] pent-5-ylmethyl and 2,5-cyclohexadiene-1,4- dione, 2, 5- dihydroxy-3-methyl -6- (1-methylethyl) bicycle heptan -3- one.[12] Lupeol isolated from the methanolic extract of leaves of *Andrographis echioides* was reported.[13] However, no studies have been done to assess the antimicrobial activities of

this isolated compound from the *Andrographis echioides* (leaves). Therefore, in the present study, the antimicrobial and antioxidant activities of isolated 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one from the methanolic extract of leaves of *Andrographis echioides* were evaluated employing *in vitro* assay methods.

MATERIALS AND METHODS

Collection of plant material

The leaves of *Andrographis echioides* were collected in the month of May from the mullipatti, pudukkottai, Tamil Nadu, India. The plant was identified and leaves of *Andrographis echioides* were authenticated and confirmed from Dr. S. John Britto, Director, Rapinat herbarium, St. Joseph College, Tiruchirapalli, and Tamil Nadu for identifying the plants. The voucher specimen number SGP001 (7.06.2017).

Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid and acarbose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soluble starch, sodium potassium tartarate, sodium dihydrogen phosphate (NaH2PO4), Di-sodium hydrogen phosphate (Na2HPO4) sodium chloride, sodium hydroxide, potassium ferricyanide, ferric chloride (FeCl3) were from Merck Chemical Supplies (Damstadt, Germany). All the chemicals used including the solvents, were of analytical grade.

Collection of test organisms

To examine the antimicrobial activity of isolated compound, five strains [*Escherichia coli* (MTCC 25922), *Enterococcus aerogenes* (MTCC 29212). *Pseudomonas aeruginosa* (MTCC 27853), *Staphylococcus aureus* (MTCC 25923) and *Proteus vulgaris* (MTCC 7299)] were prepared as test organisms. The clinical fungal test organisms used for study are *Candida albicans* (MTCC 282), *Candida tropicalis* (MTCC No.184) *Aspergillus niger*, (MTCC 227), *Aspergillus clavatus* (MTCC 1323) and *Aspergillus flavus* (*MTCC*-3396). All the strains were procured from the Microbial Type Culture and Collection (MTCC) at Chandigarh, India.

Preparation of methanol extracts

The leaves of *Andrographis echioides* were washed in running water, cut into small pieces and then shade dried for a week at 35-40ºC, after which it was grinded to a uniform powder of 40 mesh size. The methanol extracts were prepared by soaking 1.5 kg each of the dried powder plant materials in 1.5 L of methanol using a soxhlet extractor continuously for 10 hr.

The extracts were filtered through whatmann filter paper No. 42 (125mm) to remove all unextractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The entire extracts were concentrated to dryness using a rotary evaporator under reduced pressure. The final dried samples were stored in labeled sterile bottles and kept at -20ºC. The filtrate obtained was used as sample solution for the further isolation.[14]

Isolation of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one by column chromatography

The condensed methanol extract of leaves (786 g) of sample was subjected to column chromatography over TLC grade silica gel. Elution of the column first with n-hexane, increasing amount of ethyl acetate in n-hexane and finally with methanol yielded a number of fractions. The preparation of solvent systems used to obtain 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (283mg/786g) were ethyl acetate-methanol (70:30) from fraction 9. The compounds were detected on TLC plates by spraying with Libermann-Burchard reagent and heated at 100°C for 10 minutes.[15]

Purification of isolated compounds by High-performance liquid chromatography (HPLC)

The analytical HPLC system (Shimadzu) was equipped with a diode array detector, a 20µl loop, 200 x 4.6 mm C18 column, methanol (HPLC grade, 0.2mm filtered) used as a mobile phase. The isolated 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one compounds were separated using a mobile phase of methanol: water (75:25 v/v) at a flow rate of 1.0 ml/min, column temperature 30 °C. Injection volume was 40 µl and detection was carried out at 346 nm.[16]

Structural elucidation study of isolated compound

Different spectroscopic methods including ^{1}H NMR and ^{13}C NMR were used to elucidate the structure of isolated compounds. ^{1}H and ^{13}C NMR spectra were acquired on Bruker WP 200 SY and AM 200 SY instruments (^{1}H, 200.13 MHz; ^{13}C, 50.32 MHz) using TMS as internal standard and $CDCL_3$ as solvent.[14-16]

Determination of Antioxidant activity (DPPH free radical scavenging activity)

The antioxidant activity of the isolated compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one was examined on the basis of the scavenging effect on the stable DPPH

free radical activity.[17] Ethanolic solution of DPPH (0.05 mM) (300 µl) was added to 40 µl of isolated compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one with different concentrations (20 - 100 µg/ml). DPPH solution was freshly prepared and kept in the dark at 4°C. Ethanol 96% (2.7 ml) was added and the mixture was shaken vigorously. The mixture was left to stand for 5 min and absorbance was measured spectrophotometrically at 517 nm. Ethanol was used to set the absorbance zero. A blank sample containing the same amount of ethanol and DPPH was also prepared. All determinations were performed in triplicate. The radical scavenging activities of the tested samples, expressed as percentage of inhibition were calculated according to the following equation.[18]

Percent (%) inhibition of DPPH activity = [(A – B) / A] x 100

Where B and A are the absorbance values of the test and of the blank sample, respectively. A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined and represented as IC50 value for each of the test solutions.

Determination of antibacterial activity of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one from leaves of *Andrographis echioides* (disc diffusion method)

Antibacterial activity of isolated compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one was determined using the disc diffusion method. The petridishes (diameter 60 mm) was prepared with Muller Hinton Agar and inoculated with test organisms. Sterile disc of six millimeter width were impregnated with 10 µl of isolated compound at various concentrations of 20-100 µg/ml respectively. Prepared discs were placed onto the top layer of the agar plates and left for 30 minute at room temperature for compound diffusion. Negative control was prepared using the respective solvent. The dishes were incubated for 24 h at 37ºC and the zone of inhibition was recorded in millimeters and the experiment was repeated twice.[19]

Determination of antifungal activity of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one from leaves of *Andrographis echioides*

Antifungal activity of isolated compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one was determined using the disc diffusion method The petridishes (diameter 60 mm) was prepared with Sabouraud's dextrose agar (SDA) and inoculated with test organisms. Sterile disc of six millimeter width were impregnated with 10 µl of isolated

compound at various concentrations of 20-100 µg/ml respectively. Prepared discs were placed onto the top layer of the agar plates and left for 30 minute at room temperature for compound diffusion. The dishes were incubated for 24 h at 37ºC and the zone of inhibition was recorded in millimetres.[20]

Statistical analysis

All assays were conducted in triplicate. Statistical analyses were performed with SPSS 16.0 for an analysis of variance (ANOVA) followed by Duncan's test. Differences at $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Structural Elucidation of isolated compounds

2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one is yellow compound with melting point 314-315°C, MW: 302.238 g/mol which correspond to the molecular formulae $C_{15}H_{10}O_7$. In the proton ^{1}H NMR spectra of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (fig 1) showed 9.61 (1H, s, OH-3), 12.50 (1H, s, OH-5), 6.19 (1H, d, *J* = 2.0 Hz, H-6), 10.79 (1H, s, OH-7), 6.40 (1H, d, *J* = 2.0 Hz, H-8), 7.67 (1H, d, *J* = 2.0 Hz, H-2'), 9.32 (1H, s, OH-3'), 9.39 (1H, s, OH-4'), 6.87 (1H, d, *J* = 8.5 Hz, H-5'), 7.53 (1H, dd, *J*=2.0, 8.0 Hz, H-6').

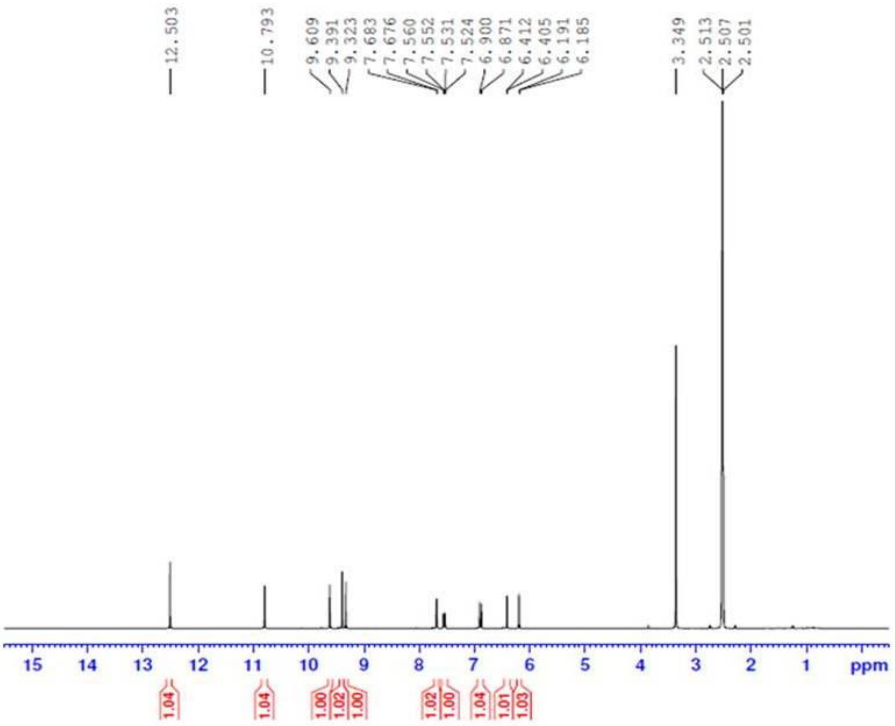

Fig. 1: ^{1}H- NMR spectra of the isolated compound.

In the proton ^{13}C NMR spectra of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (fig 2) showed 147.2 (C-2), 136.1 (C-3), 176.3 (C-4), 161.1 (C-5), 98.6 (C-6), 164.3 (C-7), 93.8 (C-8), 156.5 (C-9), 103.4 (C-10), 122.4 (C-1'), 115.5 (C-2'), 145.25 (C-3'), 147.2 (C-4'), 115.5 (C-5'), 120.4 (C-6'). The structure was confirmed by comparison with literature data.[21-23]

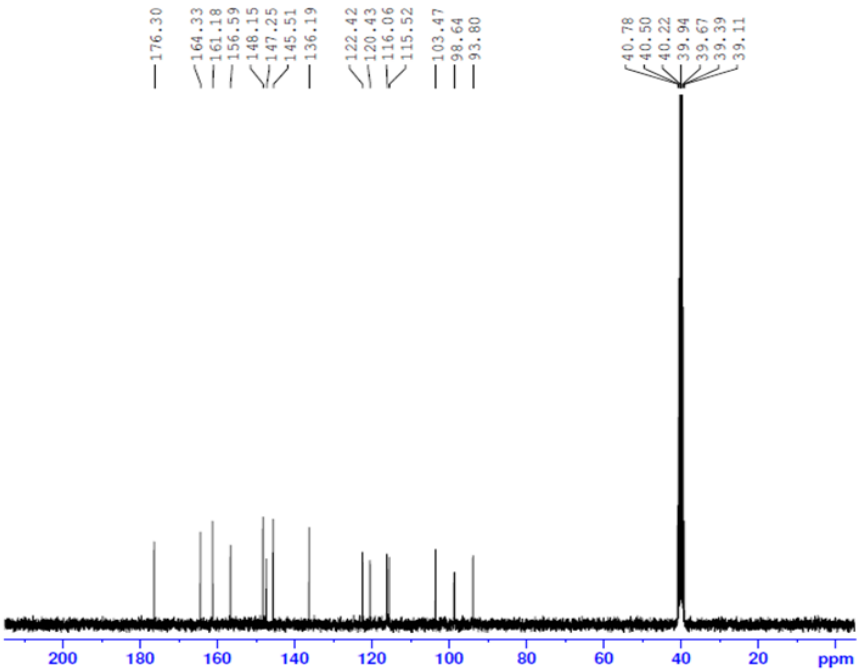

Fig. 2: ^{13}C -NMR spectra of the isolated compound.

The Retension time of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one isolated from the methanolic extract of sample was about 8.42 was shown by HPLC peak (fig 3).

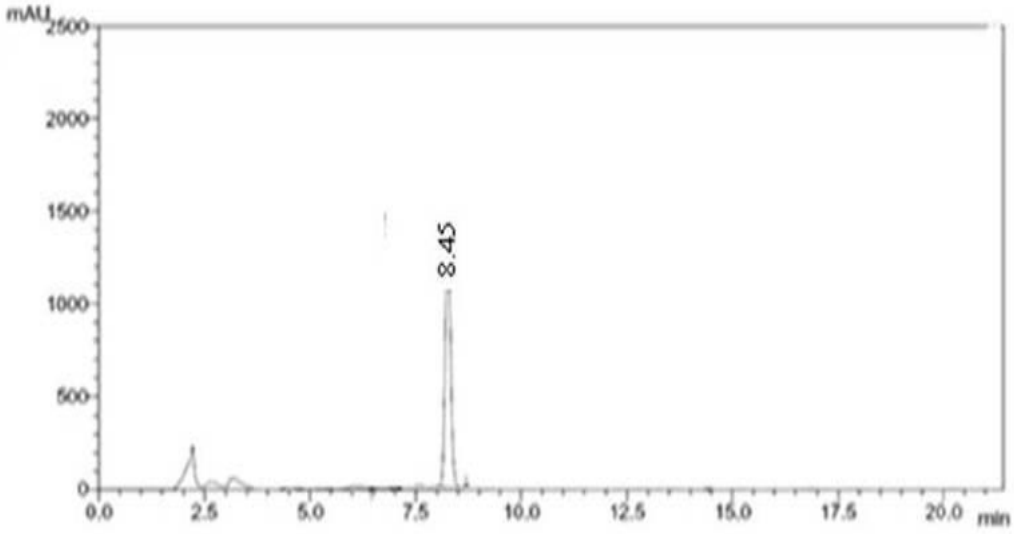

Fig. 3: HPLC spectra of purity of the isolated compound.

Antioxidant activity of isolated compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one by DPPH method

The result showed that the compound had better percentage antioxidant activities at high concentrations when compared with ascorbic acid (Table 1). The isolated compound showed 92.47 % activity at 100 µg/ml while ascorbic acid gave 95.79 % at the same concentration (fig. 4). The previous study suggested that the 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one has antioxidant properties by scavenging free radicals, decreasing lipid peroxidation and increasing the endogenous blood antioxidant enzymes levels.[24]

Table 1: Antioxidant activity of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one by DPPH method and comparison with standard drug ascorbic acid.

S. No.	Concentrations (µg/ml)	Scavenging Effect (%)	
		2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one	Ascorbic acid
1	20	30.37±1.49	30.50±1.33
2	40	58.63±1.26	64.35±1.37
3	60	78.52±1.42	74.73±1.42
4	80	86.46±1.44	85.24±1.47
5	100	92.47±1.28	95.79±1.50
	IC50	43.81	47.4

Note: Each value was obtained by calculating the average of three experiments and data are presented as mean± SEM.

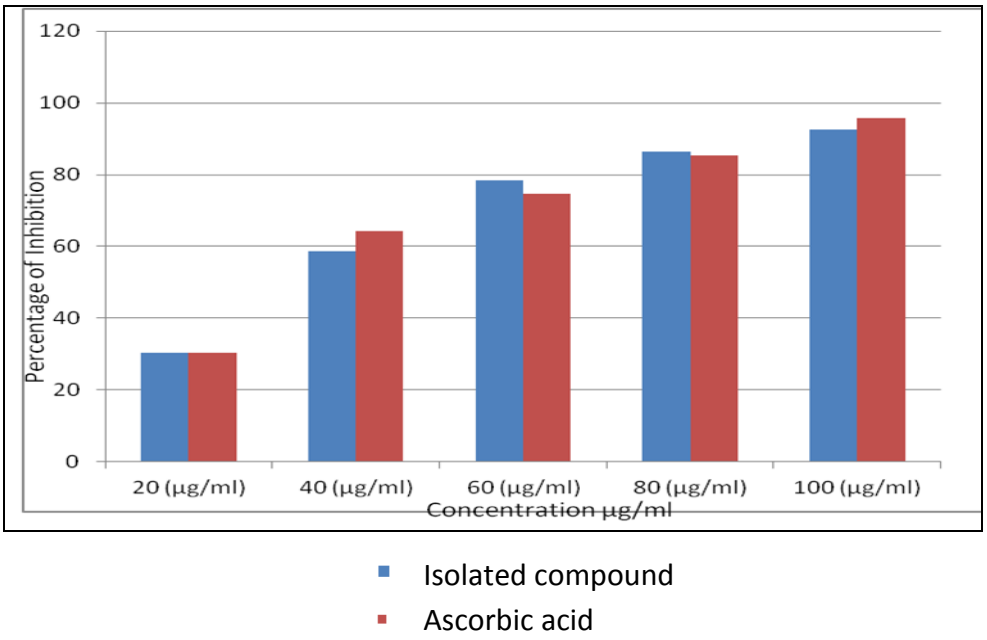

Fig. 4: Anti-oxidant activity of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one by DPPH activity.

Antibacterial activity of isolated compounds 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one by disc diffusion assay method

The results of the antibacterial activity of isolated compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy- 4H -chromen-4-one were tested against pathogens by disk diffusion method are shown in (Table 2). The isolated compound showed growth inhibitory activity against *Staphylococcus aureus* (11 mm), *Proteus vulgaris* (10 mm) at concentration 100µg/ml. At concentration 80 µg/ml, the compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one exhibited the antibacterial activity all the five bacteria, but was more susceptible against *Staphylococcus aureus* (9mm), *Escherichia coli* and *Enterococcus aerogenes* (7 mm) *Proteus vulgaris* (9 mm). However, the 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one showed better inhibitory actions against pathogens at a concentration 60, 80 and 100 µg/ml than at lower concentration (fig 5). As the concentration of isolated compound increased from 20-100 µg/ml, the inhibitory actions of the isolated compound increased towards all the strains used in this study. Previous study suggested that the 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one inhibited *S. aureus, P. aeruginosa* at concentration 20 mcg/mL while *P. vulgaris* and *E. coli* were inhibited at concentration 300 mcg/mL and 400 mcg/mL respectively.[25]

Table 2: Antibacterial activity of isolated compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one from leaves of *Andrographis echioides.*

	Concentrations (µg/ml)	**Organisms/Zone of inhibition (mm)**				
		2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one isolated from *Andrographis echioides* (leaves)				
		Escherichia coli	***Staphylocus aureus***	***Enterococcus aerogenes***	***Pseudomonas aeruginosa***	***Proteus vulgaris***
Isolated compound	20	0	0	0	0	7
	40	0	0	0	0	7
	60	0	8	0	0	8
	80	7	9	7	0	9
	100	9	10	8	8	10
Control (Methanol)	10 µl/disc	0	0	0	0	0

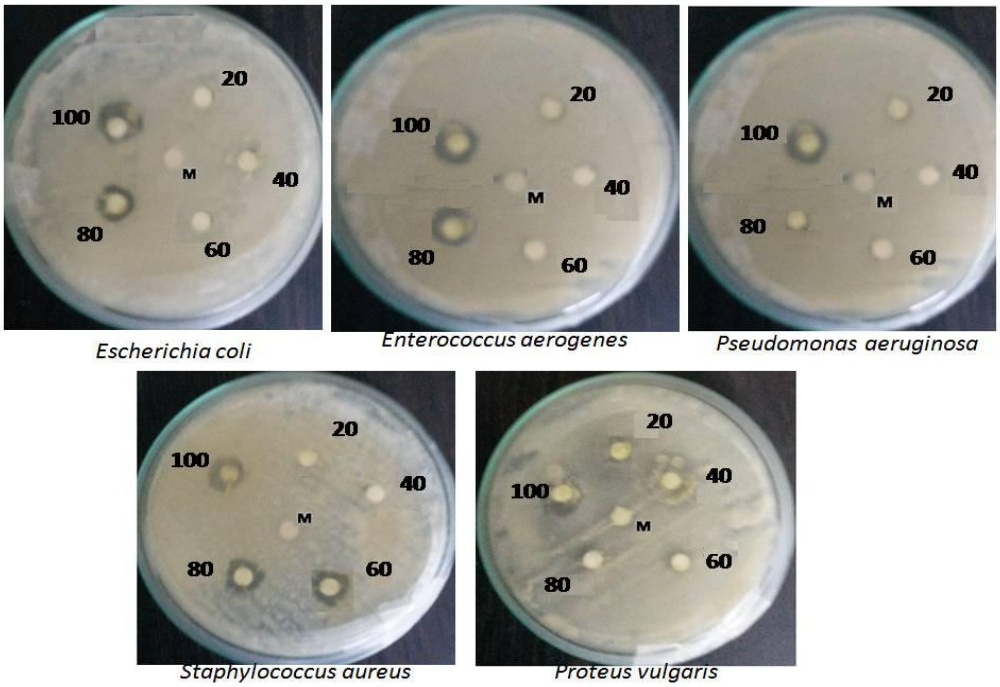

Fig. 5: Antibacterial activity of isolated compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one from leaves of *Andrographis echioides.*

The antifungal susceptibility test of the different concentration of isolated compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one and against the test organisms (Table 3). From the result, the 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one isolated from *Andrographis echioides* were the most effective and the highest activity was demonstrated against *Candida albicans* (10 mm zone of inhibition) at 100 µg/ml, followed by the highest activity against *Aspergillus flavus* and *Candida tropicalis* (9 mm zone of inhibition) at 100 µg/ml and against *Aspergillus niger* (8 mm zone of inhibition) at 100 µg/ml (fig 6). At concentration 80 µg/ml, the 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one exhibited the antifungal activity all the five bacteria, but was more susceptible against *Candida albicans* (9 mm). However, the 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one showed better inhibitory actions against pathogens at a concentration 60, 80 and 100 µg/ml than at lower concentration. As the concentration of isolated compound increased from 20-100 µg/ml, the inhibitory actions of the 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one increased towards all the strains used in this study.

Table 3: Antifungal activity of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one isolated from the leaves of *Andrographis echioides*.

	Concentrations (µg/ml)	Organisms/Zone of inhibition (mm)				
		2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one isolated from *Andrographis echioides* (leaves)				
		Candida albicans	*Candida vulgaris*	*Aspergillus flavus*	*Aspergillus niger*	*Candida tropicalis*
Isolated compound	20	0	0	0	0	0
	40	0	0	6	0	0
	60	0	0	7	0	0
	80	9	6	8	0	8
	100	10	7	9	8	9
Control (Methanol)	10 µl/disc	0	0	0	0	0

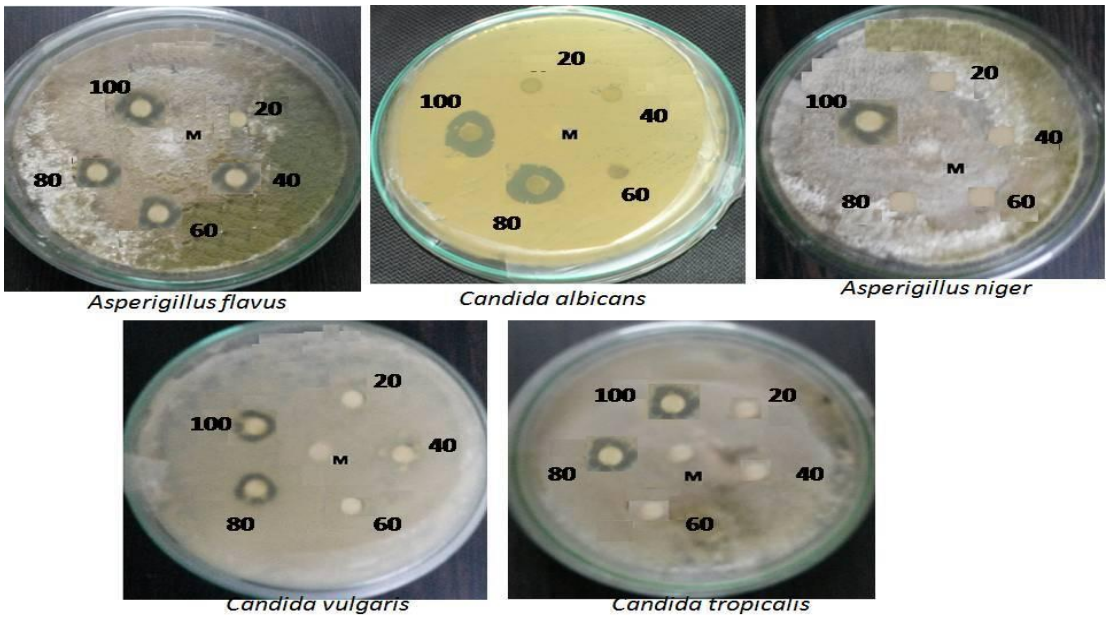

Fig. 6: Antifungal activity of isolated compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one from leaves of *Andrographis echioides.*

CONCLUSION

These results suggest that the 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one isolated from the methanolic extract of leaves of *Andrographis echioides* have good antibacterial and antifungal activity against selected pathogens. The isolated constituent of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one was identified through ^{1}H and ^{13}C NMR spectroscopy. The isolated compound 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one showed increased antioxidant activity with an increase in the treated concentrations. *Andrographis echioides* can be used as potential source for the development of antimicrobial and antioxidant agents.

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AUTHOR CONTRIBUTION

All authors contribute equally to this manuscript.

CONFLICTS OF INTERESTS

The authors declare that they have no conflict of interest. It has not been published elsewhere. That it has not been simultaneously submitted for publication elsewhere. All authors agree to the submission to the journal.

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