



**IN VITRO ANTI-MICROBIAL ACTIVITY OF
(1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl 1,2,3,4,5,6,7,7A,9,10,11,11B,12,13,13A,13B hexadecahydrocyclopenta[a]chrysen-9-ol
ISOLATED FROM METHANOLIC LEAF EXTRACT OF *ANDROGRAPHIS
ECHIOIDES***

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ABSTRACT

Objective:

The present study was designed to evaluate the *in vitro* antimicrobial activity of (1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol (Lupeol) isolated from methanolic leaf extract of *Andrographis echioides*.

Methods:

The anti microbial activity was determined by using the disc diffusion method against selected pathogens. The isolation was done using chromatography techniques. Isolated compound was subjected to spectral analysis. Structural elucidation was carried out by Interpretation of spectral data. The pure isolated compound from methanolic leaf extract of *Andrographis echioides* was confirmed to be (1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol.

Results:

The obtained results indicated that the isolated compound (1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol was more efficient against selected pathogens at higher concentration. The antimicrobial potential of methanolic leaf extract of isolated compound from *Andrographis echiooides* determined on the basis of mean diameter of zone of inhibition around the disc in millimeters. The isolated compound (1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol exhibited antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus aerogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and anti-fungal activity against *Candida albicans*, *Candida tropicalis*, *Aspergillus flavus*, *Aspergillus niger* and *Candida vulgaris*.

Conclusion:

The isolated compound (1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol showed good anti-bacterial and anti-fungal activity against selected pathogens.

Keywords: *Andrographis echiooides*, leaves, anti-bacterial, anti-fungal, Isolated compound

INTRODUCTION

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major intimidation to public health, in spitefulness of the magnificent progress in human medicine. Their impact is predominantly large in emergent countries due to the relative unavailability of medicines and the emergence of prevalent drug resistance. Medicinal plants have been a source of bioactive compounds to indulge many diseases. Traditionally used medicinal plants produce a variety of compounds by means of known therapeutic properties.

Plants are the potential source of antimicrobial agents in different countries [1]. About 60 to 90% of populations in the developing countries use plant-derived medicine. Traditionally, crude plant extracts are used as herbal medicine for the healing of human infectious diseases [1–3]. Plants are rich in a variety of phytochemicals including tannins, terpenoids, alkaloids, and flavonoids which have been found *in vitro* to have antimicrobial properties [4, 5]. Global prevalence of infectious diseases caused by bacteria is a major public health problem [3, 8]. The bacterial agents including

Staphylococcus aureus, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Proteus vulgaris* cause several human infections [9, 10]. Recent surfacing of antibiotic resistance and related toxicity issues limit the use of antimicrobial agents [11] and is prompting resurgence in research of the antimicrobial role of plants against resistant strains due to comparable safety and efficacy [1].

Andrographis echioides L. is an annual herb present in throughout South Indian. However, information on the chemical composition and bioactivity of this species is very rare [12, 13]. The plant from genus *Andrographis* is used in goiter, liver diseases, fertility problems, bacterial [14] malarial and fungal disorders [15]. Leaf juice boiled with coconut oil had controlled the falling and graying of hair [16]. From the leaves extract of *Andrographis echioides* various chemical constituents were Lupeol isolated from the methanolic leaf extract of *Andrographis echioides* was reported [17]. Therefore, in the present study, the antimicrobial activities of isolated

(1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol

(Lupeol) from the methanolic leaf extract 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 recognized 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).

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 35-40°C, after which it was grinded to a uniform powder of 40 mesh size. The methanol extracts were prepared by soaking 100 g each of the dried powder plant materials in 1 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 -20°C. The filtrate obtained was used as sample solution for the further isolation [18].

Isolation of Phytochemical constituent by column and Thin layer chromatography

The condensed methanol extract of leaves (986 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 (1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol (104 mg/786g) were n-hexane-ethyl acetate (30:70) from fraction 5. The isolated compounds were detected on TLC plates by spraying with Libermann-Burchard reagent and heated at 100°C for 10 minutes [19].

Purification of isolated compound by High performance liquid chromatography:

The analytical HPLC system (Shimadzu) was equipped with a diode array detector, a 20 µl loop, 200 x 4.6 mm C18 column, methanol (HPLC grade, 0.2mm filtered) used as a

mobile phase. The isolated (1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-enyl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol 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 [20, 21].

Collection of test organisms:

Test for Bacterial strains:

To examine the antibacterial activity of isolated compound (1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol exhibited five strains against *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. All the strains were procured from the Microbial Type Culture and Collection (MTCC) at Chandigarh, India. Bacterial strains were cultivated at 37°C and maintained on nutrient agar (Difco, USA) slant at for 4°C.

Test for Fungal strains:

The clinical fungal test organisms were used for the study are *Candida albicans* (MTCC 282), *Candida tropicalis* (MTCC No.184) *Aspergillus niger*, (MTCC 227), *Candida vulgaris* and *Aspergillus flavus* (MTCC-3396) were procured from National Chemical Laboratory (NCL), Pune, Maharashtra, India.

Determination of Antibacterial activity by disc diffusion method

Antibacterial activity of isolated compound 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 at 20, 60 and 80 µg/ml respectively. Prepared discs were placed onto the top layer of the agar plates and left undistributed 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 37°C and the zone of inhibition was recorded in millimeters and the experiment was repeated twice [23].

Determination of antifungal activity by disc diffusion method

Antifungal activity of isolated compound 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, 60 and 80 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 37°C and the zone of inhibition was recorded in millimeters [24].

RESULTS AND DISCUSSION:

Purification of isolated compound by HPLC:

The Retention time of (1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol isolated from the methanolic extract of sample was about 3.750 was shown by HPLC peak (**Figure 1**).

The microbial resistance of various strains against common antibiotics, particularly in developing countries, has resulted in wonderful selective pressure on antibiotics. These have prompted the need to explore the antimicrobial potential of traditional medicinal plants against

pathogenic strains of both bacterial and fungal strains. Disc diffusion method was used for the determination of anti-microbial activity of isolated compound and the results were presented in **Tables 1 and 2**. The diameter of zone of inhibition obtained against isolated compound by a disc diffusion method was also compared to those obtained against standard antibiotics Amoxicillin (for bacteria) and fluconazole (for fungal).

The results of these bacterial bioassays were given in **Table 1** and **Figure 2**. The isolated compound exhibited antibacterial activity in the methanolic leaf extract of *Andrographis echinoides*, which showed higher activity against *Escherichia coli* and *Staphylococcus aureus* with 8 mm and 7 mm at the concentration of 80 µg/ml whereas when the concentration was decreased the antibacterial activity of inhibition was also decreased. This antibacterial assay revealed that out of the five different bacteria, the isolated compound from *Andrographis echinoides* possesses highest anti-bacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Even though the significant antibacterial activity was observed then other two bacteria such as, *Enterococcus aerogenes* (6 mm) and *Pseudomonas aeruginosa* (6 mm). The

isolated compound was found to be moderate active against *Proteus vulgaris* (5 mm). Amoxicillin is a standard antibiotic for antibacterial activity. Standard (Amoxicillin) at a concentration of 10 µl/disc showed zone of inhibition with an 8mm compared with isolated compound. Among, the isolated compound exhibited more prominent activity.

The results of these fungal bioassays were given in **Table 2** and **Figure 3**. The methanolic leaf extract of isolated compound from *Andrographis echinoides* was tested for zone of inhibition for fungal strains containing *Candida albicans*, *Candida tropicalis*, *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus clavatus*. The antifungal activity of isolated compound had highest degree zone of inhibition against *Candida albicans* (7mm) and *Candida tropicalis* (6mm) respectively at the concentration of 80 µg/ml. Isolated compound had shown significant response against *Aspergillus niger* (5mm) whereas other fungal organisms had minimum zone of inhibition against *Aspergillus flavus* (4mm) and *Candida vulgaris* (4mm). Fluconazole is a standard antibiotic for antifungal activity. Standard (Fluconazole) at a concentration of 10 µl/disc showed maximum zone of inhibition was recorded against the fungal strains at 9mm.

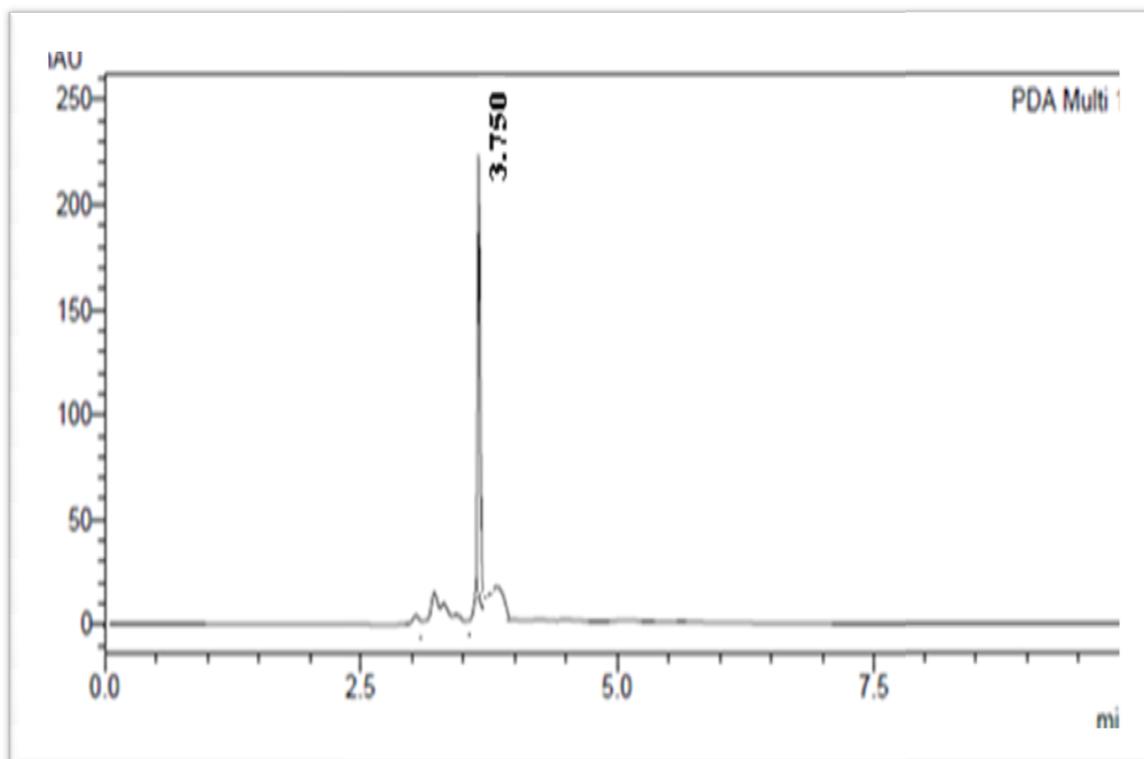


Figure 1: HPLC spectra of purity of the isolated compound

Table 1: *In Vitro* Anti-bacterial activity of isolated compound from methanolic leaf extract of *Andrographis echioides*

	Concentrations ($\mu\text{g/ml}$)	Organisms/Zone of inhibition (mm)				
		(1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol isolated from <i>Andrographis echioides</i> (leaves)				
		<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Enterococcus aerogenes</i>	<i>Pseudomonas aeruginosa</i>	<i>Proteus vulgaris</i>
Isolated compound	60	5	0	3	2	3
	80	6	6	4	5	4
	100	8	7	6	6	5
Control (Methanol)	10 $\mu\text{l/disc}$	0	0	0	0	0
Standard (Amoxicillin)	10 $\mu\text{l/disc}$	9	9	9	10	9

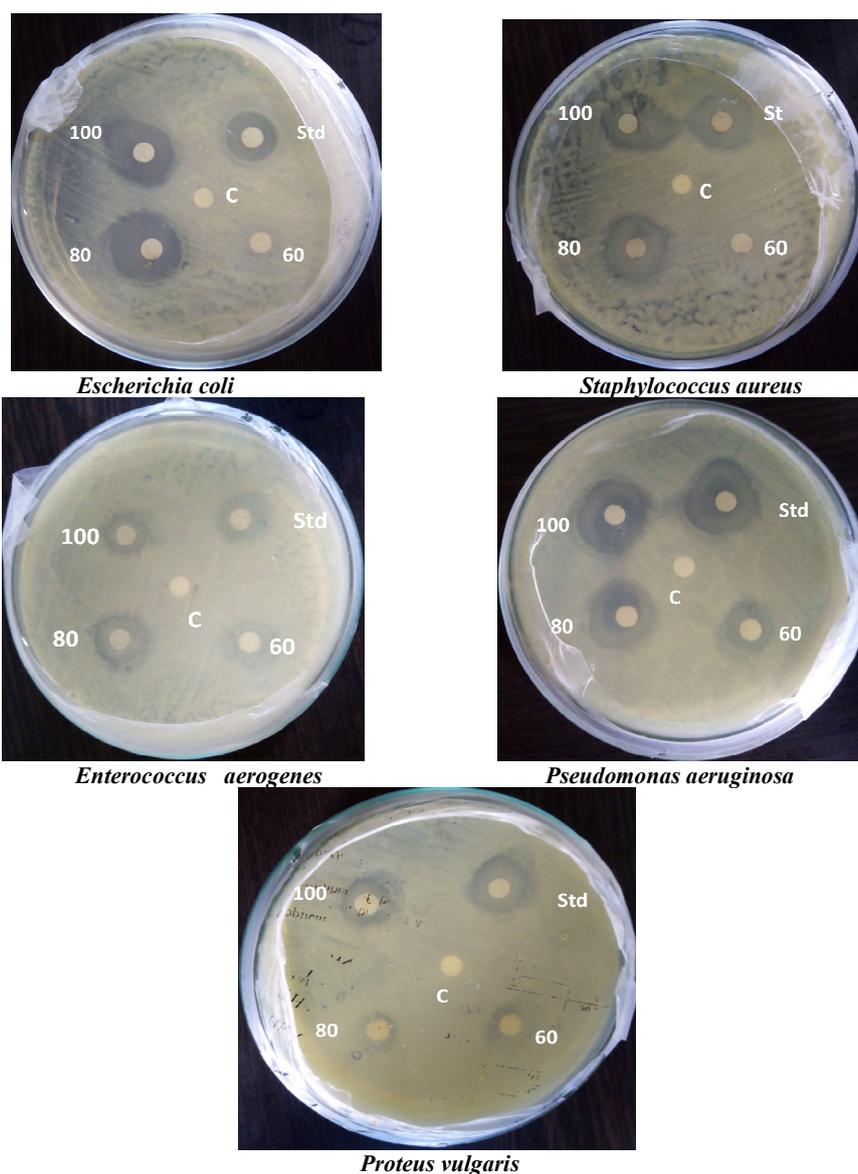


Figure 2: Anti-bacterial activity of isolated compound from methanolic leaves extract of *Andrographis echioides*

Table 2: Anti-fungal activity of isolated compound from methanolic leaves extract of *Andrographis echioides*

	Concentrations (µg/ml)	Organisms/Zone of inhibition (mm)				
		(1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol isolated from <i>Andrographis echioides</i> (leaves)				
		<i>Candida albicans</i>	<i>Candida tropicalis</i>	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Candida vulgaris</i>
Isolated compound	60	0	0	0	3	0
	80	6	5	3	4	0
	100	7	6	4	5	4
Control (Methanol)	10 µl/disc	0	0	0	0	0
Standard (Fluconazole)	10 µl/disc	9	9	9	9	9

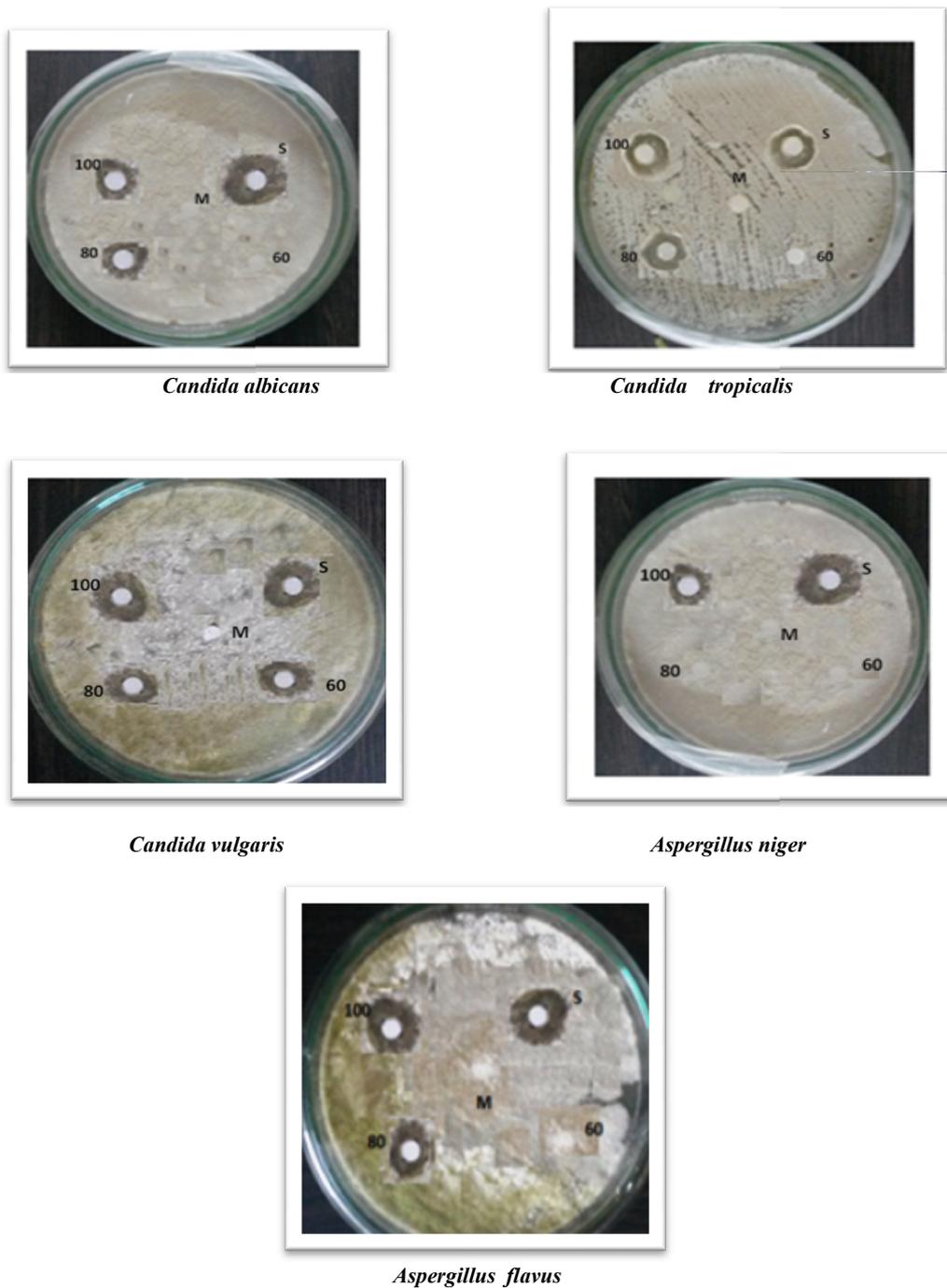


Figure 3: Anti-fungal activity of isolated compound from methanolic leaves extract of *Andrographis echioides*

CONCLUSION

It has been suggested that the isolated compound (1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol from methanolic leaf extract of *Andrographis echiioides* had strong antibacterial and antifungal activity against selected pathogens. The Purification of isolated compound (1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol (Lupeol) was identified through $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ spectroscopy and HPLC analysis. Thus, isolated compound might be a prospective source of alternative antimicrobial agents and may play an important role in the discovery of new drugs for the treatment of a wide range of pathogenic microorganisms in the near future.

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