DOI: 10.21276/ijabpt.2016.7.4.22

INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com ISSN : 0976-4550

Volume-7, Issue-4, Oct-Dec-2016 Coden IJABFP-CAS-USA Copyrights@2016

Received: 6th Sept 2016 *Revised: 18th Oct 2016* *Accepted: 20th Oct 2016*

DOI: 10.21276/Ijabpt, http://dx.doi.org/10.21276/ijabpt ***Research article***

A POTENTIAL SOURCE OF METHYL-EUGENOL FROM SECONDARY METABOLITE OF *RHIZOPUS ORYZAE* 6975

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ABSTRACT: Fungi have great opportunities for the discovery of novel compounds. Taxol, an anti-cancer compound was produced by *Taxomyces andreanae* (an endophytic fungus) isolated from yew tree (*Texus brevifolia*); similarly, *Muscodor vitigenus* produce an insect repellent (naphthalene) and methyl eugenol produced by *Alternaria* spp isolated from Rosa damascene. Likewise, many other reports are available on the production of secondary metabolites by fungal endophyte. It has been reported that some fungal endophytes produce the samebioactive compounds as their host plants, which can be helpful in reduction of harvesting of slow growing and possibly limited plants. It would contributed to reduction of worlds diminishing plant biodiversity along with curtailing its market price. Here, we investigated a volatile compound produced by an endophytic fungus *Rhizopus oryzae* isolated from *Holarrhena pubescens* tree is grown for their ornamental beauty, fragrant flowers and produce of aromatic compounds. The research work done to affirming of production of methyl eugenol by endophytic fungus *Rhizopus oryzae*. Therefore, to proven this concept 21 isolates of six different *Rhizopus* spp were selected which was isolated from various sources/background.

Key words: Natural products, Biomaterials, Chromatography, Food effects, Mass Scpectroscopy, Molecular diversity, Antiinfectives

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INTRODUCTION

More than 450 plant species contains methyl eugenol in various amounts and its use in various forms as this chemical plays in nature, especially in the interactions between tephritid fruit flies and plants (Tan and Nishida, 2012). Kurchi or bitter oleander (*Holarrhena pubescens (*Buch. Ham.) Wall. Ex G. Don. (Synonym: *H. antidysentica* L. Wall.) belongs to family Apocynaceae is a large shrub to small deciduous tree with white flowers and found throughout the dry forests of Indian subcontinent which produced about 30 steroidal alkaloids (Bhattachartya et al., 2009) . This phenylpropene compounds such as eugenol, iso-eugenol and methyl-eugenol are synthesized by plants to serve in defense themselves against insect-pests, pathogens and to attract pollinators. The phenylpropenes compound is mainfloral scent bouquet of many plant species. Such as the *Clarkia breweri* flowers emits a mixture of volatiles that include eugenol, isoeugenol, methyleugenol and methylisoeugenol (Raguso and Pichersky, 1995). This naturally occurring methyl eugenolfounds in oils and fruits and in various foods as flavoring agent (Sudhakar et al, 2009). As an essential oil Methyl eugenol is sold for use in aromatherapy, massage oils and alternative medicines (Government of Canada, 2010).

Like plants, some fungi have great possibility for the discovery of novel bioactive compounds. Taxol is an anti-cancer compound produced by *Taxomyces andreanae* (endophytic fungus) which was isolated from yew tree, *Texus brevifolia* (Stierle et al, 1993; Stone, 1993, 2001); *Muscodor vitigenus* produces naphthalene an insect repellent(Stone, 2001) and methyl eugenol produced by *Alternaria* spp isolated from *Rosa damascene* Mill (Kaul, et al, 2008). Similarly, many other reports are available on the production and isolation of secondary metabolites by fungi (Raviraja, 2005; Zang et al, 2006).

The horizontal gene transfer (HGT) was common in many cases as fungal endophytes within same plants could probably also able to produce the bioactive compounds produced by the plants (Chandra, 2012). Though, biosynthetic gene clusters have been described as common features in bacterial and fungal genomes (Zang et al., 2004). A few reports of clustered metabolic pathways found in plants has exit and spread over larger genomic regions than their microbial counterparts (Field et al., 2011; Chu et al., 2011). However, a rare evolutionary event of HGT occurs between distantly-related organisms which is also constrained by the amount of genetic information transferred and genetic barriers involving ill-assorted regulation and codon usage. This disparities with the widespread observation of Taxol biosynthesis in many different endophytic fungi (Kurland et al, 2003).

It has been reported that some fungal endophytes produce the same, rare and potential bioactive compounds as their host plants, which can be reduce the need to harvesting of slow growing and possibly limited plants. It would contributed to reduction of worlds diminishing plant biodiversity along with curtailing its market price. Here, we undertake this piece of work on a concept of production of same volatile compounds by their endophytic fungus as it produced by their plant counterpart after long association. *Holarrhena pubescens* tree is grown for their ornamental beauty, fragrant flowers and produce of aromatic compounds. The main objective of the research work being to affirming of production of methyl eugenol by endophytic fungus *Rhizopus oryzae* and its applications. Therefore, to proven this concept 21 isolates of six different *Rhizopus spp* were selected which was isolated from various sources/background.

MATERIALS AND METHODS

Source of fungus culture: Strains of *Rhizopus spp* (21 strains) were selected and taken from Indian Type Culture Collection, Division of Plant Pathology, ICAR-IARI, New Delhi. The strains were chosen for this study having different background sources on which it have been associated are given in Table 1.

Total 21 isolates were taken (14 isolates of *Rhizopus oryzae*, two isolates of *R. stonilifer,* and one each of *R. nigricans, R. microspores* var. *rhizopodifermis, R. nodorus, R. arrhizus* and two of unknown species of *Rhizopus)* and screened for the production of volatile metabolites. Production of the secondary metabolites was done by solid state fermentation. The selected fungal isolates were grown on potato dextrose agar and actively growing mycelial discs were inoculated into 500 ml Erlenmeyer flasks containing 30 gram autoclaved maize grains. Such inoculated flasks were kept at 26^0C in a B.O.D. incubator for 21 days. Extraction of secondary metabolites from solid state fermentation were done as maize kernels were transferred to a screw-cap vials and then added 10 mL of solvent mixture of methanol: Dichloromethane: ethyl acetate(1:2:3) containing 1% (v/v) formic acid. These vials were exposed to ultra-sonication for 15 minutes. The extracted material were transferred to clean vials and do freeze dry with nitrogen gas. The brown crude extract of all samples were subjected to use for thin layer chromatography and three of them were selected forGC-MS analysis.

Gas Chromatography-Mass Spectrometry Analysis

The analysis of fungal extract was carried out using Focus-DSQ GC/MS (Thermo) equipped with TG-5MS capillary column (30m x 0.25mm i.d.; film thickness 0.25 μm).Chromatographic conditions were as follows: injector temperature was 260 °C, helium as carrier gas at a flow-rate of 1 ml/min and injection volume was 0.5μl (10 mg extract in 10ml hexane), respectively. The column temperature was held at 60 °C and programmed at 5°C/min to 250 °C and held for 5 minute with split ratio of 1:20. The MS transfer line and source temperatures were 270 °C and 230 °C. The GC column was coupled directly to single quadrupole mass spectrometer in EI mode at 70eV with the mass range of 35-400 a.m.u at 1 scan/s. The identification of individual compound were carried out on the basis of retention time by comparing their mass spectra with NIST Mass Spectral Library (Ver. 2, 2005) and literature (Adams 2007).

RESULTS

All the samples were used for thin layer chromatography (TLC). On the basis of TLC two similar and on different metabolic profile of crude metabolites of three strains of *Rhizopus oryzae* was selected to analyze through GC/MS. The total chromatogram was obtained for each sample.

The base peak of each spectrum was compared with the base peak of the chemical components in the NIST Ver.2005 MS data library through on-line and comparing the spectrum obtained through GC/MS the compounds present in the crude toxin sample were identified. All the experiments were carried out in triplicates to confirm the reproducibility of the results. GC/MS analysis of metabolites yielded several compounds. List of compounds are tabulated below.

Identification of chemical constituents from *Rhizopus* extract

The major volatile compounds identified from fungal extract were methyl eugenol, 2,4-di-ter-butyl phenol, dodecane, heptadecane and nonadecane. 4.84 % of methyl eugenol was estimated to be present in the extract (Table 2 and 3). For the first time, we have been reporting the presence of methyl eugenol in fungal extract. It is reported to have antimicrobial, insecticidal and nematicidal properties.

This strain of *R. oryzae* is producing methyl eugenol which is an important secondary metabolite have liquid state, colourless to pale yellow, melting point of -4^{0}C and boiling point 254.7^{0}C.Generally, volatile oils obtained from plant sources have commercial significance. Though this compound biosynthesis in plants is regulated at the subcellular level and endophytes could be involving in that.

Table 1. List of strains of *Rhizopus spp* used in this study.

S.No.	Strain no.	*Rhizopus* species	Sources
1.	128	*R. arrhizus*	*Malus sylvestris* Quella
2.	594	*R. oryzae*	NRRL 1528
3.	1584	*R. nigricans*	Contaminant
4.	1778	*R. nodosus*	Ant, New Delhi
5.	2412	*R. stolonifer*	*Dolichos sp* seed
6.	3068	*R. oryzae*	CBS
7.	3070	*R. microspores* var. *rhizopodiformis*	CBS
8.	3072	*R. oryzae*	CBS
9.	4408	*R. oryzae*	*Momordica charantia*
10.	**4409**	*R. oryzae*	Unknown
11.	4410	*R. oryzae*	Fermented *Shorghum*
12.	4423	*R. stolonifer*	IMI 090610 UK
13.	4698	*R. oryzae*	Red cotton insect
14.	4699	*R. oryzae*	Kisan tomato puree
15.	5280	*Rhizopus sp.*	Unknown
16.	**5281**	*R. oryzae*	Jam
17.	5725	*Rhizopus sp.*	Unknown
18.	6003	*R. oryzae*	Soils from Rorkee
19.	6457	*R. oryzae*	Unknown
20.	6458	*R. oryzae*	Unknown
21.	**6975**	*R. oryzae*	*Holarrhena pubescens*

Table 2. Compounds identified from strains of *Rhizopus oryzae* through GC/MS

S.No	RT	Name of the compound	4409	5281	6975
	4.641	methyl cyclopentane		+	
	17.03	Benzene, 1,2-dimethoxy-4-(2-propenyl)-			+
	28.028	1-(tert-Butyl)-3-cyclohexylcarbodi	+		
	29.00	Benzothiazole, 2-(2-hydroxyethylthio)			+
	36.073	9,12-Octadecadienoic acid, methyl ester,	+		
	44.511	5,6,8,9- Tetramethoxy-2- methylpepper		+	
	44.546	1-(1-Adamantylcarbonyl)-4-(2-furyl carbonyl)piperazine		+	
	45.652	2,4-diphenyl-5-(p-methoxybenzoyl)- 1,2,3-triazole	+		
	46.282	2-(2-Amino-benzoimidazol-1-yl)-1-4-methoxy-phenyl)-ethanone		+	

Table 3. Volatile compounds identified from fungal extract.

S. No.	RT	Compounds
1	16.9	Dodecane
2	17.05	Methyl eugenol
3	19.75	2,4-di-ter-butyl phenol
4	24.03	Heptadecane
5	28.24	Nonadecane

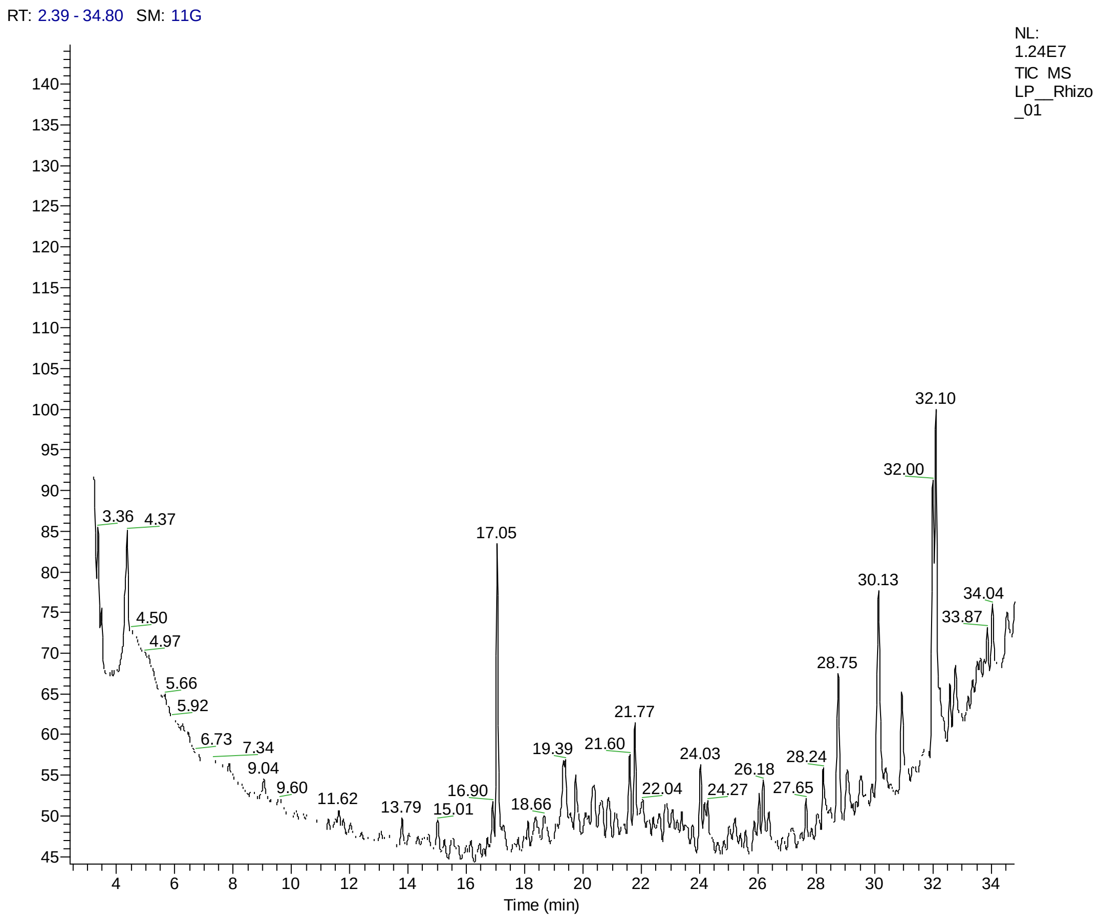

Fig. 1: Total ion chromatogram (TIC) of fungal extract produced *Rhizopusoryzae* (6975) isolated from *Holarrhenapubescens* and mass spectrum of methyl eugenol at the retention time of 17.03 minutes of the mixture.

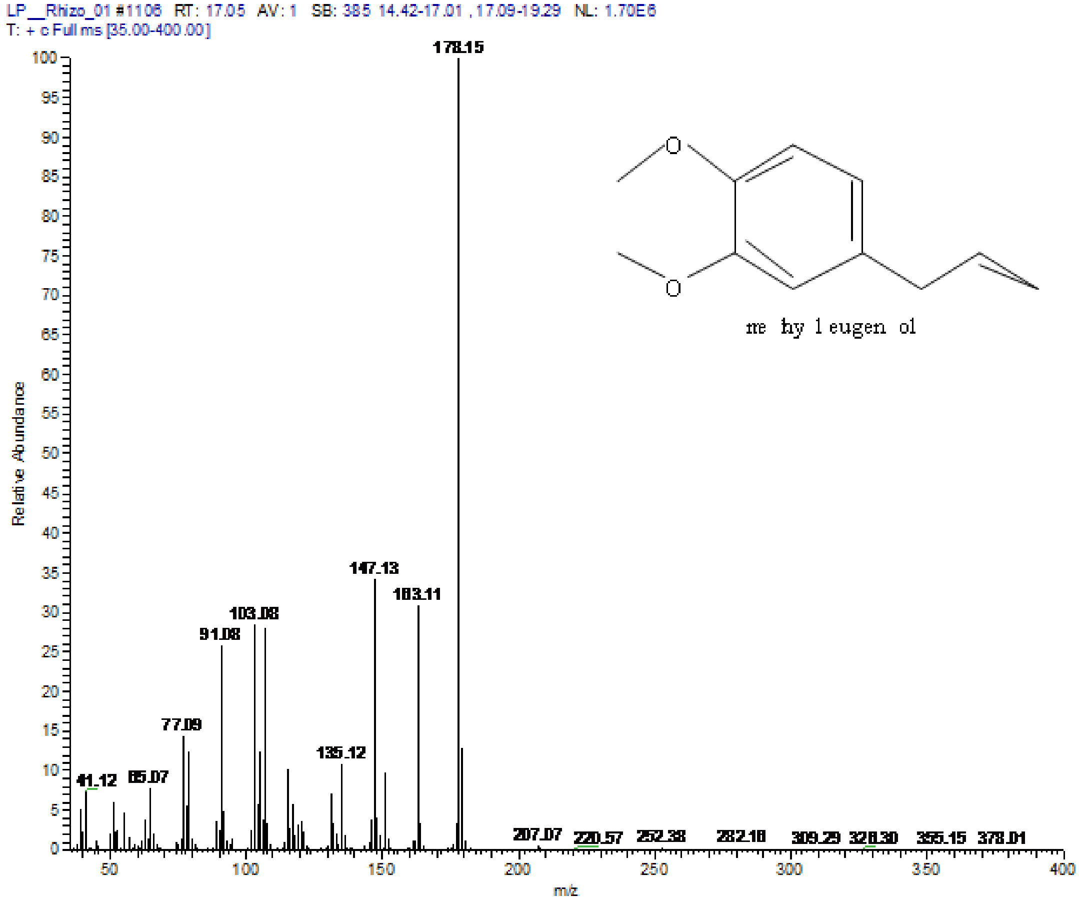

Fig. 2 Mass spectra of methyl eugenol present in fungal extract

DISCUSSION

Methyl eugenol have many important applications such as cosmetics, aromatherapy, flavoring agent in jellies, baked foods, non-alcoholic beverages, chewing gum, candy, ice-cream, pudding, and relish. Its antifungal activity was studied 16 aroma chemicals was *in vitro* against five seed-borne fungal pathogens and found that methyl eugenol was active antifungal compounds (Dev *et al*, 2004). An experiment on methyl eugenol in inhibiting *A. flavus* colonization and aflatoxin production on peanut pods and kernels was conducted and found the spray of methyl eugenol (0.5%) on peanut pods and kernels checked the colonization of *A. flavus* and aflatoxin synthesis. This chemical can be used as both prophylactic or post infection spray on peanut pods before storage. It is the first report on the inhibition of *A. flavus* by methyleugenol on peanut (Sudhakar et al, 2009). It also have an aesthetic in rodents, well known insect pheromones and can be used as an insect attractant in combination with insecticides. Though, there sufficient availability of synthetic methyl eugenol, which has been tested as harmful nature.

CONCLUSION

As per our knowledge it is first report of this compound from the fungus-*Rhizopus oryzae*, which is a saprophytic and endophytic in nature. It can be said that aroma chemicals hold immense potential for pharmaceutical and cosmetic industry. This Methyl eugenol are very useful food additive, antifungal, antibacterial, insect-attractant (insect sex-hormone), therefore, it have great economic significance.

ACKNOWLEDGEMENT

The Authors are thankful to Head Plant Pathology for all kind of facility and Dr N.A. Shakil of Division of Agricultural Chemicals for GC-MS analysis.

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ISSN : 0976-4550

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