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IDENTIFICATION AND CHARACTERIZATION OF FUMIGANT FUNGITOXIC PRINCIPLE ISOLATED FROM THE ESSENTIAL OIL OF AJWAIN SEEDS

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ABSTRACT

Essential oils were extracted from different parts of ten angiospermic plants belonging to eight different families by hydro-distillation technique in a Clevenger's apparatus. These oils were tested for their fungitoxicity against *Rhizoctonia solani* Kuhn by Poisoned Food Technique. The oil isolated from the Ajwain seeds showed 100% fungitoxicity against the test fungus at a concentration 0.5 X 10^3µl/l. To isolate the active fungitoxic principle from the essential oil of Ajwain seeds (*Trachyspermum ammi*), the oil was mixed thoroughly with 10% sodium hydroxide solution in a separating funnel. The fraction, soluble with NaOH solution was separated and extracted with ether in a separating funnel. The non-ethereal fraction was acidified with acetic acid and again extracted with ether. The ethereal fraction was separated from non-ethereal aqueous fraction. The ethereal fraction was then distilled under reduced pressure in order to remove ether. After removal, a pungent smelling oily liquid was obtained. Repeated harvesting of the liquid yielded a yellow crystalline compound. The compound was characterized on the basis of spectroscopy analysis, using UV, IR and NMR spectra.

KEYWORDS: Essential Oil, Ajwain Seeds, Fumigant Fungitoxic Principle, *Rhizoctonia solani*

India is rich in medicinal plants biodiversity. In our country more than eight thousand species of plants are distributed throughout the different geographical regions. These plant species have been used by local population for the treatment of diseases in human, animals and plants since centuries and recently these are being used in the formation of various medicines, drugs and food (Bakhru, 1998).

"Ayurveda" the Indian system of medication, is the oldest known system of medication throughout the world. The word Ayurveda" is a Sanskrit word, which means 'The science of life'. The knowledge about herbs and their role in treatment of several diseases is well documented in the Atharva Veda. The pesticidal potentialities of higher plants and the products derived from them were recognized even in prehistoric periods (Mohammed, 1983) these herbs and their products are now being commercialized in various formulations for the treatment of several diseases and about twenty five percent of them are derived from higher plants.

Angiospermic plants represent a rich source of antimicrobial agents. Eessential oils derived from plants have been used for centuries as fumigants and may act as antifungal fumigants (Burt, 2004). Essential oils, the volatile secondary metabolites of plants, are biodegradable, eco-friendly and have least or no side effects on human health. Therefore, these can be used as fungitoxic agents to control plant diseases (Park *et al.*, 2005; Akhtar *et al.*, 1997). The volatile nature makes the essential oil more penetrating and therefore helps in eradication of deep-rooted pathogens. Essential oils contain several components out of them some are active antimicrobial components.

Trachyspermum ammi is a member of the family Apiaceae, which comprises more than 2,500 members. The commonly used Hindi name of *Trachyspermum ammi* is 'Ajwain'. The seeds of *Trachyspermum ammi* mostly found in Indian cooking system to add pleasant flavour to food. It is considered to be very useful in aroma therapy.The essential oil extracted from the seeds of *Trachyspermum ammi* showed hundred percent fungitoxic efficacy against *Rhizoctonia solani* Kuhn., the causal fungus of several plant diseases like black scurf of potato tubers, damping off of soybean seedlings, base rot of lettuce, root rot of sugar beet, belly rot of cucumber, sheath blight of rice, wire stem of tomato and crucifer seedlings etc.

Keeping this in view, the present project was under taken to investigate the fungitoxic efficacy of *Trachyspermum ammi* essential oil and to characterize active fungitoxic constituent from the essential oil. This project was also aimed to explore the possibility of developing plant-based formulations for the control of *Rhizoctonia solani* Kuhn. and thereby several diseases caused by this fungus.

MATERIALS AND METHODS

Extraction of Essential Oil

Three hundred gram seeds of *Trachyspermum ammi* was placed in a sterilized flask containing 2% sodium hypochlorite solution for about five minutes for surface sterilization and then rinsed in sterilized distilled water to remove the traces of sodium hypochlorite

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solution. These seeds are then subjected to hydro-distillation process in a Clevenger's apparatus for about 8-10 hours, as a result two different layers, the upper oily layer and the lower colourless watery layer, was appeared. The oily layer was collected whereas colourless watery layer was discarded. The collected oil was stored at 4 ± 1°C for future use.

Culture of Test Fungus

The essential oils were assayed for fungitoxic efficacy against the fungal pathogen *Rhizoctonia solani* Kuhn. (MTCC No. 4633) obtained from Microbial Type Culture (MTCC), Chandigarh. This fungus was grown on PDA plate at 27^0C ± 2^0C and maintained with periodic sub-culturing at 4^0C.

***In vitro*Screening of Essential Oil against Test Fungus**

The study of fungitoxic efficacy of essential oil was done by Poisoned Food Technique (New *et al*., 1971). 1 ml desired concentration of the essential oil was mixed with 9 ml of molten Potato Dextrose Agar medium in a sterilized Petridish to prepare treatment sets. The essential oil and the medium both were mixed thoroughly to get a homogenous solution. In the similar way control sets were prepared, however in these sets the essential oil was replaced by the same amount of sterile distilled water. A fungal disc of 5 mm in diameter was cut with the help of flame sterilized cork borer, from the 7 days old culture of *Rhizoctonia solani* Kuhn and placed in the centre of the Petridish. All the Petridishes were incubated at 27 ± 2°C for 7 days. Three replicates were used for experiments. After seven days of incubation, the diameters of the colony were measured in mutual perpendicular directions and the fungitoxic efficacy of oil was calculated in terms of the percent mycelial inhibition with the help of following formula (Singh and Tripathi, 1999).

$$\text{Percent inhibition of mycelial growth} = \frac{dc - dt}{dc} \times 100$$

Where:

dc – Average diameter of fungal colony in control sets.

dt – Average diameter of fungal colony in treatment sets.

Isolation of the Active Fungitoxic Constituent from Essential Oil of *Trachyspermum ammi*

Five ml essential oil of *Trachyspermum ammi* was thoroughly shaken with 25 ml of 10% sodium hydroxide solution in a separating funnel. The NaOH soluble fraction was separated from the remaining insoluble fraction. The NaOH soluble fraction was extracted with 50 ml of ether in a separating funnel. The ethereal fraction was separated from the non-ethereal (aqueous) fraction. The ethereal fraction was distilled under reduced pressure in order to remove ether. The non-ethereal fraction was acidified with acetic acid and again extracted with ether. Two fractions i.e., ethereal fraction and non-ethereal aqueous fractions were obtained. Only ethereal fraction showed fungitoxicity. On removing ether from the ethereal fraction, a pungent smelling oily liquid was obtained.

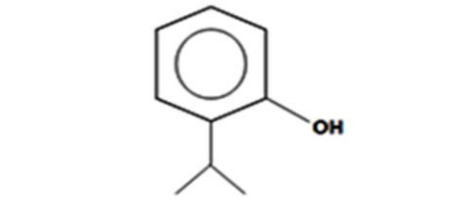
After 24 hours of storage, shining yellow crystals appeared at the bottom of the glass container. Repeated harvesting of the liquid yielded a yellow crystalline compound was collected and then recrystallized by hexane/acetone mixture. On re-crystallization the isolated yellow crystalline compound became colourless. The remaining yellow oily liquid was stored separately. The isolated crystalline compound was subjected to TLC using ethanol/acetone (3:7) as solvent. The dried chromatogram developed in the iodine chamber, showed a spot equivalent to Rf. 0.67. The melting point of isolated compound was determined and found to be 51.0°C.

RESULTS

The essential oils extracted from different parts of angiospermic plants by hydro- distillation process.These oils were then prepared in three different doses i.e., 0.5 x 10^3 µl/l, 0.7 x 10^3 µl/l and 1.0 x 10^3 µl/l and tested against *Rhizoctonia solani* Kuhn. The growth of mycelia of the test fungus was completely checked only by the essential oil of *Trachyspermum ammi.* The Minimum Inhibitory Concentration (i.e., MIC) of the essential oil against the test fungus was recorded 0.5 X 10^3 µl/l (Table: 1).

The GLC of the isolated crystals exhibited a single peak indicating the purity of the compound (Figure 1). The UV spectrum of the compound (Figure 2) showed peaks at 275 nm and 207 nm indicating thereby the presence of an aromatic nucleus. The IR spectrum (Figure 3) of the compound exhibited a band at 3350cm^{-1} showing the presence of -OH group in the molecule. The band at 2900cm^{-1} indicated the presence of -CH_3 groups. Bands at 1630cm^{-1}, 1440cm^{-1}, 1300cm^{-1}, 1260cm^{-1}, 1175cm^{-1}, 1110cm^{-1} and 970cm^{-1} indicated that the compound is a 1,3,4 substituted benzene derivative. The NMR spectrum (Figure 4) of the compound exhibited multiplet signal in the region δ6.4 to δ7.0 which might be

due to presence of aromatic protons (3H). Multiplet signals in the region δ3.05 to δ3.35 as observed in the spectrum were due to the proton attached to the tertiary carbon of isopropyl group (1H). Singlet at δ2.1 is due to the three proton of methyl group (3H). Two distinct singlets at δ1.15 and δ1.05 observed in the spectrum indicated the presence of 3H of -CH_3 of isopropyl group. On the basis of melting point and spectroscopic analysis, the structure of the isolated compound was tentatively assigned as follow,



2-Isopropyl-5methylphenol (Thymol)

[FIGURE : A]

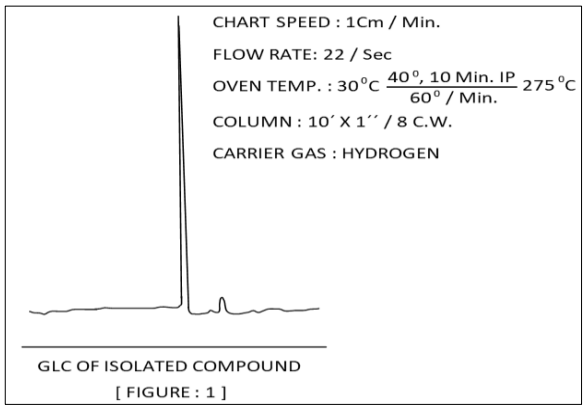
The identity of isolated compound was finally confirmed as thymol (Figure: A) by:

(1) Co- chromatography of the authentic sample and the isolated crystals using ethanol: acetone (3:7) as solvent. The Rf values of both the samples were identical i. e. 0.67.

(2) The authentic sample of thymol was mixed with the isolated crystals and the mixture was subjected to melting point determination. There was no deviation in melting point (m. p. 51°C).

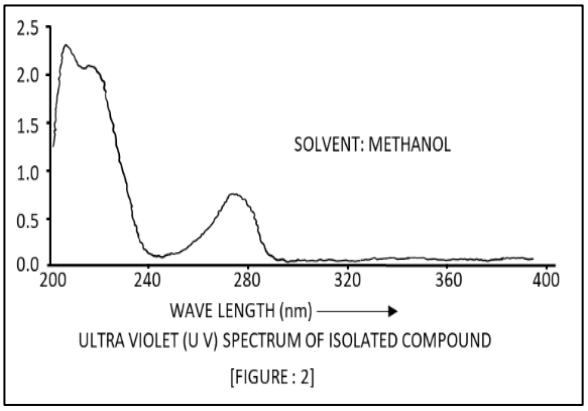
Table 1: Fungitoxic Efficacy of Essential Oils against *Rhizoctonia solani* Kuhn.

Sl. No.	Essential Oil Tested	Family	Parts Used	Yield (%)	Percent Inhibition of Mycelial Growth		
					Doses of essential oil (in µl/l)		
					0.5×10^3	0.7×10^3	1.0×10^3
1.	*Ageratum conyzoides* L.	Asteraceae	Leaves	0.02	25	35	75.1
2.	*Amomum subulutum* Roxb.	Zingiberaceae	Fruit	0.6	0.6	25.3	30.3
3.	Cannabis sativa L.	Cannabaceae	Leaves	0.1	19	35	58
4.	*Carum carvi* L.	Apiaceae	Fruit	0.8	15.4	30.5	61.1
5.	*Lantana indica* Roxb.	Verbenaceae	Leaves	0.5	19.8	22.3	53.2
6.	*Murraya koenigi* L.	Meliaceae	Leaves	0.5	22.3	29.2	62.2
7.	*Ocimum americanum* L.	Labiatae	Leaves	0.4	28.3	62	83
8.	*Polyalthia longifolia* Benth. & Hook. F.	Annonaceae	Leaves	1.1	19	36.2	56
9.	*Tagetes erecta* L.	Asteraceae	leaves	0.6	21.2	31	52.5
10.	*Trachyspermum ammi* L.	Apiaceae	Seeds	2.0	100	100	100



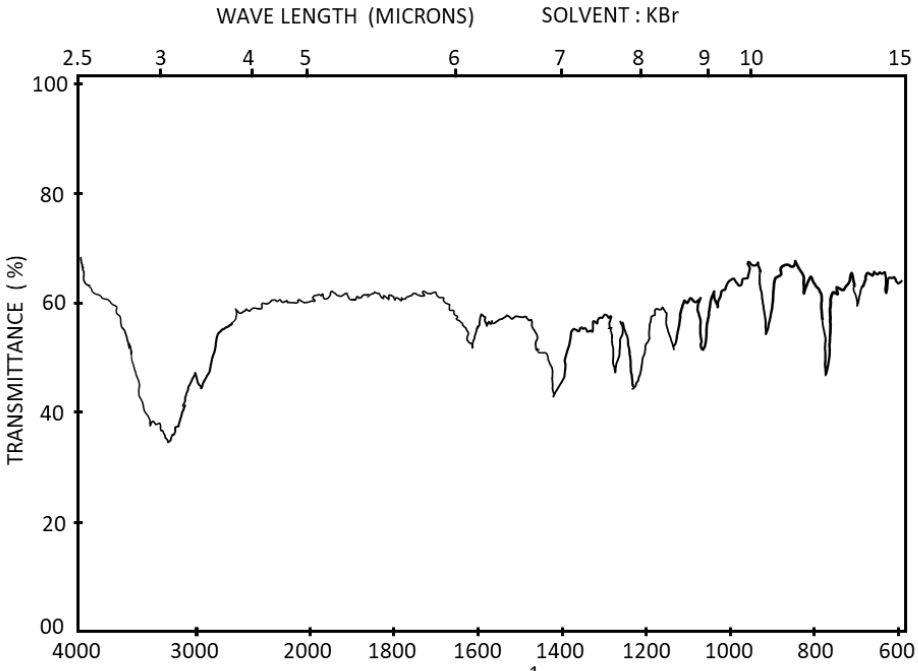
GLC OF ISOLATED COMPOUND

[FIGURE : 1]



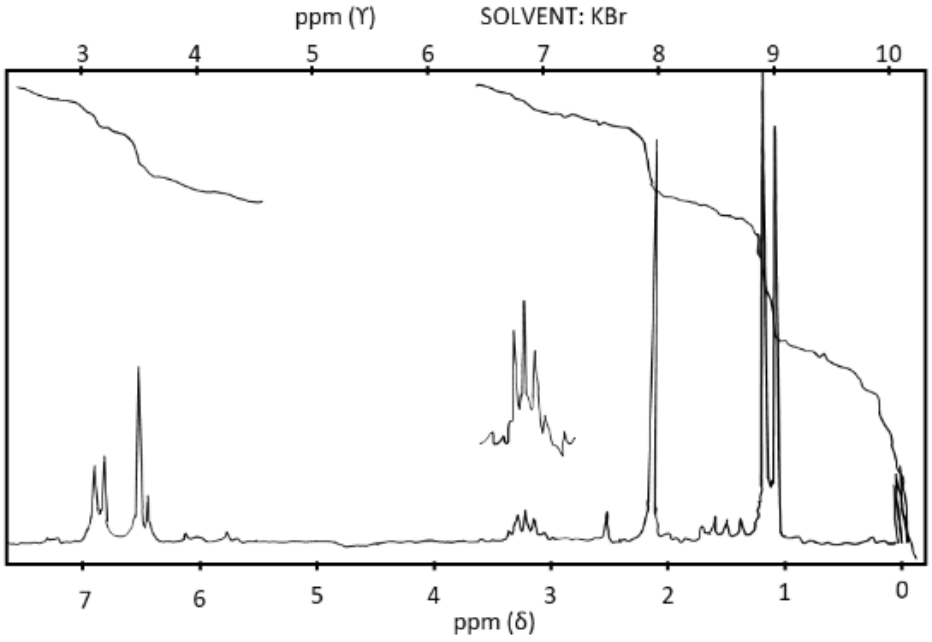
ULTRA VIOLET (U V) SPECTRUM OF ISOLATED COMPOUND

[FIGURE : 2]



INFRA RED (IR) SPECTRUM OF ISOLATED COMPOUND

[FIGURE : 3]



NUCLEAR MAGNETIC RESONANCE (NMR) SPECTRUM OF ISOLATED COMPOUND

[FIGURE : 4]

DISCUSSION

Higher plants have been proved to be the reservoirs of various biologically active substances (Dhar *et al.*, 1973; Swaminathan, 1978). In the past, several higher plants have proved their usefulness against a number of fungi (Dutta *et al.*, 2004). It has also been shown that substances extracted from various parts of plants, are largely non- toxic to humans, non-pollutive and biodegradable in nature (Mahadevan, 1982). Such substances have enough potentialities as pesticides and

can easily be procured from our natural renewable resources.

Several angiospermic plants are reported to release volatile substances especially essential oils which make the environment free from pathogens, therefore such oils have been used for hundreds of years for traditional medicine and aromatherapy. A number of studies have been carried out to prove the ability of essential oils to control phytopathogens (Beye, 1978; Daferera *et al.,* 2003).

Essential oils consist of a number of compounds that help to create resistance to plant pathogens (Lahlou, 2004). The active antimicrobial constituents of essential oils have been evaluated by several workers (Wink, 2003). The active fungitoxic constituents *viz.,* geraniol, furfurol, neuraol and pinene isolated from the flowers of *Helichrysum italicum,* inhibited the mycelial growth of *Candida albicans* (Preuss *et al.,* 2005). The active antimicrobial constituent *viz.,* estragol isolated from *Feronia elephantum* oil, was proved toxic to *Alternaria helianthi, Colletotrichum capsici, Fusarium moniliforme, F. Solani, Helminthosporium oryzae, H. Turcium, Pythium vexans, Pyricularia setariae, Rhizoctonia bactaticola and R. Solani* (Chirkina and Osipova, 1974). Carvone was reported as antifungal factor of *Nigella sativa* oil and was toxic to *Fusarium moniliforme* and *Fusarium solani* (Sharma, 1978). The fungitoxic constituents *viz.*, citral, isopimpeneyin and limetin were isolated from the oil of lemon peel and identified (Rathee *et al.*, 1982; Barkai-Golan, 2001). Methyl chavicol and linalool was isolated from the essential oil of *Ocimum sanctum* and proved as the active antifungal constituents against *Candida* species. The essential oil of *Pelargonium graveolens* reported to contain citronellol as an active antifungal constituent and effective significantly against *Rhizoctonia solani*.

In the present study, investigation on the fungitoxic efficacy of essential oil extracted from the seeds of *Trachyspermum ammi* was carried out against the test fungus *viz., Rhizoctonia solani* Kuhn. The active antifungal constituent was isolated from the essential oil and characterized as 2-Isopropyl-5-methyl phenol *i.e.*, 'Thymol' on the basis of GLC, UV, IR and NMR spectroscopy analysis.

CONCLUSION

The main objective of the present study was to develop plant based natural fungicides, which are natural, biodegradable, non-toxic, non-pollutive and eco-friendly. The essential oil extracted from the seeds of *Trachyspermum ammi* proved to be 100% toxic against *Rhizoctonia solani* Kuhn at 0.5 X 10^3µl/l (v/v) concentration under *in vitro* condition. The active fungitoxic constituent of the oil was isolated and characterized as 'Thymol'. Thus the essential oil of *Trachyspermum ammi* and its active fungitoxic constituent 'Thymol' can play important role in controlling the diseases caused by *Rhizoctonia solani* Kuhn to many of our agricultural and horticultural crops worldwide such as black scurf of potato tubers, damping off of soybean seedlings, base rot of lettuce, root rot of sugar beet, belly rot of cucumber, sheath blight of rice, wire stem of tomato and crucifer seedlings etc. However, before making any commitment regarding the practical use of the oil and its active constituent 'thymol' as natural fungicides, *in vivo* trials are required.

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