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Antimicrobial and larvicidal potential of endophytic fungi isolated from *Tragia involucrata* Linn.

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Abstract: Endophytic fungi are one of the untapped resources of therapeutic compounds for various diseases. The present study focused on the antimicrobial as well as larvicidal activity of ethyl acetate extract of endophytic fungi isolated from *Tragia involucrata*. The ethyl acetate extract of *Penicillium citrinum* CGJ-C1 (GenBankNo.KT780618), *Penicillium citrinum*CGJ-C2 (KP739821), *Cladosporium*sp. (KP739822), and *Cryptendoxyla hypophloia* CGJ-D2 (KT780619) was subjected to antimicrobial activity against a panel of microorganisms by disc diffusion method, larvicidal activity against *Culex quinquefasciatus*. All the extracts showed significant antimicrobial activity against the tested organisms ranging from 8±0.32 to 13±2.11mm (zone of inhibition). The extent of activity was comparable to the standard drugs. The larvicidal potential of the endophytes was superior to *T. involucrata* extract. The larvicidal activity was found to be dose and time dependent with LC_{50} value ranging from 4.25- 158.06 ppm after 24hrs of treatment. This is the first report on the bioactivity of the endophytes isolated from *T. involucrata*. Further studies on the bio-guided isolation of lead compound will benefit the people suffering from microbial diseases.

Key words: *Tragia involucrata*; Endophytic Fungi; Antimicrobial; Antiyeast; Larva.

Introduction

The demand for new antibiotic is escalating day by day due to the development of drug resistance even though many novel molecules are released to the market. Chemical synthesis and engineered biosynthesis are the major contributors to the several drugs. Although this method is extensively exploited for the new generic molecules, nature still astonishes us with the availability of versatile compounds. Even the chemical synthesis depends on the structure available in nature. So, an antibiotic discovery from nature is ever-increasing field of study (Supaphon *et al.,* 2013).

Most of the rural people in the developing and under developing countries still depend on natural products or its derivatives for primary healthcare (Tamokou *et al.,* 2013). Plants are the major providers of remedy for primary health associated disorders. They have been extensively used for treating various ailments since ages. Of late, researchers are focusing their attention on the myriads of untapped endophytic microorganisms symbiotically associated with plants *(Cui et al.,* 2011). Endophytic fungi have the capability to produce the secondary metabolites similar to that of the host plant (Zhao *et al.,* 2010). These microbes are reported to produce several therapeutic molecules which are studied against various ailments (Cui *et al.,* 2011; Higginbotham *et al.,* 2013; Strobel and Daisy, 2003). Endophytes have several advantages over the host plants in which they exist such as less complexity and easy to grow in mass culture. These properties make endophytes a new avenue for discovering novel drugs (Cui *et al.,* 2011).

Tragia involucrata L. (Indian stinging nettle, Family Euphorbiaceae) is a medicinal plant having multifarious uses. Different parts of this plant has been reported to show cytotoxic (Joshi *et al.,* 2011a), antitumor (Joshi *et al.,* 2011b), antifertility (Joshi and Gopal, 2011), antimicrobial, larvicidal (Bhattacharya and Chandra, 2014), and antidiabetic (Farook and Atlee, 2011). Even though *T. involucrata* have been extensively studied for various medicinal properties, not even a single report was available on theisolation and biological properties of endophytes associated with this plant. This observation prompted us to isolate the endophytic fungi from*T. involucrata* and to study antimicrobial and larvicidal activity.

Materials and Methods

Chemicals

Potato dextrose agar (PDA), chloramphenicol, agarose, Sodium hypochlorite, ethyl acetate, potassium acetate and dimethyl sulfoxide (DMSO), were procured from Merck, India. All other chemicals used for the study were of analytical grade.

Collection of plant material

Fresh stem parts of *T. involucrata* were collected from in and around Mangalore, Karnataka, India (2013) in order

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to study the endophytic fungi associated and transported to the lab in the sterile polyethylene bags. The Global Positioning System (GPS) locations were 12.5581° N, 75.3892° E.

Isolation of endophytic fungi from *T. involucrata*

Tragia involucrata stem was washed free of adhering foreign particles with running tap water and cut into small pieces (0.6-0.8cm). Stem samples were surface sterilized by 70% alcohol, 0.5% sodium hypochlorite followed by washing thrice with sterile distilled water. The samples were inoculated in PDA medium supplemented with chloramphenicol (150mg/L)and incubated at (28±1) °C for seven days (Higginbotham *et al.,* 2013).

Microscopic observation of endophytic fungi.

Colony characters of pure cultures of isolated endophytic fungi were studied and observed under the Inverted Microscope (Magnus, INVI, Japan.).

Preparation of fungal fermentation broth and Extraction of secondary metabolites from the endophytic fungal culture

Extraction of secondary metabolites was carried according to the procedure described by Higginbotham *et al.,* (2013) with slight modification. The liquid culture with evident growth of the fungus was extracted with an equal volume of ethyl acetate (100%). The homogenate was filtered and extracted twice with ethyl acetate. The ethyl acetate extracts of *P. citrinum* CGJ-C1*, P. citrinum* CGJ-C2*, Cladosporium* sp. CGJ-D1 and *C. hypophloia* CGJ-D2 were labeled as EF1, EF2, EF3, and EF4 respectively.

Antimicrobial Activity

Bacterial cultures used in the present studies were obtained from microbial type culture collection (MTCC) and yeast was isolated from coffee effluent and submitted to GenBank (Dr. Anuappaiah). The bacterial strains were *Escherichia coli* (MTCC 118)*, Staphylococcus aureus* (FRI722)*, Bacillus cereus* (F4433)*, Micrococcus luteus* (ATCC9341)*, Listeria monocytogenes* Scott A and reference strains of *Pichia kudriviavzevii*P1 (KC841145), *Pichia kudriviavzevii* P2(KC841146), *Pichia kudriviavzevii* P3 (KC841147), *Candida tropicalis* P4 (KC841148).

Antimicrobial activity of the ethyl acetate extract of the four-endophytic fungi was tested by disc diffusion method. The plates containing brain heart infusion medium (Himedia, India) and yeast extract peptone dextrose medium were inoculated with a 24h grown inoculum of bacteria and yeast respectively. Paper discs impregnated with 200µg of endophytic fungal extracts were placed on the bacteria and yeast inoculated plates. The plates were incubated at 37°±2°C for bacteria and at 28°C for yeasts for 24 h. The diameter of the zone of inhibition was measured (mm). Standard Ampicillin sodium salt (20µg) was used for antibacterial activity and Nystatin (50µg/disc) was used for the antifungal activity(Arora and Kaur, 1999; Supaphon *et al.,* 2013).

Collection and identification of Larvae of *C. Quinquefasciatus.*

Larvae (*Culex quinquefasciatus*) were collected from the stagnant water areas of Kodagu District, Karnataka, India (The Global Positioning System (GPS) locations were 12.46700 N 75.96700 E) in rectangular trays at an average temperature of 24°C ± 2° C. The identification of morphological characters of larvae was determined by comparing with the literature (Darsie and Samanidou-Voyadjoglou, 1997; "Genus Culex - Florida Medical Entomology Laboratory," n.d.)

Larvicidal activity

Larvicidal activity of the endophytic fungal extract was carried out according to WHO protocol with slight modifications (Matasyoh *et al.,* 2011). The crude extracts of endophytic fungi were dissolved in dimethylsulphoxide (DMSO, analytical grade, Merck) and diluted to the required concentration with distilled water. The concentration of DMSO was kept below 1%. The colonies of larvae were maintained in plastic trays containing tap water. The experiments were carried out at 27 ± 2°C and 75–85 % relative humidity under 14:10 h light and dark cycles. The bioassays were performed with third instar larvae of *Culex quinquefasciatus* and carried out in triplicate using 20 larvae for each replicate assay. The larvae were placed in 50 mL disposable plastic cups containing 15 mL of the test solution and were fed a diet of Brewer's yeast, dog biscuits, and algae in a ratio of 3:1:1, respectively. The number of larvae surviving at the end of 12, 24, 36, 48 and 60hrs was recorded and the percentage mortality was determined by using mentioned below formula.

The percentage of mortality =

(No. of larva dead /No. of larvae) X100

Results and Discussion

Plants protect themselves from the pests and pathogens by symbiotically associating with the endophytes where plants provide the shelter to endophytes and in turn endophytes protect the plants against the pathogens. Endophytes with defensive secondary metabolites are selected by the host (Katoch *et al.,* 2014). Antimicrobial secondary metabolites from microorganisms have several advantages such as no destruction of resources, sustainable use, large scale industrial productions and quality control (Liang *et al.,* 2012). In the present study, we have isolated four endophytic fungi namely, *P. citrinum* CGJ-C1 (GenBank No. KT780618), *P. citrinum* CGJ-C2 (GenBank No. KP739821), *Cladosporium* sp. CGJ-D1 (GenBank No. KP739822), and *C. hypophloia* CGJ-D2 (GenBank No. KT780619). Each fungus was extracted with ethyl acetate and the characteristics of the extracts are given in Table1. Ethyl acetate extract of endophytic fungi (EF) showed a different level of inhibition of pathogenic bacteria and fungi. EF2 showed broad spectrum activity against tested organisms than other extracts. EF3 was mild antibiotic against the tested organisms. However, the extent of antimicrobial activity of other two extracts was in between the EF2 and EF3

(Table 2). The different level of antimicrobial activity of the fungal extracts may be attributed the structure and composition of chemicals present in that fungus (Teke *et al.,* 2011). Our study is in agreement with the earlier studies on endophytic fungi containing secondary metabolites with different level of antimicrobial activity (Teke *et al.,* 2011).

Table 1: Characteristics of the ethyl acetate extracts of fungi isolated from *T.involucrata*

Fungal extract	% of yield (g)	Color	Odour	Nature
EF1	0.13	Light orange	Characteristic	Crystal
EF2	0.10	Yellow	Characteristic	Crystal
EF3	0.14	Colourless	Characteristic	Crystal
EF4	0.11	Brown	Characteristic	Sticky

Table 2: Antimicrobial activity of ethyl acetate extracts of endophytic fungi.

Name of the organism	Zone of inhibition (mm) (Mean±standard Deviation)					
	EF1	EF2	EF3	EF4	NYS	AP
E.coli	10 ± 0.12	10 ± 2.1	NS	NS	NT	NT
S. aureus	NS	NS	9 ± 0.25	12 ± 0.76	NT	NT
B.cereus	NS	NS	NS	12±1.43	NT	NT
M.luteus	11 ± 0.23	12 ± 3.0	11 ±0.83	11 ± 1.22	NT	NT
L.monocytogenes	10 ± 0.11	NS	NS	13±2.11	NT	NT
P.kudriviavzevii(P1)	11 ± 0.98	12 ± 0.41	NS	NS	18 ± 2.39	14 ± 0.43
P. kudriviavzevii (P2)	8 ± 1.20	10 ± 0.17	9 ± 0.56	NS	18 ± 1.43	15 ± 0.56
P. kudriviavzevii (P3)	8 ± 0.32	10 ± 0.67	9 ± 1.15	NS	18 ± 2.32	15 ± 1.32
C. tropicalis (P4)	NS	9 ± 0.42	9 ± 1.61	9 ± 0.76	24 ± 0.98	13 ± 1.76

NYS- Nystatin; AP-Ampicillin; NS-Not Sensitive; NT-Not tested. (n=3)

Table 3: Percentage mortality of mosquito larva (*Culex Quinquefasciatus*) by EF.

EF	No hours	Larvicidal activity of endophytic fungal extract % Mortality *at different concentration (In ppm) (%, Mean ±standarddeviation) (n=3)				
		100ppm	200ppm	300ppm	400ppm	500ppm
EF1	12	0±0	5± 2.35	38.33±2.35	78.33±2.35	91.66±2.35
	24	0±0	36.66±2.35	61.66±2.35	90±4.08	100±0
	36	11.66±2.35	86.66±2.35	90±0	100±0	100±0
	48	16.66±2.35	95±4.08	100±0	100±0	100±0
	60	21.66±2.35	93.33±2.35	100±0	100±0	100±0
EF2	12	13.33 ±2.35	13.33±2.35	31.66±=2.35	61.66±2.35	70±0
	24	31.66±2.35	31.665±2.35	55±0	80±4.08	86.66±2.35
	36	33.3±2.35	40±0	60±4.08	81.66±2.35	90±0
	48	43.33±2.35	41.66±2.35	65±6.23	81.66±2.35	98.33±2.35
	60	55±0	61.66±2.35	68.33±2.35	83.3±2.35	100±0
EF3	12	5±2.35	38.33±2.35	60±4.08	81.66±2.35	100±0
	24	15±0	88.33±6.23	91.66±2.35	83.33±2.35	100±0
	36	15±0	93.331±2.35	95±4.08	100±0	100±0
	48	16.66±2.53	95±0	96.65±2.35	100±0	100±0
	60	30±4.08	98.33±2.35	100±0	100±0	100±0
EF4	12	0±0	6.66±2.35	28.33±2.35	35±4.08	46.66±2.35
	24	6.66±2.35	15±4.08	35±4.08	36.66±2.35	48.33±2.35
	36	41.66±2.35	35±0	43.33±2.35	80±4.08	91.66±2.35
	48	50±4.08	61.66±2.35	61.66±2.35	85±4.08	93.33±6.23
	60	63.3±2.35	63.33±2.35	66.66±2.35	93.33±2.35	95±4.08

Table 4: % Mortality of larva (*Culex Quinquefasciatus*) by EF.

Endophytic fungal extracts	Period of Treatment	LC_{50} (ppm)	LC_{90} (ppm)	Regression	R^2
EF1	12	26.74	126.976	Y=25.665x-34.331	0.95
	24	14.84	71.952	Y=25.334x-18.338	0.97
	36	4.68	38.436	Y=19.002x+20.658	0.64
	48	3.05	31.374	Y=17.168x+30.828	0.75
	60	2.66	30.814	Y=16.335x+33.993	0.56
EF2	12	42.18	500.6964	Y=16.167x-10.505	0.92
	24	12.911	161.466	Y=15.834X+9.494	0.93
	36	9.88	130.320	Y=15.506x+14.474	0.97
	48	6.91	99.504	Y=15x+20.996	0.94
	60	2.410	86.747	Y=11.164x+40.166	0.95
EF3	12	14.88	82.599	Y=23.33x-13.001	0.98
	24	4.25	47.990	Y=16.5x+26.114	0.57
	36	3.54	34.055	Y=17.667x+27.665	0.57
	48	3.17	32.622	Y=17.168x+30.158	0.55
	60	1.619	27.248	Y=14.167x+43.165	0.51
EF4	12	179.84	4817.449	Y=12.166x-13.168	0.97
	24	158.06	7138.800	Y=10.5x-3.17	0.95
	36	11.30	178.394	Y=14.5x+14.83	0.80
	48	3.16	120.073	Y=11x+37.33	0.92
	60	1.19	86.834	Y=9.34x+48.304	0.81

Mosquitoes and its associated microorganisms are responsible for various diseases in people living in tropical regions (Bhattacharya and Chandra, 2014). Anti-mosquito medicines derived from natural products were well received by the people due to easy biodegradation and less side effects (Rajasekaran and Duraikannan, 2012). In our study, the endophytic extracts showed a moderate to a significant level of larvicidal activity. All the extracts were active against larva even at 100ppm with EF4 extract showing the highest activity. The toxicity towards the larva was showing a positive relation with the time of incubation and concentration of EF.100 percent larval death was observed in all the extract when the concentration was scaled up to 500ppm. All the larvae were almost dead at 500ppm in EF1 and EF3 extract after 12h treatment (Table 3). The EF3 extract showed a significant larvicidal activity with anLC_{50} and LC_{90} values of 4.25 and 47.990 ppm (at 24h) respectively (Table 4). The extent of the larvicidal potential of EF3 was highest among the ethyl acetate extracts of the endophytes. Interestingly, Bhattacharya and Chandra (2011) have studied the larvicidal activity of *T. involucrata* extract (Bhattacharya and Chandra, 2014). However, the larvicidal potency of the endophytic extract was even better than the *T. involucrata* extract. Our study supports the view that the endophytes share the bioactive properties of the host plants as put forwarded by *Zhao et al.,* (2010). The endophytic extract in crude form or the bioactive principle present in it can be sprayed on the stagnant sewage water which is the breeding ground for mosquitoes. But understanding the mechanism of action of the leads present in the extract will help in designing the better strategy for mosquito menace. The endophytic extract of *T. involucrata* showed better activity than the extracts of endophytes of different plants (Matasyoh *et al.,* 2011) as well as some medicinal plants reported earlier (Bhattacharya and Chandra, 2014).

Conclusion

Overall the present study for the first time established the possible role of endophytes of *T. involucrata* in the development of antimicrobial and larvicidal agents. All the endophytic extracts selected in this study showed varying degree of bioactivity. Further studies on the isolation, characterization and understanding the mechanism of action of lead molecules will aid in the formulation of a novel as well as cost effective drugs against microbe-caused diseases.

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