H. Sheeba et al /J. Pharm. Sci. & Res. Vol. 11(5), 2019, 1826-1833

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Bioactive compounds and antimicrobial activity of fungal crude extract from medicinal plants

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Abstract

Aim: The present study was conducted to indentify anti-microbial and phytochemical screening of fungal crude extracts of secondary metabolites derived from *Hybanthus enneaspermus, Ziziphus mauritiana and Mukia maderaspatna.*

Methods: The isolated endophytic fungi were fermented for 21 days in Potato Dextrose Agar (PDA). The bioactive compounds were determined using ethyl acetate and further screened for phytochemical analysis, TLC profiling and anti-microbial analysis.

Results: In TLC profiling using 2 solvent methods, Solvent 2-Benzene: Ethyl acetate (1:1) with Rf values of H3-0.790; A2-0.76, 0.397; A13-0.166, 0.93; X-0.54, 0.906. Solvent 3-Butanol: Ethyl acetate (1:1) with Rf values of H3-0.1612, 0.790; A2-0.54, 0.76, 0.1505; A13-0.755, 0.76 and X-0.767, 0.72. From 16 endophytic fungal crude extract, various endophytes showed presence of terpenoids, alkaloids, tannins, phenols, flavonoids when comparing to coumarins and anthraquinones and absence of steroids under phytochemical analysis. The 11 crude extracts were tested for antibacterial activity namely *Trichoderma viride* , *Pinkish white sterile mycelia, Nigrospora sphaerica, Paecilomyces carneus, White sterile mycelia, Alternaria alternata, Botyritis cinerea, Aspergillus nidulans, Humicola grisea, Ascochyta pisi* and *Sterile mycelia* against *Salmonella typhi* "H", *Enterococcus* species, *Pseudomonas aeuroginosa, Staphylococcus aureus, Salmonella paratyphi "A", Proteus species, Klebsiella pneumoniae, Salmonella paratyphi "B", Enterococcus species* and *Bacillus species.*

Conclusion: From this study it has been observed that only 7 extracts were found to be capable of antibacterial activity at a concentration of 50 and 100 µg/ml. Hence these studies help to access a good source of novel compound to treat as an effective anti-microbial agent.

Keywords: Crude extract, Antimicrobial, PDA, Phytochemical, TLC

INTRODUCTION

Endophytes generally belong to microbial colony residing inside of healthy tissues in the plants. Most of the microbial endophytes predominantly occur in both monocotyledonous and dicotyledonous plants species [1]. Endophytic fungi have been recognized as a wide producer of phytochemical components with extensive array of biological activity [2]. Many researchers have been reported that more than one million species of endophytic fungi was naturally seen among many medicinal plants. The endophytic fungi are rich in source of flavonoids, terpenoids, alkaloids, phenols, tannins, steroids, amines, organic acids, rosmarinic acid, myoinositol, volatile ester, range of fatty acids etc [3].

Among these only few endophytic fungi are distributed throughout the host with mutual interaction beneficially. According to Hallmann *et al.*, the bio-diversity of endophytic fungi which plays a dynamic role for the enhancement of plant growth, also produce more number of secondary metabolites with novel compounds naturally. Majority of the endophyte contains novel compounds are becoming extinct more rapidly and screening of biological product has become expensive and difficult process. There is an extensive search of novel and effectual anti-microbial compounds. The research institutions are investing to find novel resources with considerable pharmacological activity [4] [5].

Researchers have more attention towards medicinal plants due to the presence of natural compounds relatively known as herbal drugs which are cost-efficient with significant side effects [6] [7]. In the present study stems and leaves of *Hybanthus enneaspermus, Ziziphus mauritiana, Mukia maderaspatna, Cardiospermum helicacabum* and *Cleome viscosa* were investigated for the isolation of endophytic fungi and potential evaluation of bioactive metabolites. All these selected plants have major pharmacological activities namely, anti-diabetic, anti-inflammatory, anti-viral, anti-microbial, anti-oxidant, anti-cancer, anti-diuretic, anti-arthritic, anti-infertility, anti-helminthic, anti-parasidal and nephro-protective activities.

MATERIALS AND METHODS

Collection of plant materials

The leaves and stems of *Hybanthus enneaspermus, Ziziphus mauritiana, Mukia maderaspatna, Cardiospermum helicacabum and Cleome viscosa* were collected in sterile bags from Thirupporur forest. The identification of plant species was authenticated by Prof. Dr. M. Syed Ali, Department of Biotechnology, Mohamed Sathak College of Arts and Science, Chennai and Prof. Dr. Subashini, Department of Plant Biotechnology, Quaidhamillath College of Arts and Science for Women, Chennai. The collected plant materials were stored at 4°C until use.

Isolation of endophytic fungi

The collected leaves and stems were surface sterilized and the isolation procedure was carried out using standard method of Petrini (1986) with slight modifications [8]. It involves 3 steps, namely (a). Removal of debris (b). Surface sterilization (c). Inoculation and incubation.

(a). Removal of debris

The plant samples were initially washed with running tap water for 5 minutes for removal of debris and other particles and rinsed twice with double distilled water.

(b). Surface sterilization

After rinsing, the tested samples were surface sterilized to 70% ethanol for one minute, 3% sodium hypochlorite for four minutes, and 70% ethanol in 30 seconds and finally washed thrice with double distilled water for 60 seconds.

(c). Inoculation and incubation

After surface sterilization**,** the leaf and stem parts were cut into 5 mm x 2 mm and transferred into petriplate containing Potato dextrose agar (PDA) medium aseptically. In order to suppress the growth of bacteria 100 µg/ml of tetracycline was supplemented in the PDA medium. Finally the plates were incubated under room temperature for 3-5 days. The observed individual fungal colonies from all the plant materials was counted from the PDA plate and each colony was isolated and sub-cultured on PDA medium separately for obtaining pure culture [9].

Identification of endophytic fungi

The endophytic fungus identification can be performed by cultural and morphological characteristic observation. The macroscopic view of endophytic fungi has been identified based on the cultural characteristics like growth of mycelium on PDA, surface texture, colony morphology and pigmentation. The microscopic examination of endophytic fungi was done using LPCB stain in which a portion of mycelium were teased and observed under 45 X magnification. Based upon the morphology of the spores and fruiting bodies like conidia, mycelia growth and conidiophores, the fungal species has been identified [10].

Data analysis

Isolation rate (IR), can be calculated by measuring the fungal sample such as number of isolates from segments of tissue divided by the total number of segments. The colonization frequency was calculated according to Suryanarayanan et al., [11].

$$\% CF = \frac{\text{Number of tissue segments colonized by a fungus}}{\text{Total number of screened tissue segments}} \times 100$$

Mass cultivation and extraction of secondary metabolites

The isolated individual endophytic fungi were inoculated in 500 ml of Erlenmeyer flask containing sterilized potato dextrose broth medium. The flask was incubated at 28°C room temperature for 21 days with periodical shaking. After incubation period the culture was filtered using sterile muslin cloth and the obtained filtrate was preserved and the mycelia part was removed. For the extraction of secondary metabolites, equal volume of filtrate and equal volume of ethyl acetate solvent was taken in the separating funnel and shaken continuously for 30 minutes and allowed to stand for 5 minutes until the formation of two immiscible layers [12] [13]. The upper portion of the solvent was separated using separating funnel and kept for evaporation. After evaporation, the obtained compounds were dissolved using DMSO to yield crude extract with concentration of 1mg/ml and stored at 4°C for further studies.

Phytochemical analysis

Alkaloids

0.5 ml of crude extract was taken in the test tube, add 0.2 ml acetic acid with few drops of dragendorff reagent and shaken well. Appearance of orange brown precipitate indicates presence of alkaloids.

Terpenoids

1 ml of crude extract was taken in the test tube, to that1 ml of chloroform and 2 to 3 ml of acetic anhydride was added. Finally 1 to 2 drops of concentrated sulphuric acid was added. Appearance of dark pink or red color refers to terpenoids.

Steroids

1 ml of crude extract and 1 ml of chloroform along with 2 to 3 ml of acetic anhydride was added. Then 1 to 2 drops of concentrated sulphuric acid was added finally. Appearance of green colour indicates presence of steroids.

Tannins

In a test tube 2 to 3 ml of crude extract was taken and a few drops of 10% of alcoholic ferric chloride were added. Presence of dark blue or greenish grey indicates tannins.

Coumarins

Few ml of crude extract and 2 ml of ethanol were added. Finally few drops of alcoholic sodium hydroxide were added. Dark yellow color appearance indicates presence of coumarins.

Quinones

0.5 ml of crude extract and 1 ml of 10% sodium hydroxide was added. Presence of blue green or red color indicates quinones.

Flavonoids

To the 1 ml of crude extract a few drops of 10% lead acetate was added. As a result yellow color precipitates indicating flavonoids.

TLC Analysis

Thin layer chromatography (TLC) was done to characterize the bioactive compounds present in the ethyl acetate crude extract.TLC plates were made using silica gel. The prepared silica gel slurry was spread using TLC spreader, allowed to stand for 5 minutes until it completely dries. The prepared plates were activated in hot air oven for 1 hour at 110°C. Subsequent to activation the TLC plates were taken out and kept at room temperature [14]. Using the capillary tubes the crude extract of *Trichoderma viride (H3)*, *Pinkish sterile mycelia (A2)*, *Nigrospora sphaerica (X)*, *Sterile mycelia* (A13) was spotted on the pre-coated TLC plates and kept in TLC chamber using 2 solvent method i.e. Chloroform: Methanol (8:2), Benzene: Ethyl acetate (1:1) and Butanol: Ethyl acetate (1:1) ratio. After 20 minutes the TLC run plates was allowed to dry and observed initially under visible light followed by UV light and also kept under iodine containing chamber for 1-2 minutes for detecting the bands [15]. The movement of active compound was expressed based on its retention factor (Rf) values for calculated from the obtained crude samples.

$$Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}} \times 100$$

Antibacterial activity by agar well diffusion method

The obtained crude extract from the potential endophytic fungus were tested against both gram positive *S. aureus* and gram negative urinary tract pathogens namely *E.coli, P. aeruginosa, Proteus species, K. pneumoniae, Enterococcus fecalis* and typhoid causing pathogen bacteria namely *Salmonella typhi H, Salmonella paratyphi AH, Salmonella paratyphi BH,* and *Bacillus species*. The human pathogens were spread on Muller Hinton Agar (MHA) plates by lawn culture. Using sterile cork borer wells were bored on the MHA plate and the crude extract with two different concentrations of 50 and 100 µl was poured along with the control Ciprofloxacin. Then the plates were incubated at 37°C for 24 hours [16] [17]. After incubation, zone of inhibition was measured using measuring scale and compared with positive and negative control.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi from medicinal plants:

During these study 40 segments from each healthy plant stem and leave (5 mm x 2 mm) of *Hybanthus enneaspermus, Ziziphus mauritiana, Mukia maderaspatna, Cardiospermum helicacabum* and *Cleome viscosa* were obtained and screened for presence of endophytic fungus. Apparently 38 fungal isolates were identified from 3 plants 18 species in *Ziziphus mauritiana,* 13 species in *Hybanthus enneaspermus* and 7 species in *Mukia maderaspatna* comparing *Cardiospermum helicacabum* and *Cleome viscosa.* Based on the morphological characteristics like color, mycelium growth on the PDA the endophytes were determined. The colonization frequency (CF) of endophytic fungi of *Z. mauritiana, Hybanthus enneaspermus* and *Mukia maderaspatna* in leaf and stem was calculated accordingly (Figure 1).

$$\% CF = \frac{\text{Number of tissue segments colonized by a fungus}}{\text{Total number of screened tissue segments}} \times 100$$

Screening of phytochemical constituent:

The phytochemical analysis was carried out using 38 crude extracts of endophytic fungi. Among 38 fungal isolates 16 crude extracts showed significant phytochemical constituents. In this process terpenoids, coumarins, alkaloids, anthraquinones, phenols, flavonoids, tannins, quinones and steroids were screened (Table 1). Terpenoids, tannins, coumarins and anthraquinones were present in the species of *Trichoderma viride.* The presence of coumarins, flavonoids and terpenoids are rich in white *sterile mycelia.* Alkaloids and phenols were found in *Paecilomyces carneus.* The presence of tannins and quinones were found in *Nigrospora sphaerica.* Alkaloids, terpenoids, tannins, coumarins and quinones were found in *Botyritis cinerea.* In the species of *Alternaria alternata, phytochemical compounds such as alkaloids, terpenoids, tannins, phenols and flavonoids was observed.* Tannins were observed in *Humicola grisea. Aspergillus nidulans shows the presence of* alkaloids and phenols. Tannins, phenols and flavonoids are present in *Fusarium oxysporum.* Absence of steroids among all crude extracts (Table 1).

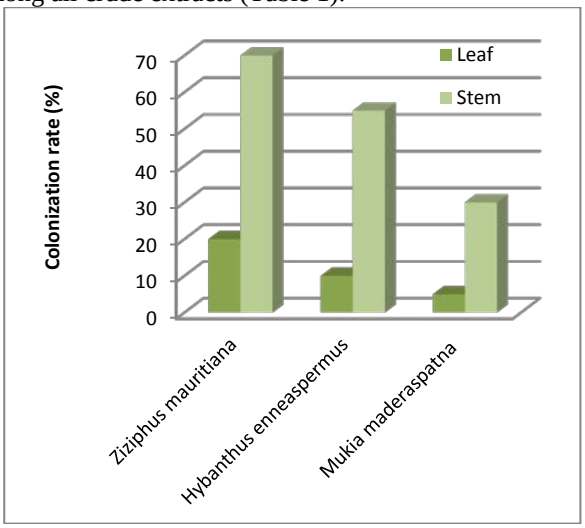


Figure 1: Colonization frequency of fungal species from medicinal plants

Table: 1 Qualitative analysis of phytochemical screening from the fungal metabolites

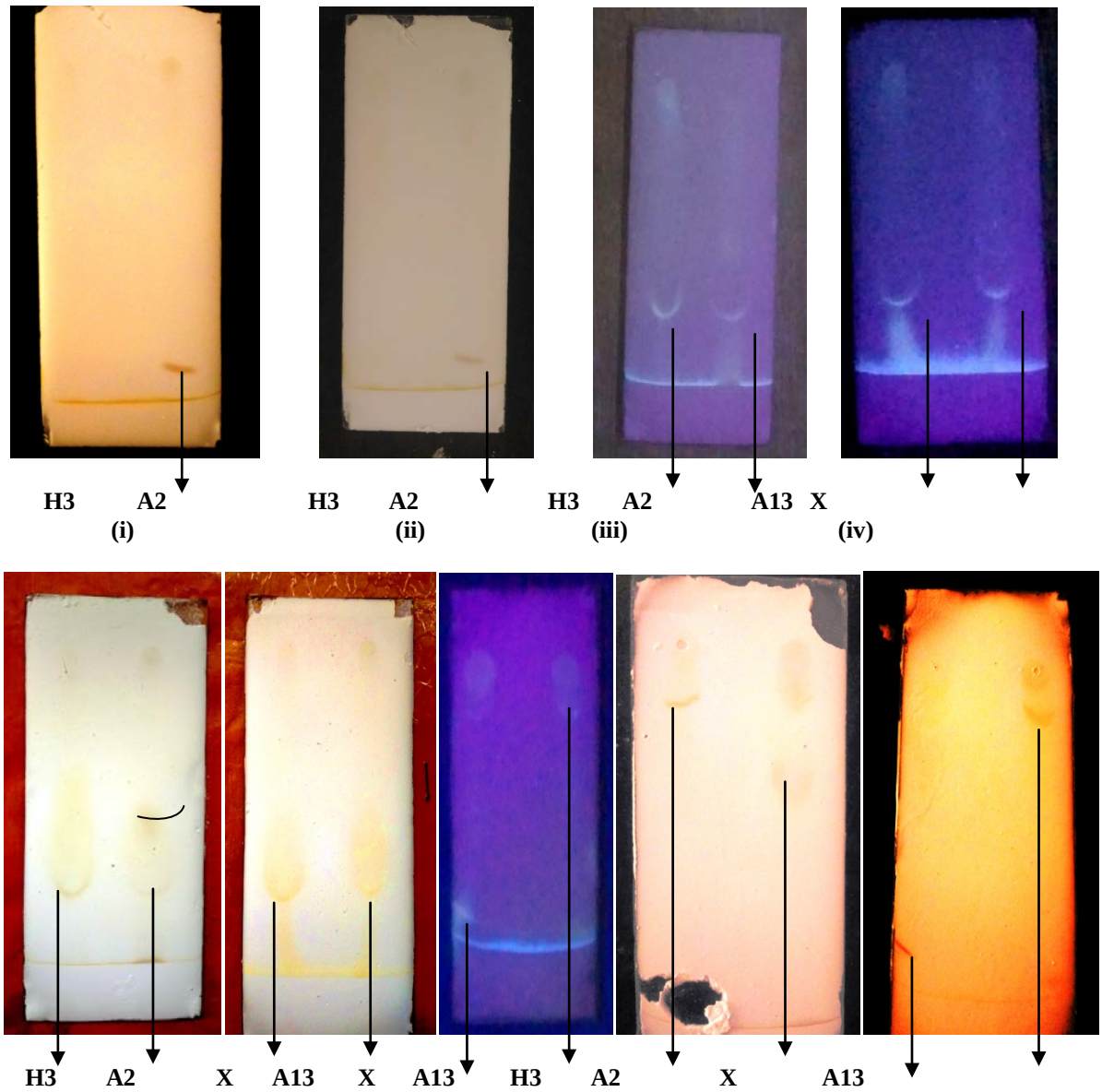
Name of the endophytic fungus	Alkaloids	Terpenoids	Steroids	Tannins	Coumarins	Anthraquinones	Phenols	Quinones	Flavonoids
B2	+	-	-	-	-	-	+	-	-
H3	-	+	-	+	+	+	-	-	-
A2	-	+	-	-	-	-	-	-	-
U	-	+	-	-	+	-	-	-	+
X	-	-	-	+	-	-	-	+	-
F	+	+	-	+	+	-	-	+	+
N	-	-	-	-	-			-	-
WC	+	+	-	+	-	-	+	-	+
J	-	-	-	+	-	-	-	-	-
X2	-	+	-	-	-	-	-	-	-
A12	-	+	-	-	-	-	+	-	-
A13	+	-	-	-	-	-	-	-	+
AN	-	-	-	+	-	-	-	-	-
X14	+	-	-	-	-	-	+	-	-`
P	-	-	-	+	-	-	+	-	+
T	-	-		+	-	-	-	-	-

B2-Paecilomyces carneus, H3-Trichoderma viride, A2-Pinkish white sterile mycelia, U- White sterile mycelia, X-Nigrospora sphaerica, F-Botyritis cinerea, N-Ascochyta pisi, WC-Alternaria alternata, J-Humicola grisea, X2-Sterile mycelia, A12- Grey sterile mycelia, A13-Sterile mycelia, T-Aspergillus flavus, X14-Aspergillus nidulans, P-Fusarium oxysporum, AN-Aspergillus niger **+ Present, - Absent**

TLC Profiling analysis of the crude extract

In this study 3 types of solvent systems were chosen to obtain a good result. From the Chloroform: Methanol (8:2), the crude extract of *Pinkish white sterile mycelia* revealed 1 compound with Rf values of 0.880. In the Benzene: Ethyl acetate (1:1), the crude extract of *Trichoderma viride* revealed the presence of 1 compound with Rf values of 0.790 and 2 compounds from *Pinkish white sterile mycelia* Rf values of 0.76, 0.397; 3 spots were detected from *Sterile mycelia* with Rf values of 0.166, 0.93, 0.89; the crude extract of *Nigrospora sphaerica* revealed with Rf values of 0.54 , 0.906, 0.52 respectively. Finally in Butanol: Ethyl acetate (1:1) the crude extract of *Trichoderma viride* develops 2 bands from iodine and UV with Rf values of 0.1612, 0.790, from the extract of *Pinkish white sterile mycelia*-3 spots with Rf values of 0.54, 0.76, 0.1505, from *Sterile mycelia* -2 spots with Rf values 0.755 and 0.76 were identified, and in the extract of *Nigrospora sphaerica* 2 spots were obtained Rf values 0.767 and 0.72 were detected (Figure 2) (Table 2).

Figure 2: Schematic representation of TLC using 2 solvent methods from fungal crude extract of *Trichoderma viride-H3, Pinkish white sterile mycelia-A2, Nigrospora sphaerica-X, Sterile mycelia-A13.*



(i) Chloroform (8): Methanol (2)-Iodine; (ii) Chloroform (8): Methanol (2)-Visual light; (iii) Butanol (1): Ethyl acetate (1)-UV; (iv) Butanol (1): Ethyl acetate (1)-UV; (v) Butanol (1): Ethyl acetate (1)- Iodine; (vi) Butanol (1): Ethyl acetate (1)-Ioidine; (vii) Benzene (1): Ethyl acetate (1)- UV; (viii) Benzene (1): Ethyl acetate (1)- Iodine; (ix) Benzene (1): Ethyl acetate (1) - Iodine;

Table 2: TLC solvent system with Rf values from fungal crude extract

S.No	Crude Extract	Solvent I Chloroform : Methanol (8:2)				Solvent 2 Benzene : Ethyl acetate (1:1)				Solvent 3 Butanol : Ethyl acetate (1:1)			
		V	UV	I	Rf	V	UV	I	Rf	V	UV	I	Rf
1	H3	-	-	-	-	-	-	1	0.790	-	1	1	0.1612 0.790
2	A2	1	-	1	0.880	-	-	2	0.76 0.397	-	1	2	0.54 0.76 0.1505
3	A13	-	-	-	-	-	1	2	0.166 0.93 0.89	-	1	1	0.755 0.76
4	X	-	-	-	-	-	1	2	0.54 0.906 0.52	-	1	1	0.767 0.72

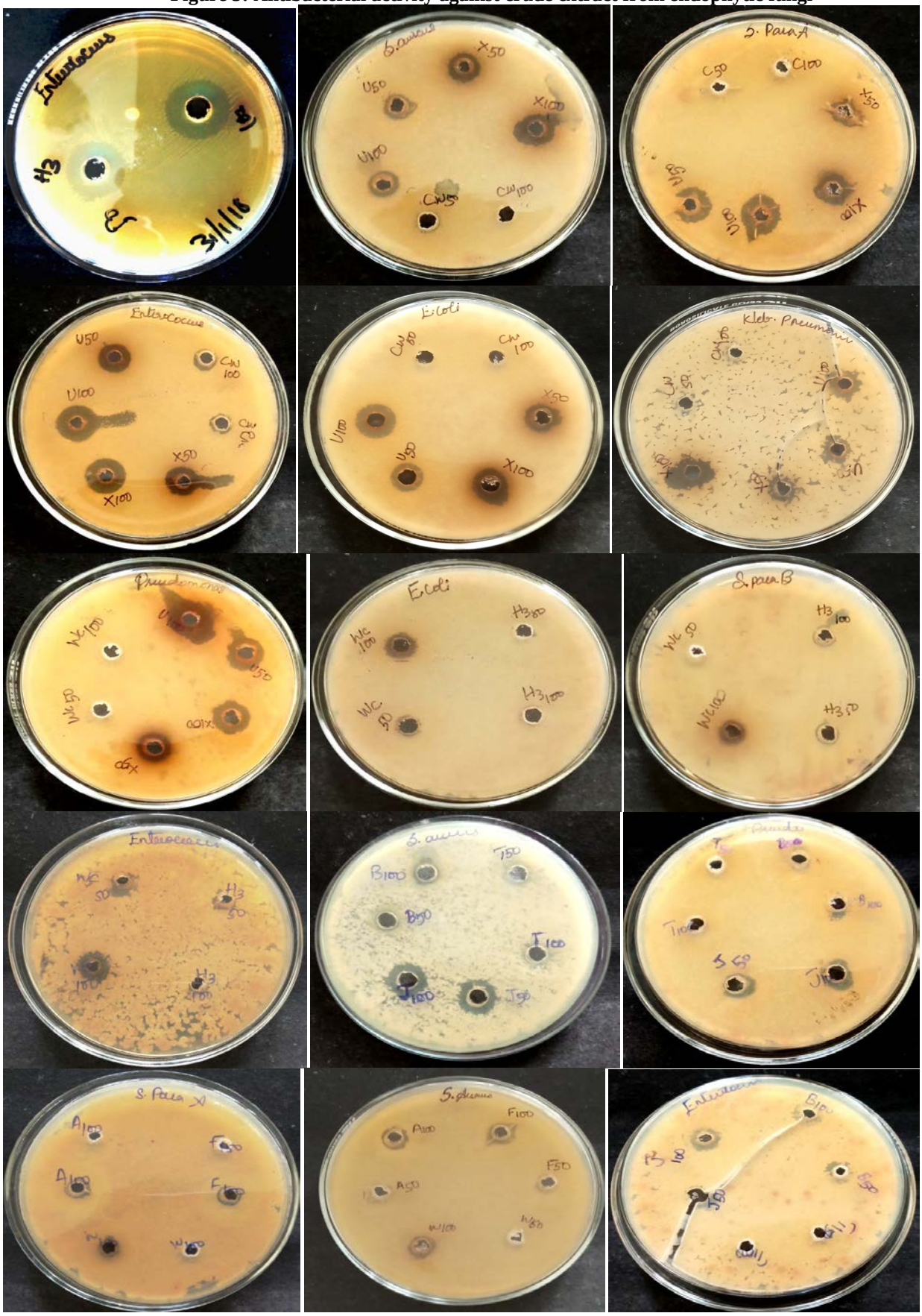
Antibacterial activity of endophytic fungi

The 16 endophytic fungal extracts were screened for antibacterial activity including gram positive and gram negative by agar well diffusion method. Among 16 only 12 crude extracts namely *Trichoderma viride* , *Pinkish white sterile mycelia, Nigrospora sphaerica, Paecilomyces carneus, White sterile mycelia, Alternaria alternata, Botyritis cinerea, Aspergillus nidulans, Humicola grisea, Ascochyta pisi* and *Sterile mycelia* showed maximum zone of inhibition against *Salmonella typhi* "H", *Enterococcus* species, *Pseudomonas aeuroginosa, Staphylococcus aureus, Salmonella paratyphi "A", Proteus species, Klebsiella pneumoniae, Salmonella paratyphi "B", Enterococcus species* and *Bacillus species* (Figure 3) (Table 3).

Table 3: Determination of zone of inhibition from fungal crude extract

S.No	Medicinal plants	Endophytic fungi	*E.coli*	*Klebsiella pneumoniae*	*Salmonella typhi "H"*	*Enterococcus species*	*Pseudomonas aeroginosa*	*S.aureus*	*S. paratyphi "A"*	*S. paratyphi "B"*	*Bacillus species*	*Proteus species*
1	*Ziziphus mauritiana*	*Tichoderma viride*	-	-	20	20	13	17	15	-	-	-
2	*Ziziphus mauritiana*	*Sterile mycelia*	-	-	-	-	16	-	-	-	-	-
3	*Ziziphus mauritiana*	*Pinkish white sterile mycelia*	-	-	20	9	7	18	13	-	8	-
4	*Ziziphus mauritiana*	*Paecilomyces carneus*	-	-	-	-	8	-	-	-	-	12
5	*Ziziphus mauritiana*	*White sterile mycelia*	14	13	-	17	-	10	14	15	-	-
6	*Ziziphus mauritiana*	*Nigrospora spherica*	18	17	-	14	14	15	13	15	18	-
7	*Ziziphus mauritiana*	*Alternaria alternata*	11	11	-	13	12	-	-	9	-	-
8	*Mukia maderaspatna*	*Humicola griseae*	-	-	8	9	10	-	-	-	18	18
9	*Mukia maderaspatna*	*White sterile mycelia*	-	8	10	10	6	13	-	-	-	-
10	*H. enneaspermus*	*Botyritis cinereae*	-	-	-	-	-	10	11	10	-	12
11	*H. enneaspermus*	*Aschocyta pisi*	14	16			12	-	-	-	-	-
12	*H. enneaspermus*	*Aspergillus nidulans*	17	-	-	15	-	13	-	-	-	10

Figure 3: Antibacterial activity against crude extract from endophytic fungi



DISCUSSION

In the healthy plants endophytic fungi inhabit in the core of stems, leaves, bark, roots and various parts of the plants by naturally protecting them without any cause [18]. The comparative study was made among the parts of various plants, through which maximum number existence of endophytic fungi were found to be in stem parts on the basis of more nutrients compared to leaves [19]. Based on various research these endophytic fungi were successfully used in pharmaceutical industry, biological pests control and plant diseases. These endophytic fungi have immense medicinal values with numerous active biological compounds as a novel drug which helps towards the betterment of human health issues [20] [21].

In this present study 38 fungal extracts were obtained from *Ziziphus mauritiana, Hybanthus enneaspermus* and *Mukia maderaspatna,* among them 16 crude extracts showed significant phytochemical properties such as terpenoids, flavonoids, tannins, coumarins, phenols, alkaloids and anthraquinones. From this study it has been observed that from 16 crude extracts only 7 extracts especially *Trichoderma viride, Pinkish white sterile mycelia, Nigrospora sphaerica, Paecilomyces carneus, White sterile mycelia, Alternaria alternata* and *Botyritis cinerea* showed maximum zone of inhibition comparing to other extracts. The previous research work has been reported that *Aspergillus, Alternaria spp, Cladosporium spp* isolated from *Ziziphus mauritiana* was agreed with the same endophytic fungi in their study [22]. Certain endophytic fungi are highly host specific while others are usually dispersed in plants [23].

According to Akanksha et al (2015) phytochemical analysis of fungal crude extracts obtained from Spikes of *Pinus roxburghii* revealed presence of flavonoids, alkaloids, phenols, saponins, steroids, tannins, terpenoids and he also observed excellent antimicrobial activity was obtained from crude ethyl acetate extracts against human pathogenic bacteria namely coli, *Staphylococcus aureus, Salmonella typhimurium, Candida albicans* [24]. Li et al, *(2005)* isolated 130 endophytic fungi and study on anti-tumor and antifungal activities from their extracts showed 9.2% showed anti-tumor activity and 30% showed anti-fungal activity which indicates the presence of active fungal compounds [25].

Similarly Poorani et al (2015) also observed significant efficacy of anti-microbial activity from endophytic fungi using methanolic extract [26]. Hormazabal and Piontelli (2009) found best anti-microbial activity from endophytic fungi *Curvularia protubera* on *Staphylococcus aureus, Bacillus subtilis,* with maximum of inhibition 16 mm and 12 mm respectively [27]. In the study of Nayak et al (2017), 25 fungal species were isolated from the *Avicennia marina* a mangrove plant founded endophytic fungi of *Aspergillus niger, Colletotrichum sp., Phoma sp., White sterile mycelia, Cochilobolus victoria, Curvularia lunata, Fusarium oxysporum, Glomerella sp, P. oxalicum* etc. Similarly Vizcaino et al isolated 4 taxonomic groups of endophytic fungi were screened for anti-bacterial, anti-fungal, anti-yeast activity [28].

CONCLUSION

It has been concluded that on the basis of secondary metabolite compound, the results were directing towards the existing of numerous phyto constituents. It is very important evidence for understanding the polarity of the phytochemicals substances thereby it helps for the assortment of appropriate solvent for pure compound separation. The compound with the different Rf values reflects the idea about their nature of polarity. However further studies has to be carried out to ascertains its bioactivity and toxicity study. This potential effect of fungal extracts provides optional methods for drug discovery naturally with cost efficient, economical, and environmental secure and reliable.

Acknowledgments

The authors are very thankful for their encouragement and valuable support to Department of Biotechnology, Mohamed Sathak College of Arts and Science, Chennai.

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