In vitro antioxidant and antimicrobial activity of isolated flavonoid from the methanolic extract of *Andrographis echioides* leaves

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Abstract

The aim of this study was to isolate the active flavonoid component found in the entire Andrographis echioides plant. The isolation of flavonoids from a plant component was investigated using a methanolic extract from the leaves of A.echioides. The presence of flavonoid components in methanol extract was identified by phytochemical studies. TLC and column chromatography were used to purify the flavonoid component that had been isolated. UV-Visible, FT-IR, C^{13}, H^1NMR and Mass spectroscopy were used to characterize the structures of the isolated compounds.

As a result, the isolated molecule was named 9-methoxy-6a, 11a-dihydro-6H-benzofuro [3, 2-C chromen-3-ol] i.e. medicarpin. For the isolated flavonoid found in this plant, additional biological research is required. Flavonoids are a unique group of secondary metabolites found in plants that have been utilized as traditional remedies with scientifically confirmed pharmacological advantages. They support a wide range of medical actions that could lead to unique and potentially therapeutic findings in drug development. Recent study has focused on flavonoids functional activity as antioxidants in the presence of oxidative stress.

Keywords: *Andrographis echioides*, separation of flavonoids, medicarpin, antioxidant activity.

Introduction

Andrographis echioides is a plant in the Acanthacae family used for a variety of medical purposes in South Asia, particularly India and China. According to the literature, this plant has antimicrobial activity, anti-inflammatory activity, diuretic, anthelmintic, analgesic, antipyretic, hepatoprotective activities and antioxidant effect. It is high in phytochemicals like flavonoids, flavones, steroids, tannins, carbohydrate, glycosides and alkaloids[1,18]. *Andrographis echioides* leaf juice is used to treat fevers. The Andrographis genus of plants is used to treat a variety of diseases including goiter, liver disease and fertility issues, bacterial, malarial and fungal infections[26].

Boiling *Andrographis echioides* with coconut oil is used to reduce hair fall and graying[12]. The primary goal of this research was to isolate active components, primarily flavonoids, from the entire *Andrographis echioides* plant and characterize them using spectroscopy techniques such as UV-Visible, FT-IR, C^{13} and H^1 NMR and mass Spectroscopy. Antioxidants are substances that can neutralize free radicals by accepting or donating electrons, thereby suspending the oxidation reaction chain[2].

According to numerous studies[19,23,24], natural antioxidants not only protect against reactive oxygen species (ROS), but they can also cause lipid per oxidation. A chemical that inhibits the oxidation process is referred to as an antioxidant. Natural antioxidants can protect the human body from the harmful effects of free radicals. Antioxidants found in foods and medications help to slow the progression of many chronic diseases and lower lipid oxidation activity. Secondary metabolites from plants such as phenolics and flavonoids have been shown to be powerful free radical scavengers. They can be found in plants' leaves, fruits, seeds, roots and bark[13].

Traditional plant-based medicine has been shown to be clinically beneficial and less harmful than currently available pharmaceuticals[3]. The extraction technique's solvent has a significant impact on the success of determining physiologically active chemicals from plant material[7]. Proteins, lipids, DNA, carbohydrates and other biological substances can be harmed by free radicals. Free radicals can also cause cellular damage and disrupt immune function[16]. A number of studies have discovered that the biological effects of these chemicals are linked to their antioxidant capacity[8]. Diabetes complications, as well as other neurological disorders such as Parkinson's disease, are caused by reactive oxygen species[15]. It has been discovered that phenolic molecules are more active than other natural antioxidants[25]. The antioxidant and antimicrobial activities of *Andrographis echioides* leaves were investigated in this study.

Material and Methods

Collection of Plant material: *Andrographis echioides* was collected in its entirety from Archampatty, Karur District, Tamil Nadu, India. The plant was identified and the leaves of *Andrographis echioides* were authenticated and confirmed by Dr. Arulanandam, Director, Rapinat Herbarium, St. Joseph College, Tiruchirappalli, Tamil Nadu. SDD003 is the voucher specimen number. The entire *Andrographis echioides* plant was dried in the shade, separated, pulverized with a mechanical grinder and passed through a 40 mesh sieve.

Extraction Method: The powdered plant materials were extracted for 48 hours with hexane (35-650C) in a Soxhlet apparatus using a hot continuous percolation process. The extract was filtered and concentrated using a rotary evaporator. The residual marc was then immersed in chloroform for 48 hours before being extracted with hexane. In the same way, ethyl acetate and methanol extracts were collected and concentrated. All the extracts were freeze-dried in a lyophilizer until they were dry powder and then stored in screw cap vials at 4°C until needed.

Separation of flavonoid from the methanolic extract of *Andrographis echioides:* Because the flavonoid test in the methanol extract yielded a good result, the extract was carried further for separation. On preparative TLC (Preparative Thin Layer Chromatography), a few spots of methanol extract were detected. Methanol, glacial acetic acid and water (90:5:5) make up the system's mobile phase, whereas benzene, acetic acid and water make up the stationary phase (60:30:10). The spot R_f value was recorded and TLC was viewed in a UV-Chamber for spot detection.

Isolation of flavonoid by using the Column Chromatography: To achieve consistent mixing, 20 gms methanolic extract of *Andrographis echioides* leaves was mixed with 20 gms silica gel (60/120 meshes). 200 gms silica gel (70/325 meshes) was placed into an appropriate column and packed very carefully without air bubbles; slurry preparation was done using petroleum ether. For one hour, the column was set aside to allow for careful packing. The admixture was then added to the top of the stationary phase and the compounds were separated by eluting with the various solvent mixes listed in the TLC. Under reduced pressure, all of the column fractions were collected separately and concentrated. Structure analysis was performed on the concentrated fraction.

Chemical identification of flavonoids: For the purpose of confirming the presence of flavonoid fraction, the following assays were carried out[6,9,17].

Shinoda Test: Magnesium ribbon was added to a small amount of test solution in alcohol followed by drops of strong hydrochloric acid; the creation of pink colour shows the presence of flavonoids.

Zn- Hydrochloride Reduction Test: Add a mixture of zinc dust and concentrated hydrochloric acid to the solution. Heat the solution until the color changes to red after a few minutes.

Aluminum Chloride Test: Two drops of 1 percent aluminum chloride were added to a small amount of test solution and the yellow coloration indicates the presence of flavonoid.

Characterization of Flavonoid: A phytochemical screening test identified the separated compound which was then subjected to spectral studies for characterization. The UV-visible absorption spectrum of CD-1 was recorded and methanol was used as a control solvent. FT-IR was used to identify functional groups and it was done with a small amount of CD-1 mixed with spectroscopic grade KBr and then well ground before preparing the pellet. BRUKER-AMX 400MHz spectrophotometer was used to analyze proton NMR (H^1-NMR) and carbon-13 NMR (C^{13}-NMR). The solvent and internal standards were DMSO and TMS respectively. Mass spectra analysis of the extract was performed using a JEOL GC MATE-11 HR Mass spectrometer[11,20,21].

Antioxidant activity (DPPH free radical scavenging activity) determination: The antioxidant activity of the isolated compound medicarpin was investigated using the DPPH free radical scavenging effect. A 300µl ethanolic solution of DPPH (0.05 mM) was added to 40µl of isolated compound medicarpin at various concentrations (20 - 100 µg/ml). The DPPH solution was freshly prepared and stored at 4°C in the dark. Ethanol 96 percent (2.7 ml) was added and the mixture was vigorously shaken. After 5 minutes of standing, the absorbance was measured spectrophotometrically at 517 nm. The absorbance was set to zero using ethanol. A control sample with the same concentrations of ethanol and DPPH was also prepared. All measurements were made in triplicate[22]. The radical scavenging activities of the tested samples were calculated as a percentage of inhibition using the following equation:

$$\text{DPPH activity inhibition percentage (\%)} = [(A - B) / A] \times 100 \quad (1)$$

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

Antimicrobial assay: Using samples, an antibiogram was performed using the disc diffusion method[4,14]. 30 ml of NA/PDA medium was poured into Petri plates. The test organism was inoculated on a solidified agar plate using a micropipette and spread for 10 mints before drying. Bacteria from a broth culture were inoculated on the surfaces of the media. A sterile cotton swab is dipped into a standardized microbes test suspension and used to evenly inoculate the nutrient agar /PDA plates' entire surface. In brief, microbial strain inoculums were spread on nutrient agar/PDA plates.

The sterile filter papers (6 mm diameter) containing 50µl, 100µl, 150µl of samples, standard solution as chloramphenicol and fluconazole 30µl and control 30µl were laid down on the surface of the inoculated agar plate using sterile forceps. The plates were incubated at 37^0C for 24 hours for bacteria and 48 hours for yeast strains. Each sample was tested three times.

Results and Discussion

Separation of Flavonoids: TLC results show that yellow bands with Rf values of 0.42 and 0.31 were obtained in solvent systems of methanol: glacial acetic acid: water (90:5:5) and benzene: acetic acid: water (60:30:10). The flavonoid was isolated from the crude methanol extract using column chromatography. Figures 1 and 2 show images of TLC and column chromatography. The compound was characterized and structurally elucidated based on phytochemical and spectroscopic studies.

Characterization of Flavonoid: The isolated compound was a pale yellow or light yellow powder. The isolated compound had a melting point of 127.5-128.5^{0}C. Figure 3 depicts the UV spectrum of this compound which showed major absorption peak in the region 282.35nm, indicating the presence of flavonoid nucleus structure.

The FT-IR spectra revealed OH stretching at 3426.21 cm^{-1}, CH_2 stretching at 2931.75 cm^{-1}, CH bonding at 2082.53 cm^{-1}, CO group at 1458 cm-1 and C-OH vibration at 1363.07 cm^{-1}. The flavonoids FT-IR results are shown in fig. 4.

1H NMR spectrum of isolated compound: In the proton 1H NMR spectra of isolated compound, fig. 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'). The H^1-NMR reveals that the isolated structure might be medicarpin i.e. 9-methoxy-6a, 11a-dihydro-6H-benzofuro [3, 2-c] chromen-3-ol.

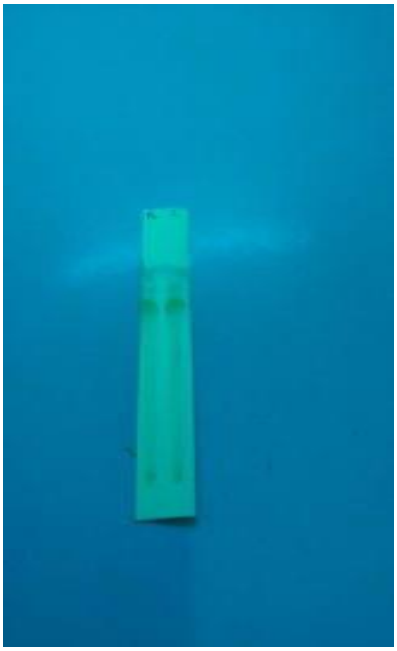


Fig. 1: TLC

Fig. 2: column chromatography

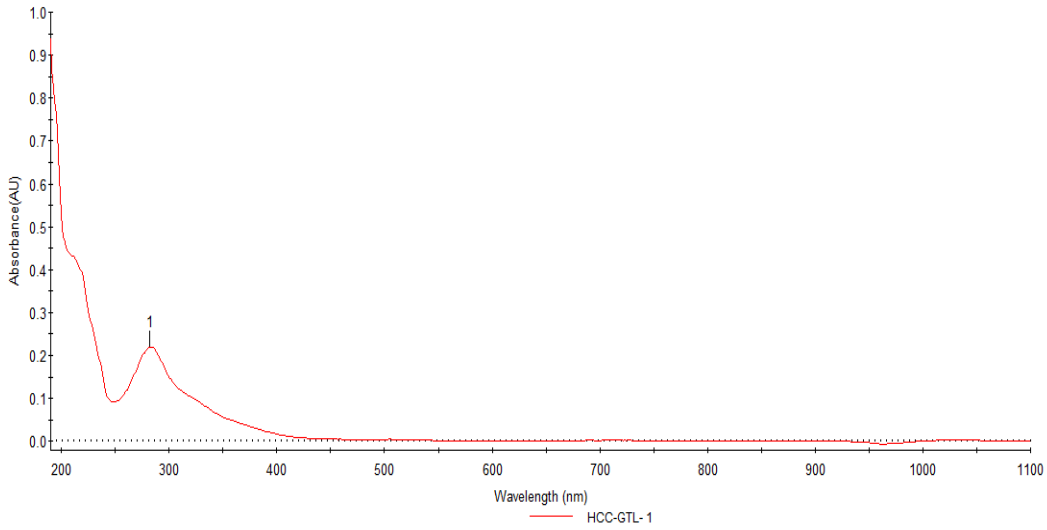


Fig. 3: UV - Visible spectrum of Flavonoid

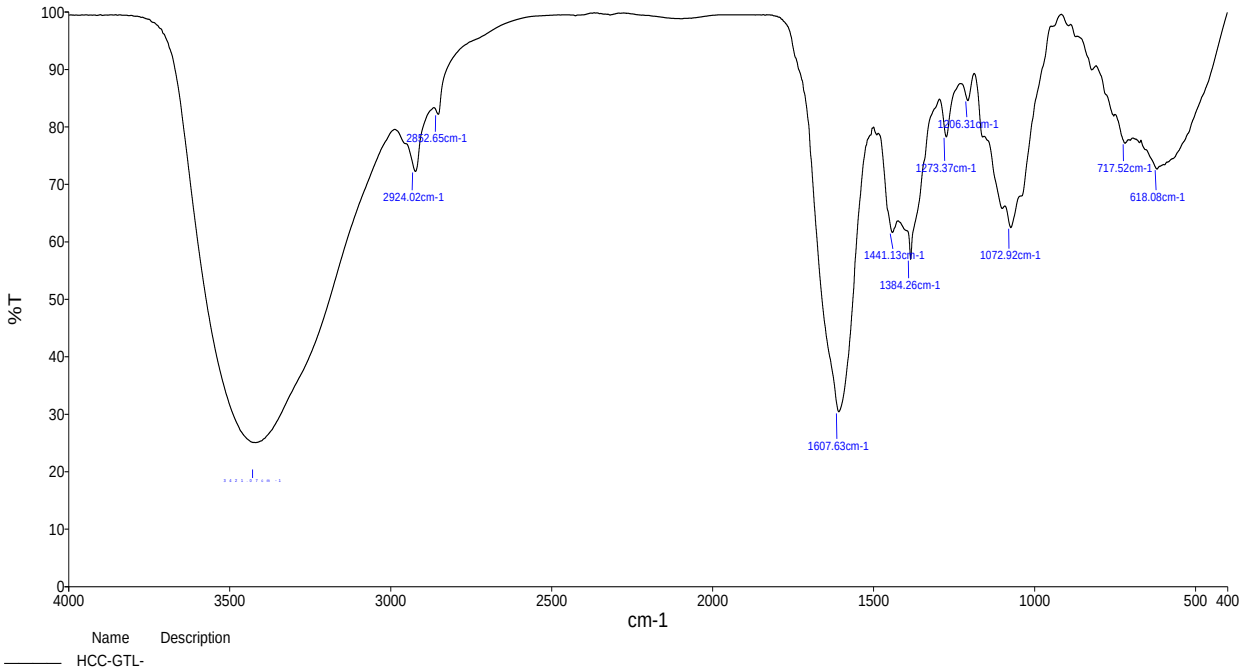


Fig. 4: FT-IR spectrum of flavonoid

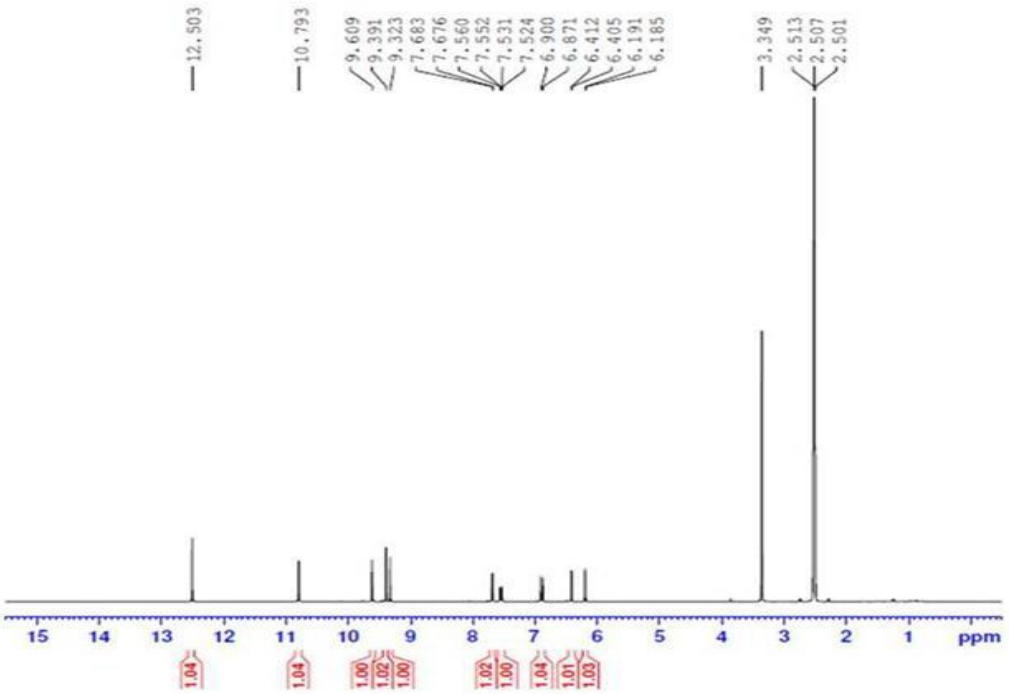


Fig. 5: H^1 - NMR spectrum of Flavonoid

^{13}C-NMR reports and interpretations are: ^{13}C NMR (DMSO) 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 peaks present in the NMR spectrum showed resemblance with the medicarpin which was also confirmed by previous reports[5,10]. Thus, it can be confirmed that the isolated compound is found to be medicarpin.

Mass spectrum of isolated compound from extract: The mass spectrum of isolated compound showed a base peak $[M]^+$ for compound at m/z 270.90 indicating the compounds as medicarpin. The molecular formula was inferred from $C_{16}H_{14}O_4$.

Isolated compound: Based on the chemical and spectral data, evidences have been characterized as isolated compound was Medicarpin (3-Hydroxy-9-methoxypterocarpan) (MF: $C_{16}H_{14}O_4$) (Figure 6).

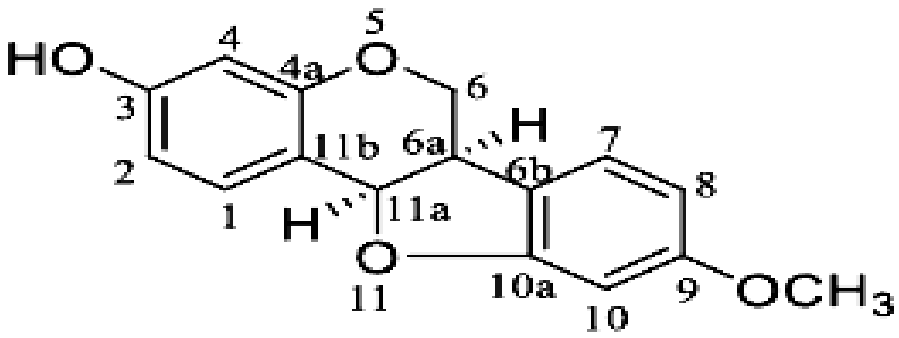


Fig. 6: Structure of Medicarpin

Table 1
***In vitro* DPPH radical scavenging activity of Medicarpin and standard ascorbic acid**

Concentrations (µg/ml)	% of inhibitions	
	Medicarpin	Std. (Ascorbic acid)
20	17.65±0.43	20.53±0.51
40	36.31±0.39	40.98±0.45
60	57.84±0.41	59.11±0.43
80	71.52±0.52	76.84±0.37
100	86.63±0.48	94.79±0.46
IC_{50} value (µg/ml)	55.38	50.83

Values expressed as Mean ± SD for triplicate. Data were statistically analyzed, and correlation coefficients were calculated. The R2 value was calculated as concentration vs. percent of inhibitions using MS-excel.

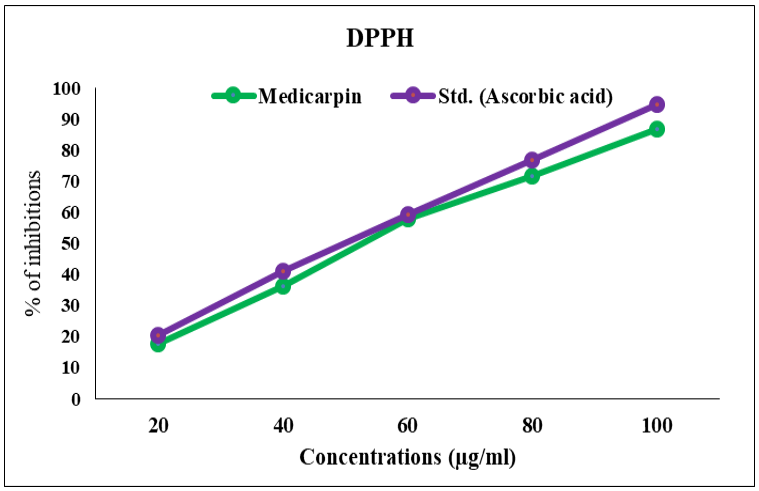
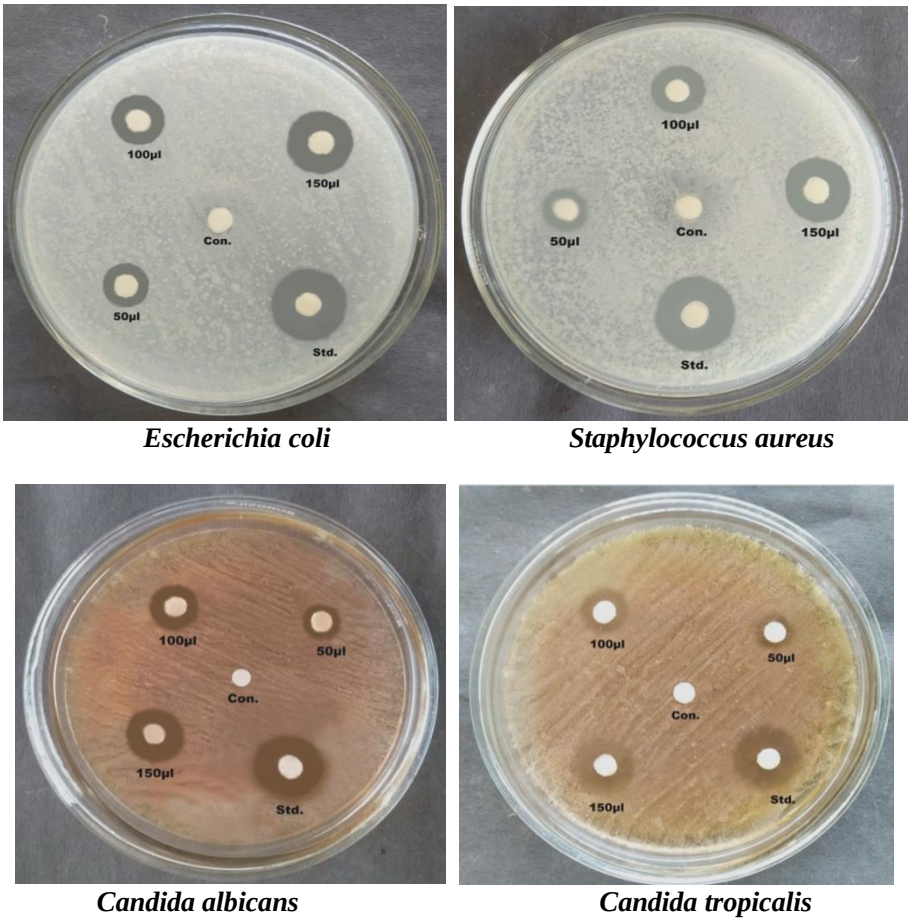


Fig. 7: *In vitro* DPPH radical scavenging activity of Medicarpin and standard ascorbic acid

Table 2
Anti-microbial activity of Medicarpin

Microbial strains	Zone of inhibition (mm) of Medicarpin				
	50µl	100 µl	150 µl	Std. (30 µl)	Con. (30 µl)
Bacterial strains					
Escherichia coli	3.20	5.35	8.80	12.25	0.05
Staphylococcus aureus	3.05	5.15	8.50	12.20	0.05
Fungal strains					
Candida albicans	2.20	4.50	6.85	10.30	0.00
Candida tropicalis	2.60	4.90	7.10	10.35	0.00

Values expressed as mm; Bacterial standard: Chloramphenicol; Fungal standard: Fluconazole; Control: Ethanol.



Escherichia coli ***Staphylococcus aureus***

Candida albicans ***Candida tropicalis***

Plate 1: Anti-microbial activity of Medicarpin

Antioxidant activity of isolated compound medicarpin by DPPH method

DPPH radical scavenging activity: DPPH, or 2, 2-Diphenyl-1-picrylhydrasyl, is a dark compound made up of stable free radical molecules. In the presence of antioxidants, the purple colour of DPPH decays. The change in absorbance at 517 nm in the presence of antioxidants can be equated with the compound's antioxidant potential.

The reduction of DPPH molecules by a hydrogen donor antioxidant in DPPH radical scavenging assays causes the colour of the solutions to change from purple to yellow. Ascorbic acid had an IC_{50} of 50.83g/ml and medicarpin had an IC_{50} of 55.38g/ml, demonstrating significant dose-dependent inhibition of DPPH assay activity. The lower is the IC_{50} value, the greater is the antioxidant activity.

Conclusion

This study concluded that methanol extracts mostly separate flavonoids from plant materials and that methanol solvent was a suitable solvent for flavonoids extraction. For the isolation of flavonoids compounds, chromatographic techniques such as TLC and column can be used. The study focuses on the presence of medicarpin in this plant. Medicarpin has antioxidant and antimicrobial properties and is used to treat a variety of diseases. Flavonoids are found in the human diet. The entire plant *Andrographis echioides* contains a high concentration of medicarpin and synthesis of medicarpin from *Andrographis echioides* may be economically advantageous and is easily accessible.

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