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HR-LCMS of phytoconstituents and antifungal activity of medicinal plants

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Abstract
Casuarina equisetifolia and *Annona squamosa* are very unique plants having large number of medicinal values. We analyzed major chemical constituents that are present in methanol extract of leaves through HR-LCMS techniques. The compounds include: Lyxosylamine, Isovaleric acid, Taurine, Minoxidil, 4-Trimethyl Ammoniobutanal, 6 beta-Naltexol-3-glucuronide, Glucosylgalactosyl hydroxylysine, Dihydromyricetin, Dihydrorobinetin, Rutin, Cosmosiin, Barbituric acid, 5-ethyl-5-(2-hydroxyethyl), 2,2,9,9-tetramethyl-undecan-1,10-diol, Dihydrodeoxystreptomycin, Hexadecanedioc acid, Ethosuximide M5, Sinomenine, Hydroxyanastrozole, 7-Desmethylpapaverine, Antifungal properties were estimated by the food poison method. The results showed that antimicrobial components were separated and identified by HR-LCMS from the leaves of *Casuarina equisetifolia* and *Annona squamosa.* Antifungal activities were found against *Fusarium oxysporum* and *Colletotrichum capsici.*

Keywords: *Casuarina equisetifolia, Annona squamosa,* HR-LCMS, *Fusarium oxysporum, Colletotrichum capsici*

1. Introduction
Medicinal plants are very important for relief from various diseases can be traced back over thousands of years in India. Due to presence of various chemical constituents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs. (Srivastava *et al*., 1996) Green plants are an important source of various chemical compounds which are used for mankind for thousands of years to cure diseases. It contain many chemical compounds such as alkaloids, flavonoids, glycosides, phenols, resins, steroids, saponins, tannins and volatile oils which were deposited in their specific parts such as flowers, fruits, bark, leaves, root and seeds etc. (Tonthubthimthong *et al*., 2001) [7]. Dandekar *et al*., (2015) [9] also evaluated the alcohol extract of *Epiphyllum oxypetalum* contains secondary compounds like Megastigmatrienone Cycloocta-1,3,6-triene,2,3,5,5,8,8,-hexamethyl; 4-((1E)-3-Hydroxy-1-propenyl)-2- methoxyphenol; 2,5-Dihydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one; by GC-MS analysis. Mustapha *et al*., (2016) reported that the isolation and identification of bioactive compounds from the crude extracts of *A. adianthifolia* and *P. angolensis* against *E. coli, P. aeruginosa, B. subtilis, S. aueus* and *C. albicans* strains.
Yong-Chun Jin *et al*., (2011) [11] reported that 63 components were separated and identified by GC/MS from the varieties of bamboo leaves. *cis*-3-Hexenol, whose content in cv. *Pubescens*, *Gracilis*, *Heterocycla* and *Ph. kwangsiensis* was 27.11%, 24.62%, 30.51% and 34.65%, respectively, which having Antioxidant and Antimicrobial Activities. Sarkar S. *et al*. (2013) [4] studied that aqueous latex extract of *Calotropis gigantea* contains alkaloid, tannin, saponin and glycosides. but it don't showed antimicrobial activity. The present investigation was revealed to identify new plant based compounds against *Fusarium oxysporum* and *Colletotrichum capsici* using *C. equisetifolia* and *A. squamosa* extracts. In this regard, Primary screening was carried out to find the Antimicrobial activity of the plant extract. Further, based on the primary phytochemical screening, high resolution liquid chromatography and mass spectrometry (HR-LCMS) was performed to separation and identification of the phytoconstituents based on their retention time and data base difference from the crude extracts showing good antifungal activity.

2. Materials and Methods
2.1 Collection of Plant Material
The fresh and Healthy leaves of both the plants were collected from campus of Government

institute of Science Aurangabad, during December 2014. The identification is done with the help of standard floras (flora of Marathwada by V.N. Naik *et al.*, 1998) [2]. The leaves were shade dried, powdered and stored in airtight container for further study.

2.2 Preparation of plant Extract

About 30 gm of leaves powder were subjected to Soxhlet extraction with 300 ml of the methanol for 8 to 10 hrs (60-70^0 c**)**, leaves powder were successively extracted with methanol.

3. Antifungal activity of plant extract

The food poisoned technique (Schmitz, 1930) [5] was used to test the antifungal activity of the extracts. The cultures were prepared and incubated at 37^0 ± 1^{0}c for 24 hours. The antifungal activity was observed on basis of inhibition zone that was compared with standard fungicides. 5% Extract was added with Potato Dextrose Agar & poured in sterile Petri plates. Fungal Disc of 4 mm of 7 days old culture were used for inoculated aseptically on Potato Dextrose Agar plates were incubated at 37^0 ± 1^{0}c for 24 hours and the diameter of zone of inhibition of fungal growth was measured in mm.

4. Antifungal activity of fungicide

The antifungal activity of the fungicide propacanozole was used to study the comparative account of fungal inhibition by using the food poisoned technique (Schmitz, 1930) [5] with reference to the antifungal activity of the plant extract.

5. High resolution liquid chromatography and mass spectrometry (HR-LCMS) analysis

The extract was prepared in methanol and then subjected to HR-LCMS analysis. The

HR-LCMS of sample was carried out in Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay, Pawai, Mumbai. Chemical finger prints of two selected medicinal plant extracts were prepared by Agilent high resolution liquid chromatography and mass spectrometry model- G6550A with 0.01% mass resolution. The acquisition method was set to be MS- minimum range 50 *(M/Z)* and maximum 1000 dalton *(M/Z)* with scanning rate each spectrum per second. Gas chromatography had maintained at 250 ^{0}C with gas flow 13 psi/minute.

Hip sampler with model- G4226A was used with auxiliary speed 100 µl/minute, ejection speed 100 µl/minute, flush out factor 5µl and 8µl injection volume used for HR-LCMS. Within 30 minutes Acquisition time, initial 2 minutes the flow of solvent composition A : B was 95 : 5.

Solvent was use for HR-LCMS.

1. 100% Water
2. 100% Acetonitrile

Table 1: Bioactive compounds in methanol extract of *Casuarina equisetifolia.*

Name of Compound	RT (min)	Mass	Formula	DB Diff (ppm)
Minoxidil	1.79	209.1267	C9 H15 N5 O	4.76
6beta-Naltrexol-3- glucuronide	8.386	519.2101	C26 H33 N O10	0.59
Pentahydroxyflavanone	9.655	304.0564	C15 H12 O7	6.15
Dihydrorobinetin	9.974	304.056	C15 H12 O7	7.55
Barbituric acid, 5-ethyl-5-(2- hydroxyethyl)-	10.568	200.0794	C8 H12 N2 O4	1.37
Dihydromyricetin	11.48	320.0509	C15 H12 O8	7.26
Idebenone Metabolite (Benzenebutanoic acid, 2,5- dihydroxy-3,4-dimethoxy-6- methyl-)	12.648	270.1102	C13 H18 O6	0.35
2,2,9,9-tetramethyl-undecan- 1,10-diol	12.806	230.2246	C14 H30 O2	0
Dihydrodeoxy-streptomycin	12.929	567.2863	C21 H41 N7 O11	0.25
Hexadecanedioic acid	14.261	286.2144	C16 H30 O4	0.1
Tetranor Iloprost	15.072	306.1809	C18 H26 O4	7.15

Table 2: Bioactive compounds in methanol extract of *Annona squamosa*.

Name of Compound	RT (min)	Mass	Formula	DB diff (ppm)
Capryloylglycine	1.784	201.1352	C10 H19 N O3	6.29
Hydroxyanastrozole	1.848	309.1587	C17 H19 N5 O	0.86
4-(2-hydroxy-3- isopropyl- aminopropyl)benzoic acid	5.725	253.1302	C13 H19 N O4	4.86
(S)-Reticuline	7.74	329.1611	C19 H23 N O4	4.96
Tranilast	8.098	327.1105	C18 H17 N O5	0.42
Anastrozole	8.213	293.1638	C17 H19 N5	0.88
Dihydromyricetin	8.513	320.0516	C15 H12 O8	5.17
Rutin	8.514	610.1518	C27 H30 O16	2.63
Sinomenine glucuronide	8.881	329.1611	C19 H23 N O4	4.81
6beta-Naltrexol	9.416	343.1768	C20 H25 N O4	4.54
7- Desmethylpapaverine	9.719	325.1305	C19 H19 N O4	2.82
Ambelline	10.912	331.1403	C18 H21 N O5	5.04
Ethoxyquin	14.897	217.1449	C14 H19 N O	7.95

Chromatogram of methanolic Extract of *Casuarina equisetifolia* and *Annona squamosa*

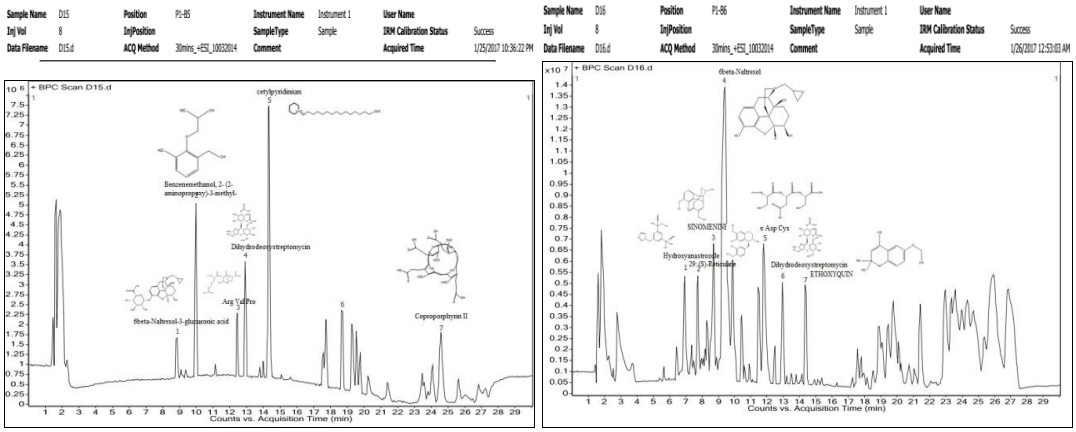


Table 3: Mycelium growth of fungal disc in mm

Fungal pathogen	7 days growth of mycelium against *C. equisetifolia* Extract (triplicate)			Mean	7 days growth of mycelium against *A. squamosa* Extract (triplicate)			Mean	7days growth of mycelium against Fungicide (Propacanozole) (triplicate)			Mean
Fusarium oxysporum	52	53	54	53	43	44	42	42	63	63	63	63
Colletotrichum capsici	30	30	30	30	20	19	21	20	31	30	32	31

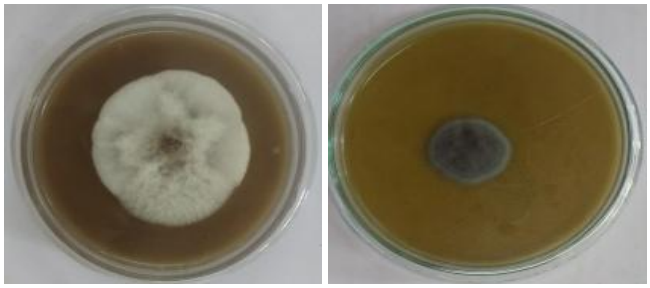


Photo Plate 1: Antifungal activity of Leaves Extract of *C. equisetifolia*

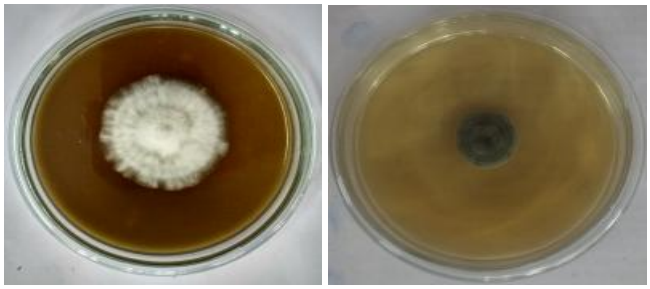
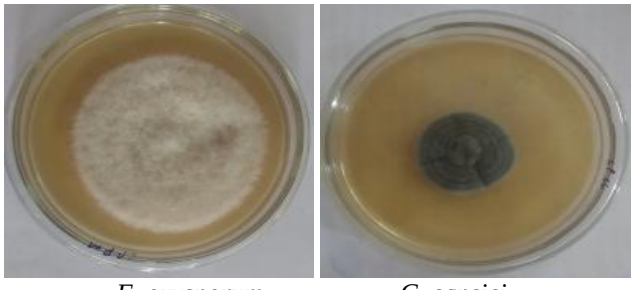


Photo Plate 2: Antifungal activity of Leaves Extract of *A. squamosal*



F. oxysporum *C. capsici*

Photo Plate 3: Antifungal activity of Fungicide (Propacanozole)

5. Phytochemical screening

Methanolic Extract of *Casuarina equisetifolia* and *Annona squamosa* recorded the presence of saponins, tannins, alkaloids, glycosides, steroids and flavonoids, through the qualitative analysis.

Table 4: Phytochemical analysis of Methanolic Extract of *C. equisetifolia* and *A. squamosal*

Phytochemical	*Casuarina equisetifolia*	*Annona squamosa*
Tannins	+++	++
Alkaloids	-	++
Saponins	++	+++
Glycosides	+++	-
Flavonoids	++	++
Steroids	+	++

+++ strongly present, ++ present, + weekly present, - absent

6. Result and Discussion

HR-LCMS analysis of methanol extract of *Casuarina equisetifolia* and *Annona squamosa* leaves showed respectively 9 and 6 major peaks indicating the presence of various phytochemical constituents. On comparison of the high resolution liquid chromatography and mass spectra of constituents with the main library all these compounds were charachterised and probably identified. Identified compounds is Dihydromyricetin, Dihydrorobinetin, Rutin, Cosmosiin, Barbituric acid, 5-ethyl-5-(2-hydroxyethyl), 2,2,9,9-tetramethyl-undecan-1,10-diol, Sinomenine, Dihydrodeoxystreptomycin, Hexadecanedioc acid, Ethosuximide M5, Hydroxyanastrozole, 7-Desmethylpapaverine, Lyxosylamine, Isovaleric acid, Taurine, Minoxidil, 4-Trimethyl Ammoniobutanal, 6 beta-Naltexol-3-glucuronide, Glucosylgalactosyl hydroxylysine. Tannins Alkaloids Saponins Glycosides Flavonoids Steroids were also reveals presece in both the plant extracts by simple phytochemical method.

Antifungal activity was carried out by food poison techniques, According the recorded antifungal assay (Table I), the plant extract showed maximum inhibitory effect against both selected plant pathogenic fungi in comparison with fungicide (Propacanozole). But it Showed excellent result against *Colletotrichum capsici* even a very good result showed against *Fusarium oxysporum* than fungicide Through the study the results clearly reveals that the plant extract play the important role in controlling the plant diseases. Ramdas *et al*. (2006) [3] revealed that the phytochemical plays an important role in the treatment of diseases without any side effects, there is a need to search new drugs from natural

sources. India is a home to a variety of traditional medicine system that relay to a very large extent on native plant species for new drug materials. Therefore now there is a need to look back towards traditional medicine which can serve a novel therapeutic agent (Chitravadivu *et al*., 2009) [1]. The pharmacognostical evaluations also give valuable information which is essential to standardize the drug.

7. Conclusion

The result of HR-LCMS analysis specifies that the methanol extract of *Casuarina equisetifolia* and *Annona squamosa* leaves contains various valuable secondary compounds which have various medicinal properties that can be useful for the treatment of various diseases. The study reveals the vital role of phytochemical which are released in the form of secondary compounds in controlling the fungal plant diseases without effecting the environment helping in reducing the soil salinity and increase the fertility.

8. References

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