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EXPLORING ENDOPHYTIC MYCOESTROGENS FROM CHHATTISGARH REGION

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ABSTRACT

Mycoestrogen are natural hormone or estrogen like compounds produced from fungi which contaminates cereals, grains etc .These fungi produce many compounds which are both helpful and harmful to human. Fusarin C and Zearalenone are some of the known compounds. In our study edible angiospermic plants of Chhattisgarh region had been chosen for exploration of endophytic mycoestrogen. The source of mycoestrogen were endophytes from five different species of plant i.e Wheat, Flax, Cucumber, Cauliflower, and Turnip. A total of 18 bacteria and 12 fungi were isolated in the study .The isolates were screened for production of lignans .The crude extract was subjected to HPTLC for confirmation of lignans.Screened extracts are subjected for their antimicrobial and antioxidant activity. Antimicrobial activity of Hexane: Ethyl acetate extract using agar disc diffusion method was evaluated against two pathogenic bacteria and two phytopathogenic fungi. All screened extracts showed inhibitory activity against at least one or more pathogenic microorganism with average zone varied between 1mm to2.5 mm against *P.avanae* and *E.coli.*

KEYWORDS: Mycoestrogen, Endophytic Fungi, Crude Extract, Antimicrobial Activity, Lignan.

Endophytes are group of microorganism that colonize inside host plant at intra cellular level (Pimentel *et al* .,2011;Singh and Dubey, 2015).Endophytes are known to provide many advantages during their mutualism with host plant .Sometimes they provide tolerance to biotic and abiotic stress and even pest and insecticide resistance. They are also capable of producing compounds like secondary metabolites and phytohormones which are of biotechnological interests (Joseph and Priya, 2011; Parthasarathi *et al.,* 2012). In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully discovered from the endophytic fungi. These bioactive compounds could be classified as alkaloids, terpenoids, steroids, quinones, lignans, phenols and lactones (Zhang HW *et al* 2006, Xu L *et al.,* 2008). Some endophytic fungi have developed the ability to produce the same or similar bioactive substances as those originated from the host plants. This is beneficial for us to study the relations between the endophytes and their host plants, and to develop a substitutable approach for efficiently producing these scarce and valuable bioactive compounds (Gunatilaka *et a.,l* 2006). Such bioactive materials are lignans. The aim of our study is to isolate and screen some endophytic fungi capable of producing lignans or its derivatives.

MATERIALS AND METHODS

Samples were randomly chosen from 15 plant growing in 3 different fields of Chhattisgarh region i.e. from Raipur, Durg and Balod district with geographical coordinates of 21.1797° N, 81.7787° E , 21.1623° N, 81.4279° E, 20.7750° N, 81.2519° E respectively . The study was conducted from the period of December 2013 to December 2014.The tissue samples from stem, leaf, and fruit/seed /tuber were collected from Flax plants *(Linum usitatissimum),* Cauliflower (*Brassica Oleraceae*), Cucumber (*Cucumis sativus),* Turnip (*Brassica rapa) and* Wheat (*Triticum aestivum*) plants respectively . Collected plant samples were washed to remove surface adherent. Approx (1x1cm) of each selected plant species. Two different sterilization protocol were referred by (Crous *et al.,* 1995) (Suryanarayan & Vijaykrishna 2001) with modification like change in sterilising agent and treatment time in order to standardizing the protocol. . Sterilized segment of plant species were imprinted on Potato Dextrose Agar and Nutrient agar media. Absence of colonies from imprint indicates successful sterilization of plant material. The sterilize segment after incubation at28+-10C and 37+- 10C were regularly observed for microbial growth. Pure endophytic culture were made and used as stock culture for further experiment.

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Statistical analysis: The data collected during study period was subjected to statistical analysis.

Colonisation rate was calculated as:

$$\frac{\text{Total no. of plant tissue segment infected one/more microbe}}{\text{Total no. of segment inoculated}} \text{ X 100}$$

(Pertini *et al* .1982)

Identification of isolates

Identification of strains that showed positive result for biochemical test were then identified at genus level on the basis of cultural morphology, pigment production, colony colour, structure of colonies, presence of wrinkle and furrow. were all taken into consideration (Holt *et al.* 1994; Silva *et al.* 2009; Verma *et al*., 2009).

Production of secondary metabolite by Liquid state Fermentation method

All 29 isolates were subjected to liquid fermentation medium in Erlenmeyer flasks (250 ml) containing 100 ml of Potato Dextrose Broth (PDB) and Nutrient Broth (NB) were autoclaved at 121°C for 15 min. After this, PDB was inoculated with mycelium plugs of actively growing cultures on PDA of the fungi. Nutrient broth was inoculated with plugs from the bacterial isolates. The flask were incubated for 3 weeks in water bath shaker at 29 ^{0}C. The culture filtrates were then extracted with ethyl acetate after 7, 12, 20 days for fungal culture and 4 and 6 days for bacterial culture respectively.

Biochemical investigation for sugar moietys in fermentation broth found in lignin

For screening of lignan producing endophyte the filtered liquid extracts after fermentation were subjected to biochemical investigation .general structure of lignan contain two sugar moiety in their ring, thus can be tested according to method adapted by (Essam F. Al-Jumaily *et al.,* 2012).The test performed were Molish, Benedict and Fehling's test.

Preparation of Crude extract

The cultures was then filtered with whatman filter paper No.1 and were dried at 40 ^{0}C .The filtered crude extract was extracted with hexane/ethyl acetate three times with 2:1 ratio of hexane / ethyl acetate and subjected to evaporation to obtain crude extract. These crude extract were then evaluated for biochemical. The strains showing positive for all three tests were selected for further secondary screening. The colur and consistency of the extract were noted .All the solvent used for the experiment were of analytical grade (Merk, Mumbai)

Identification of isolates

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Preparation of Crude Extracts

Test for lignans

0.5mL of aqueous solution of extract was added to 2mL of 2% (V/V)

furfuraldehyde in a test tube– Red color indicates the presence of flavonoids.

Qualitalive analysis of crude extract: Qualitative analysis of crude extract con

Chromatography Technique

For chromatographic technique HPTLC was chosen to perform qualitative analysis of extract**.** HPTLC is a useful analytical technique .It is combinational art of chromatographic techniques. Its main advantage is its rapidity and moderate cost.

Basic steps involved in HPTLC

- Extracts used: Hexane: ethyl acetate
- Application mode: CAMAG Linomat V.
- Development mode: CAMAG Twin Trough chamber.

Sample application

The samples were dissolved in same solvent and 10 µl quantity of sample was applied on the HPTLC silica merk 60F 254 graded plate sized 10.0 x 10.0 cm as narrow bands using CAMAG Linomat 5 injector.

Chromatogram Development

It was carried out in CAMAG Twin Trough chambers. Sample elution was carried out according to the adsorption capability of the component to be analysed. After elution, plates were taken out of the chamber and dried.

Scanning

Plates were scanned under UV at 244nm. The data's obtained from scanning were brought into integration through CAMAG software. Chromatographic finger print was developed for the detection bioactive components present in each extract.

Mobile Phase

Mobile Phase Hexane: Acetone: Ethyl Acetate (7:2:1).

Antimicrobial assay

The strains showing positive strong antioxidant activity were further analysed for their antimicrobial activity against two pathogenic bacteria *Pseudomonas aeruginosa* and *E.coli* and two fungi *Mucor* Sp. *and Apergillus* Sp.for secondary screening, agar disc diffusion method.

Disc diffusion method

The antimicrobial activity of crude extract was studied by employing agar disc diffusion method (Dilika et al 2000;Leite et al 2006).The standard inoculums prepared previously was used($5x\ 10^5$) cfu/ml . Four disc of oncentration, 200 µg/ml disc were taken to perform the test. For standard disc of Gentamycin (30 µg) (Hi media) were taken.

RESULTS

Endophytes isolated

After isolation, 31 isolates were recovered from 45samples (out of which 15 leaf, 15stem , and 15 tuber/fruit/flower).The no.of isolates and colonisation obtained with leaf were higher than those obtained from stem or fruit .The colonisation frequency of fungus in leaves were 33.3%.(Table 1).

Table 1: L-Leaf, S-Stem, F-Fruit/Flower/Tuber

Colonization Frequency of Endophytic Fungi isolated from 5 Plants															
Plant species	Wheat			Flax			Cucumber			Cauliflower			Tunip		
	COLONISING FREQUENCY (%)														
Plant Parts/ Area	L	S	F	L	S	F	L	S	F	L	S	F	L	S	F
DURG	33.3	0	0	0	0	33.3	33.3	0	0	0	0	33.3	0	33.3	0
RAHNANDGOAN	33.3	0	0	0	0	33.3	33.3	0	0	0	0	0	33.3	0	0
BALOD	0	0	33.3	33.3	33.3	33.3	33.3	0	0	33.3	0	0	0	33.3	0

Biochemical investigation for production of lignin

11Filtrates out of 29 reported positive in all three tests and were further processed for crude extract preparation.

Preparation of Crude extract

The crude extract prepared by Hexane: ethyl acetate solvent had following properties (Table 2).

Table 2: Physical Characterstics of Crude Extracts.

S.No.	Extract	Colour	Physical Nature
1	WB3	Pale brown	Powdery
2	WF2	Brownish green	Solid
3	WF3	Dark brown	Semi solid
4	FF1	Dark brown	Semi solid
5	FF2	Light brown	Crystalline
6	FF3	Brown	Solid
7	CF2	Pale brown	Powdery
8	CF3	Light brown	Solid
9	CuB3	Blackish	Sticky
10	CuF2	Dark brown	Solid
11	CuF3	Pale brown	Powdery

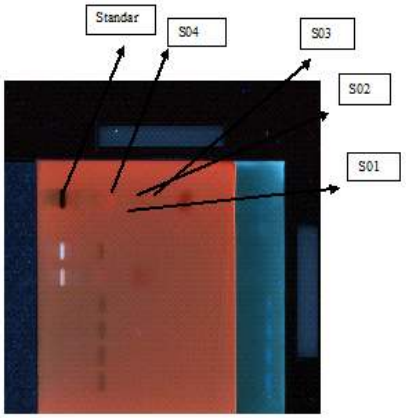
Test for lignans

Crude extracts of endophytic fungal isolates WF2, FF1, FF2, and FF3 that showed positive result for lignan test. These extract are further subjected to qualitative analysis for confirmation lignan compound in them.

Qualitaive Analysis: Chromatography Technique: For chromatographic technique HPTLC was chosen to perform qualitative analysis of extract**.** HPTLC is a useful analytical technique .It is combinational art of chromatographic techniques. Its main advantage is its rapidity and moderate cost. HPTLC method is a modern sophisticated and automated separation technique derived from Thin layer chromatography. Pre-coated HPTLC graded plates and auto sampler was used to achieve precision, sensitive, significant separation both qualitatively and quantitatively. It is a valuable quality assessment tool for the evaluating bioactive compounds efficiently and cost effectively. HPTLC method offers high degree of selectivity, sensitivity and rapidity combined with single-step sample preparation. in addition it is a reliable method for the quantitation of nanograms level of samples. thus this method can be conveniently adopted for routine quality control analysis. it provides chromatographic fingerprint of bioactive compounds which is suitable for confirming the identity and purity of raw materials. The HPLC of all crude extracts reveals significant presence of lignan in the extracts in sample 2, 3 and 4 and confirms production of mycoestrogen from fungi *aspergillus*

Figure 1: HPTLC of Extracts

Mobile Phase Hexane: Acetone: EthylAcetate (7:2:1) was used to perform HPTLC
Hptlc result suggests presence highest concentration of lignan in crude extract 04.



Antimicrobial activity

All Four extracts showed siginificant zone of inhibition against one or more microorganism. Crude extract 2 showed maximum zone of inhibition i.e 5.1 and2.8 mm against p.aeruginosa and E.coli . thus exhibit significant activity against gram negative bacteria.Regarding antifungal activities these are less significant in case of Mucor and do not show any activity against rhizopus. (Table 3.1 and 3.2).

Table 3.1: Inhibition zone of extract and Antibiotics

Crude extract Zone of Inhibition (mm)	Pathogenic bacteria	
	P.aeruginosa	***E.coli***
CE1	2	1
CE2	5.1	2.8
CE3	1.5	0.5
CE4	3.2	1.2
G	4.8	ND
CE:Crude Extract G: Gentamycin ND: Not determined		

Table 3.2: Inhibition zone of extract and Antibiotics

Crude extract Zone of Inhibition (mm)	Phytopathogenic fungi	
	Mucor	***Rhizopus***
CE1	**0.8**	**ND**
CE2	**0.4**	**ND**
CE3	**0.3**	**ND**
CE4	**0.2**	**ND**
G	**1.2**	**0.5**
CE:Crude Extract G: Gentamycin ND: Not determined		

CONCLUSION

A wide variety of endophytes have been studied as novel source of secondary metabolites. Mycoestrogen are unique class of compounds which have been in their pioneer research stage. Such compounds zearalenone, zearalenol and zearalanol (Fink-Gremmels, J.; Malekinejad, H. (October 2007) from source fungi *fusarium* had already been known.Diffrence in colonization rate had been reported in many studies and are in congruent with our findings. These difference in plant tissues of a plant like wheat *T. aestivum*) had been observed earlier(Sieber *et al.,* 1988; Larran *et al.,* 2007). Fungal taxa isolated are common

endophytes studied earlier in wheat *T. aestivum* (Larran *et al.* 2002; Larran *et al,*. 2007).As all endophytes were recovered from healthy tissues lead to suggest that they were a virulent or virulent in latent phase (Pertini, 1991).

Liquid state fermentation technique is most common method of obtaing high yield of secondary metabolite and crude extract from endophytic microbes.

Screening on the basis of sugar moiety present in extract had been investigated by other researchers also his agrees with AL-Awaad (2001) and AL-Shemary (2004) Lignans are polyphenolic compounds and researchers had investigated biochemical screening on tfor identification of sugar moiety present in extracts

Many endophytes had been reported to produce antimicrobial compound that were active aganist human pathogenic microorganism .Exhibition of such activity had been reported by (Chareprasert *et al.,*2006). Crude extract 2 showed maximum zone of inhibition i.e 5.1 and 2.8 mm against *P.aeruginosa* and *E.coli* . Thus exhibit significant activity against gram negative bacteria.. Lignan containing Crude extracts showed significant inhibition against gram positive bacteria whereas are very little or non-significant against fungi *Mucor* and r*hizopus* . .These results were supported by study made by Ioana Andreea 2014.Our study suggest that these endophytes have the potential to be a source for novel bioactive products.

The present results may lead to the conclusion that endophytes are considered to be a potential source for novel bioactive products (Strobel 2003). The data presented in this study demonstrated that extracts of endophytic fungus *isolated* from wheat and flax have antimicrobial, especially *Aspergillus* sp.1 , *Aspergillus* sp.2 and , *Aspergillus* sp.3 , Endophytic fungi might also represent an alternative source for the production of therapeutic agents that are not easily obtained by chemical synthesis, and which have a high activity against pathogenic microorganisms. However, this work will serve as a prelude to more comprehensive studies on the chemistry and biology of the bioactive natural products produced by these endophytes. Further examination can be done to learn if endophytes may have the potential to serve as a biological control or as new pharmacological agents.

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