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Research article

ANTIMICROBIAL ACTIVITY OF EXTRACELLULAR METABOLITE OF ENDOPHYTIC FUNGI *Phomopsis* spp. ISOLATED FROM FOUR DIFFERENT MEDICINAL PLANTS OF INDIA.

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ABSTRACT: The present investigation is on endophytic fungus *Phomopsis* spp isolated from four Indian medicinal plants like., *Artabotrys odoratissimus, Cassia auriculata, Guazuma ulmifolia* and *Terminalia catappa* in four different months. Antimicrobial activity of ethyl acetate extract from the culture filtrate of *Phomopsis* spp were tested against six human pathogenic bacteria. Isolated three *Phomopsis* spp. were grown in Czapex Dox Broth for 21-days. The extracellular secondary metabolites present in the culture filtrate were extracted with ethyl acetate solvent. The extracellular bio-active compounds of the isolated fungus were tested for its anti microbial potential in well diffusion method, against three, Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Escherichia coli, Klebsiella Pneumoniae* and *Pseudomonas aeruginosa.* Among all the three *Phomopsis* spp, the extract obtained from *Phomopsis* sp.2 exhibited a promising activity against the entire test bacteria. This bioactivity compounds focus on clinical pharmacology to identify a novel therapeutic targets and it can be easily scaled up for the large-scale commercial production.

Key words : *Phomopsis* spp, Bioactive compounds, Antibacterial activity, Medicinal Plants, Ethyl acetate extract.

INTRODUCTION

Fungi causing asymptomatic infections in living plant tissues has been called as fungal endophytes (Hyde and Soytong, 2008). There are numerous studies on endophyte communities of temperate, tropical and subtropical plants (Kumar et al., 2005; Arnold and Lutzoni, 2007; Oses et al., 2008; Rungjindamai et al., 2008; Sánchez Márquez et al., 2008; Tao et al., 2008) Endophyte-host represent a series of interactions ranging from strong antagonism to obligate mutualism (Saikkonnen et al. 1998; Clay and Schardl, 2002). The relationships of endophytes with single or multiple plant hosts can be described in terms of host- specificity, host- recurrence, host selectivity, or host-preference (Zhou and Hyde, 2001; Cohne, 2006). Host-specificity is the relationship on which a fungus is restricted to a single host or a group of related species, but does not occur in other unrelated plants in the same habitat (Holliday, 1998). In addition, fungal endophytes have been recognized as a repository of novel compounds of immense value in agriculture, industry and medicine (Kumar et al. 2004; 2005; Tan and Zou 2001; Strobel and Daisy 2003).

The genus *Phomopsis* is a rich source of biologically active secondary metabolites including antimicrotubule phomopsidin (Kobayashi et al., 2003), antimalarial and antitubercular phomoxanthones (Isaka et al., 2001), antifungal phomoxanthone - A (Elsaesser et al., 2005), phomodiol (Horn et al., 1994), antibacterial chromones (Ahmed *et al*., 2011), antifungal lactones (Wu SH et al., 2008) and cytotoxic sesquiterpenes (Hemtasin et al., 2001). Chemical investigation of the ethyl acetate extracts from the culture broth and the cells of the endophytic fungus *Phomopsis* sp. PSU-D15 led to isolation of three new compounds, namely phomoenamide phomonitroester and deacetylphomoxanthone B (Wagenaar and Clardy, 2001). *Artabotrys odoratissimus* R. Brown, (Annonaceae) is an evergreen perennial shrub, mostly native of tropical Africa, Eastern Asia and India (The Wealth of India, 1952). It is s used in Indian system of medicine for the treatment of vomiting, biliousness and disease of blood and heart. Leaves are used to treat some anti-fungal and antimicrobial diseases.

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The antifertility activity of *A. odoratissimus* plant has been reported in albino rats (Chakrabarti *et al*., 1968). The genus Artabotrys is a rich source of diverse secondary metabolite (Wong and Brown, 2002). *Cassia auriculata* Linn, (Caesalpiniaceae) is a common Asian beverage and medicinal plant. In Sri Lanka, where the plant is known by its common name Ranawara or Avaram. Its used for the treatment of diabetes, urinary disorders, ulcers, skin diseases and chronic fever (Gaikwad et al., 2010). *Guazuma ulmifolia* Lam, is a member of the Sterculiaceae family that grows in Ecuador, Panama and other Latin American countries from bush to tree size. In traditional medicine, the bark of *G. ulmifolia* is used in the treatment of diarrhea, hemorrhages, fever, coughs, bronchitis, asthma, gastrointestinal pain and hypertension, and as stimulant for uterine contractions (Caballero-George et al., 2001; Dom´nguez and Alcorn *et al*., 1985). Dried leaves are brewed into tea in some countries and used for kidney, gastrointestinal diseases, fever, dysentery, diabetes, and externally as an ailment for wounds, skin eruptions and even baldness. *G. ulmifolia* leaves are also traditionally boiled as a treatment for diabetes and this method has been experimentally proven to decrease hyperglycaemia in rabbits (Alarcon-Aguilara *et al*., 1998). *Terminalia catappa* Linn (combretaceae) is found throughout in the warmer parts of India and called as Indian almond, Malabar almond and Tropical Almond. It is a medium sized tree with leaves clustered towards the ends of the branches. The leaves of *T. catappa* contain many hydrolysable tannins, such as punicalagin, punicalin, terflavins A and B tergallagin, tercatain, chebulagic acid, geraniin, granatin B, and corilagin, but no caffeine (Tanaka et al., 1986).

Hence this work focuses on studying the diversity of endophytic fungi, *Phomopsis* spp. from four medicinal plants in four different seasons and exploring the efficiency of its extracellular bio-active compounds against six human pathogenic bacteria. This is an alternative and cost effective method for antibacterial drug production from mycological sources.

MATERIALS AND METHODS

Plant material

Fresh healthy stems and leaves of *A. odoratissimus, C. auriculata, G. ulmifolia,* and *T. catappa,* were collected from Chennai, Tamil Nadu, India. In February – May (2009).

Isolation of endophytic fungus

The collected samples were washed thoroughly in running water and air-dried. The cleaned stems and leaves were surface-sterilized as follows: 75% ethanol (v/v) for 3 min and 3% sodium hypochlorite (v/v) for leaves 3 min and stems 5 min, then rinsed in sterile distilled water for five times. The sterilized samples were cut into pieces of about 0.5 - 1 cm and placed on the potato dextrose agar (PDA) plates supplemented with 150 mg/L chloramphenicol and incubated at 28 °C for 3-15 days. The hypha of the endophytic fungus growing out from the plant pieces was transferred onto another PDA plate in time and incubated for 3-5 days. The fungi were identified on the basis of their morphological characteristics and sporulation.

Inoculation and culture conditions

Three isolates of *Phomopsis* spp. (1, 2 and 3) were aseptically inoculated into 250 ml Erlenmeyer flasks containing 100 ml CDB medium containing 3g Sucrose, 0.3g Sodium nitrate, 0.1g Dipotassium hydrogen phosphate, 0.05g Magnesium sulphate and 0.001g Ferrous sulphate, pH was adjusted to 6.5 with 0.1 N of NaOH or 0.1 N of HCl before autoclave at 121°C and 15 lb for 20 min. All cultures were maintained at 28±1°C for 21 days, under 16 hrs photoperiod at a photosynthetic flux of 12.6 µ mol m^{-2} s^{-1}, provided by cool daylight fluorescent lamps.

Isolation of fungal bioactivity compounds

After incubation period, the fungal culture was filtered through 2 layers of cheesecloth. The culture filtrate was extracted with two equal volumes of ethyl acetate solvents. The extracted samples were evaporated under reduced pressure using rotary evaporator.

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Anti bacterial activity of agar-well diffusion method

Each fungal extract was re-dissolved in DSMO solvent (100mg/ml). These samples were tested for its antibacterial activity by agar well diffusion method. Each bacterial suspension was spread over the surface of Nutrient Agar (Himedia, India) plates containing 4 wells of 6 mm diameter. The wells were filled with the fungal bioactive metabolites. The plates were incubated at 37°C for 24 h. The results were expressed in terms of the diameter of the inhibition zone. The respective solvents were used as negative control, (DMSO).

RESULTS

Three species of *Phomopsis* were isolated from the leaves and stems of four medicinal plants throughout the study period (February – May 2009) (Figure 1,2,3). The *Phomopsis* sp.1 dominated the endophytic assemblage of four medicinal plants whereas; *Phomopsis* sp.2 was restricted only to *Guazuma ulmifolia and Phomopsis* sp.3 was isolated from all the three plants except *Guazuma ulmifolia* (Table 1). Ethyl acetate extract of endophytic fungus showed inhibition zones ranging form 1 to 7 mm by *Phomopsis* sp.1, *Phomopsis* sp.2 exhibit 6 to 13 mm and *Phomopsis* sp.3 exhibit 1 to 12 mm in diameter (Figure 4, Graph 1). Overall, the extract of *Phomopsis* sp.2, showed more anti-bacterial activity for all the three Gram-positive and Gram-negative bacterium such as *Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Escherichia coli, Klebsiella Pneumoniae* and *Pseudomonas aeruginosa* when compared to control.

Table. 1: Endophytic fungi *Phomopsis* spp, were isolated from four Indian medicinal plants at four different months.

S.No	Name of the Fungus	Name of the Medicinal Plants															
		A. odoratissimus				*C. ariculata*				*G. ulmifolia*				*T. catappa*			
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
1	*Phomopsis* sp. 1	+	+	-	+	+	+	+	-	+	+	-	+	+	+	+	+
2	*Phomopsis* sp. 2	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-
3	*Phomopsis* sp. 3	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+

A - February, B - March, C - April, D – May, + - Present, — - Absent.

Figure. 1: Different Indian medicinal plants - a - *Artabotrys odoratissimus*, b - *Cassia auriculata*. c - *Guazuma ulmifolia* and d -*Terminalia catappa.*

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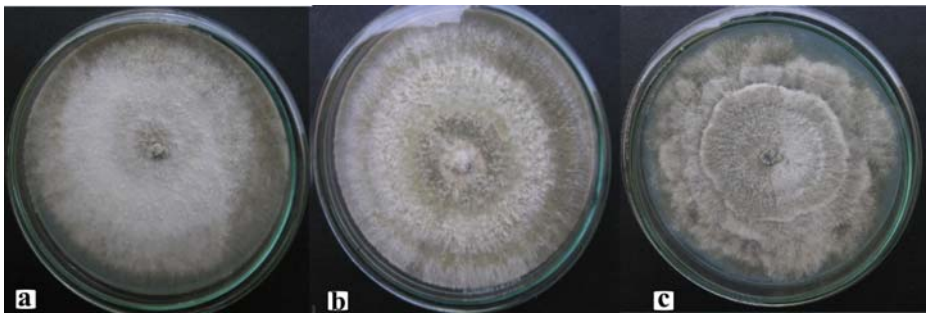

Figure. 2: Endophytic fungal *Phomopsis* spp, were grown in PDA medium: a - *Phomopsis* sp-1, b - *Phomopsis* sp-2, c - *Phomopsis* sp-3.

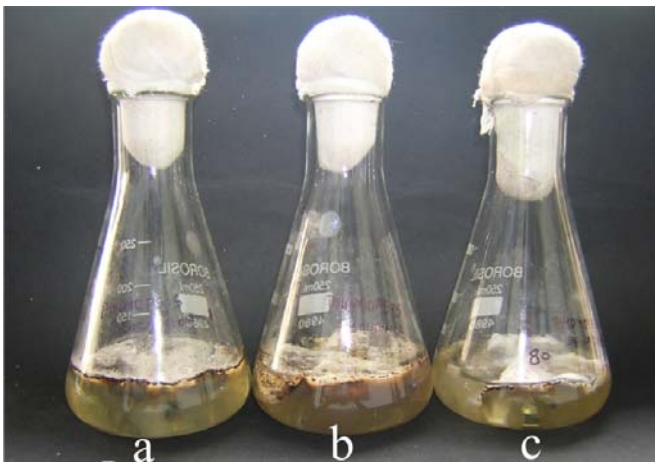

Figure. 3:Three endophytic *Phomopsis* spp, were grown in CDB medium for 21 days. a - *Phomopsis* sp-1, b- *Phomopsis* sp-2 and c - *Phomopsis* sp-3 .

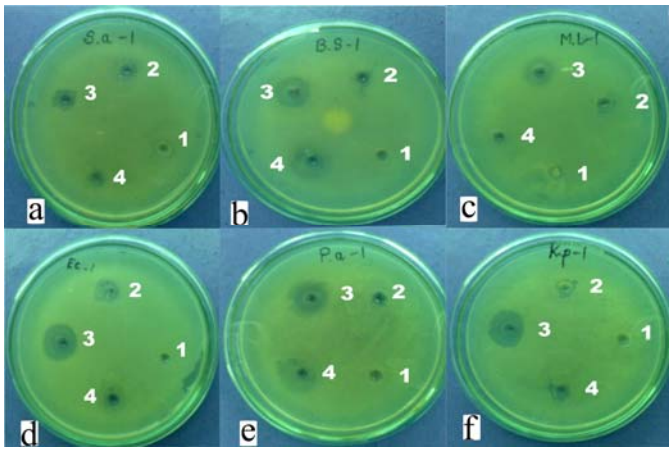

Figure. 4: Antibacterial activity of fungal bioactivity compounds against three gram positive bacterium. (a) - *Staphylococcus aureus* (b) -.*Bacillus subtilis.* (c) - *Micrococcus luteus* and gram negative bacterium (d) - *Escherichia coli.* (e) - *Pseudomonas aeruginosa.* (f) - *Klebsiella pneumonia.* 1.Control for DMSO solvent; 2. *Phomopsis* sp.1, 3. *Phomopsis* sp.2, 4. *Phomopsis* sp.3.

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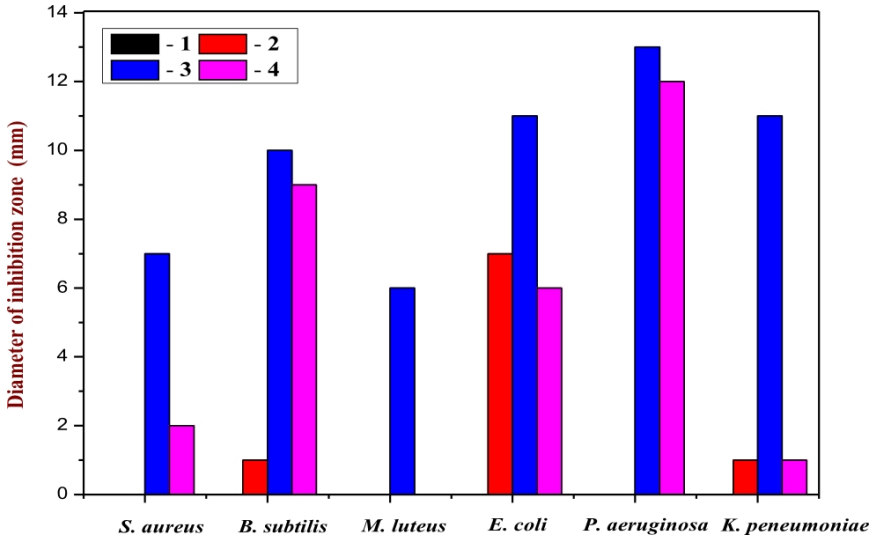

Graph. 1: Antibacterial activity of fungal bioactive compound of three *Phomopsis* spp. 1 - Control (DMSO solvent), 2 - *Phomopsis* sp-1 extract, 3 - *Phomopsis* sp-2 extract, 4 - *Phomopsis* sp-3 extract.

DISCUSSION

Phomopsis spp. used in this study, were isolated from all the medicinal plants. Similarly, in the previous study *Phomopsis* sp. were also been isolated from Mangroves and other medicinal plants (*Rhizophora apiculata, Allamanda cathartica* and *Plumeria acutifolia* (Nithya and Muthumary, 2010;2011; Buatong et al., 2011). Presence of *Phomopsis* sp. in most of the plant hosts made it as a dominant fungus in the endophytic fungal community. Since *Phomopsis* sp.2 was isolated only in *Guazuma ulmifolia* throughout the study period it was clearly understood that there must series of biochemical interactions happening between the host and the endophyte, which made it a host specific endophyte (Holliday, 1998). The extracellular metabolites produced by all the three *Phomopsis* spp. showed antibacterial activity but the range varied based on the difference in the chemical compound synthesized by the individual fungus. The secondary metabolites of the *Phomopsis* sp. contain terpene and other rich source of bioactive compound having a better antibacterial activity (Nithya and Muthumary, 2010;201). Phomol, a novel antibiotic produced by a *Phomopsis* sp isolated from the medicinal plant *Erythrina crista*-galli (Weber *et al*., 2004). *Phomopsis* sp.isolated from the plant *C. salvifolius* produced four Pyrenocine (J to M) compounds. The compounds K and M proved antifungal activity against *M. violaceum* and compounds J, K, and M against fungal *S. tritici*. Compound L had good anti-bacterial and algicidal activity (Hussain et al., 2012). Ten membered lactone compounds were isolated from *Phomopsis* sp. B27, an endophytic fungal strain isolated from *Annona squamosa* (Xiao et al., 2008). Phomoenamide isolated *Phomopsis* sp. PSU-D15 exhibited moderate *in vitro* antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra strain (Rukachaisirikul et al., 2008). *Phomopsis* sp. (strain HKI0458) isolated from the mangrove plant *Hibiscus tiliaceus* synthesized four A-seco-oleane-type triterpenes (Li et al., 2008).

CONCLUSION

The present investigation indicates that, three endophytic fungi were isolated from four healthy medicinal plants leaves and stems. The host specificity of *Phomopsis* sp.2 was observed in *Guazuma ulmifolia*. It produced bioactive secondary metabolites in the broth medium. Ethyl acetate extract of the *Phomopsis* spp. were tested against six human pathogenic microbes. The better result was obtained by *Phomopsis* sp.2 bio-active compounds. This bioactivity compounds focus on clinical pharmacology to identify a novel therapeutic targets and it is an alternative and cost effective method for antibacterial drug production from mycological sources.

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