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Evaluation of isolated active principle of *Acorus calamus* oil for its toxicity against *Fusarium oxysporum* f. Sp. *Ciceris*

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Abstract
Background: Chickpea is a good source of protein and carbohydrate, together constituting about 80% of the total dry mass of seed. Besides being a very rich source of protein, it also maintains soil fertility through biological nitrogen fixation. The fungi associated with seeds bring about several undesirable changes making them unfit for consumption, sowing and adversely affects the quality and health of seeds. The fungus *Fusarium oxysporum* f. sp. *ciceri* causes destructive wilt disease in most of the popular chickpea cultivars grown in India and abroad. The present project was undertaken to isolate and identify the active principle of *Acorus calamus* essential oil for its antifungal efficacy against the test fungus and to explore the possibility of developing plant-based formulations for the control of wilt of chickpea.
Methods: The antifungal efficacy of *Acorus calamus* essential oil was studied against the test fungus using 'Poisoned Food Technique'. In treatment sets, the molten PDA medium was thoroughly mixed with desired concentration of oil in presterilized Petri plates whereas in control sets, in place of oil, distilled water was mixed. In order to isolate the active principle, the oil was subjected to column chromatography. The column was eluted with different solvents like ethyl acetate & benzene; hexane & benzene; benzene & acetone etc. in different ratios. Elutants were collected as fractions of ten mL and tested for their antifungal activity against the test fungus employing modified paper disc techniques.
Result: The essential oil of *Acorus calamus* showed the most significant antifungal activity against *Fusarium oxysporum* f. sp. *ciceris.* The active principle of the oil was isolated and identified as α- Asarone (1, 2, 4 - Trimethoxy-5-propenyle benzene) on the basis of IR, NMR and UV spectroscopy analysis. The above investigations clearly suggest the significant potential of *Acorus calamus* oil and α- Asarone (the active principle) as antifungal agents against *Fusarium oxysporum* f. sp. *Ciceris* to control wilt of chickpea disease.

Keywords: *Acorus calamus, Fusarium oxysporum,* essential oil, active principle

Introduction

Chickpea (*Cicer arietinum*) is one of the most important pulse crops. It belongs to family Leguminosae; the second largest family of angiosperms which comprise some 690 genera and 18,000 species (Purseglove, 1968) [33]. About twenty species of this family are cultivated widely for their edible seeds which contain high percentage of protein (Aykroyd & Doughty, 1964) [4]. *Cicer arietinum* is the world's third most important pulse crop, after dry beans (*Phaseolus vulgaris* L.) and dry peas (*Pisum sativum* L.) (Vishwadhar & Gurha, 1998) [42].

Chickpea is an important component of the diets of those individuals who cannot afford animal proteins or those who are vegetarian by choice (Barman, 2014) [5]. There is a growing demand for chickpea due to its nutritional value. Chickpea is a good source of carbohydrate and protein, together constituting about 80% of the total dry seed mass (Chibbar *et al*, 2010; Geervani, 1991; Jukanti *et al*, 2012) [11, 17, 22]. In India, the consumption of chickpea is highest. India accounts for 68% of total global output of chickpea. Besides being a very rich source of protein, it also maintains soil fertility through biological nitrogen fixation.

The fungi associated with seeds at the stage of harvest and under storage bring about several undesirable changes making them unfit for consumption and sowing. Further, association of fungi adversely affects quality and health of seeds. Many fungal species have been detected in seed samples of chickpea which includes *Fusarium oxysporum* f. sp. *ciceri* which causes destructive wilt disease in most of the popular chickpea cultivars grown in India and abroad. Occurrence of *F. oxysporum* f. sp. *ciceri* was found predominant. Mc Rae (1932) as well as Prasad and Padwick (1939) [31] reported *Fusarium oxysporum* f. sp. *ciceris* as pathogenic to chickpea crop which is now accepted worldwide (Kaiser *et al*, 1994) [23]. Currently the disease is prevalent in several countries. The pathogen attacks the seedling in soil under favourable conditions and may cause damage of crops upto 90%. It causes great economic as well as labour loss to farmers. The pathogen is both seed and soil borne, facultative saprophyte and can survive in soil up to six years in the absence of susceptible host (Haware *et al*, 1986) [20]. The productivity of chickpea can be increased by 5-10% by reducing the production losses thereof caused by *Fusarium oxysporum* f. sp. *ciceris.* There are several pesticides including fungicides available in the market for chemotherapy of plant diseases but their ill effects have always been overlooked and these are being used without critical assessment of their carcinogenic, treatogenic and pollutive effects (Detroy *et al*, 1971; Howe, 1978; Agnihothrudu, 1982) [1, 13, 21]. In recent years, because of awareness of the toxicity to man and animals and disastrous effect of pesticides, the regulatory agencies have banned the use of several pesticides (Gupta, 2008 [19]; Devi & Raha, 2013) [14].

The antimicrobial potentialities of higher plants were recognized in prehistoric days (Mohammed, 1983) [27]. Plant essential oils have been used for centuries as fumigants and may act as antifungal fumigants (Burt, 2004; Park *et al*, 2005) [9, 30]. These are the by-products of plant metabolism and are commonly referred to as volatile plant secondary metabolites (Koul *et al*, 2008) [24]. Essential oils are less hazardous to the health, biodegradable and environmentally safe and could be used as alternative to control plant diseases (Chuang *et al*, 2007; Akhtar *et al*, 2007), [12, 2]. The volatile nature makes the essential oil more penetrating and therefore helps in eradication of deep rooted pathogens.

Keeping this in view, the present project was undertaken to isolate and identify the active principle of *Acorus calamus* essential oil for its antifungal efficacy against *Fusarium oxysporum* f. sp. *ciceris* and to explore the possibility of developing plant-based formulations for the control of wilt of chickpea.

Materials and Methods

Extraction of Essential Oil

The rhizome of *Acorus calamus* was surface sterilized by dipping in 2 % sodium hypochlorite solution for 5 minutes, thoroughly washed with sterilized double distilled water and chopped into small pieces. Five hundred grams of chopped rhizome was subjected to hydro-distillation process in a Clevenger's apparatus. After 10 to 12 hours, two distinct layers are formed i.e., an upper aromatic oily layer and a lower colourless aqueous layer. The amount of oil thus recovered was noted in terms of per cent recovery on fresh /dry weight basis according to the material used. The oil was refrigerated at 4 - 6^0C temperature for further use.

Test Fungus and Growth Conditions

The essential oil of *Acorus calamus* and its active principle were assayed for antifungal efficacy against the test fungus *viz, Fusarium oxysporum* f. sp. *ciceris.* The test fungus was grown on Potato Dextrose Agar (PDA) plates at 27^0C ± 2^0C and maintained with periodic sub – culturing at 4^0C.

Evaluation of Antifungal Efficacy of *Acorus calamus* Essential Oil

The antifungal efficacy of *Acorus calamus* essential oil was studied against the test fungus using 'Poisoned Food Technique' (Arora and Dwivedi, 1979) [3]. In control sets, nine ml of molten PDA medium was poured in a presterilized Petri plates and mixed thoroughly with one ml of sterilized double distilled water whereas in the treatment sets, nine ml of molten PDA medium was poured in a presterilized Petri plates and in place of double distilled water, mixed thoroughly with one ml of the essential oil. In the centre of the Petri plates, placed the test fungal disc (5 mm in diameter) with the help of flame sterilized cork-borer.

The plates were incubated for 7 days at 27 ± 2°C. On the seventh day, the diameter of the fungal colony was measured in mutual perpendicular directions. The antifungal efficacy of essential oil was recorded in terms of the percent inhibition of mycelial growth and calculated using the following formula (Vincent 1947) [41]:

$$\text{Percent Inhibition} = \frac{dc - dt}{dc} \times 100$$

Where,

dc = average diameter of fungal colony in control sets

dt = average diameter of fungal colony in treatment sets

The experiments were repeated twice and each set contained four replications.

Isolation and Identification of Active Principle from the Essential Oil

The oil was subjected to column chromatography to isolate the active principle. A clean and dried Pyrex glass column (1120 mm X 40 mm) was packed with silica gel 'A' and ten mL of *Acorus calamus* essential oil was placed at the top of the column. The column was eluted with ethyl acetate and benzene (1:1, 1:2 & 2:1); hexane and benzene (3:1, 1:1 & 1:3); benzene and acetone (3:1, 1:1 & 1:3); acetone and chloroform (2:1, 1:1 &1:2); chloroform and ethanol (1:1, 1:2 & 2;1); ethanol and diethyl ether (2:1), 1:1 &1:2). Elutants were collected as fractions of ten mL and altogether 106 fractions were collected and tested for their antifungal activity against the test fungus employing modified paper disc techniques (Tripathi & Dixit, 1977) [38]. Hexane and benzene (1:3) fractions completely inhibited the mycelial growth of the test fungus. All these fractions collected together and subjected to TLC using Hexane and benzene (3:1) as solvent. The chromatogram of the pooled fraction showed the presence of a single circular spot with Rf value 0.84. The solvents of the collected fractions were removed under reduced pressure, which resulted white shiny crystals designated as compound 'X'. The melting point of compound 'X' was determined to be 61 – 63^0C. The data obtained from UV, IR and NMR was used for the identification of compound 'X'. Confirmation of the compound isolated was made by mixed boiling point determination and super imposable I. R.

Results and Discussion

The screening of essential oils extracted from different parts of plants was done against *Fusarium oxysporum* f. sp. *ciceris* at 0.5 X 10^3 µl/l, 1.0 X 10^3 µl/l, 1.5 X 10^3 µl/l and 2.0 X 10^3 µl/l concentrations. The mycelial growth the test fungus was completely inhibited only by the oil of *Acorus calamus* at all concentrations. The Minimum Inhibitory Concentration (i.e., MIC) of oil against the test fungus was recorded 0.5 X 10^3 µl/l (Table 1).

The Nuclear Magnetic Resonance (NMR) spectroscopy (Figure: 1) of the compound 'X' demonstrated a three proton, doublet at δ1.85 for a methyl group, nine proton broad singlet at δ 3.75 – 3.85 for three methoxy group, one proton singlet at δ 6.40, 6.85, 6.71 and 6.15. Doublet at δ 6.71 (J= 16 Hz) is characteristic of a trans-olefinic proton. These NMR signals suggested that the isolated natural product contains three methoxyl groups attached to a side chain containing trans-olefinic protons and a methyl group. The InfraRed (IR) spectroscopy (Figure 2) of the compound 'X' isolated from the oil showed the presence of aromatic C-H stretching at 3020 cm^{-1} ; aliphatic C-H stretching at 2920 cm^{-1}, 2840 cm^{-1}; aliphatic C=C at 1640 cm^{-1}; aromatic C-C stretching at 1600 cm^{-1},1590 cm^{-1}, 1505 cm^{-1}, 1505 cm^{-1} and C-O stretching at 1120 cm^{-1} in the molecule. The Ultra violet (UV) spectroscopy of the compound 'X' showed λ max at 284 nm which is characteristic of aromatic conjugated transition. On the basis of these spectral data, the

compound 'X' was identified as α- Asarone (1, 2, 4 - Trimethoxy-5-propenyle benzene).

The melting point of the compound 'X' (62-63^{0}C) recorded herewith also confirmed the melting point of α- Asarone. Mixed melting point determination exhibited no depression in melting point. The InfraRed (IR) spectroscopy of authentic α- Asarone sample was found more or less super-imposable to the InfraRed (IR) spectra of the isolated compound. The identity was thus confirmed to be α- Asarone. The Minimum Inhibitory Concentration (MIC) of α- Asarone against the test fungus was recorded in table: 2.

Green plants have been recognized as reservoirs of various biologically active substances (Bhakuni *et al*, 1971 [7]; Dhar *et al*, 1973; Swaminathan, 1978) [15, 37]. It has been emphasized that substances of plant origin, besides being largely non- toxic to humans have enough potentialities as pesticides (Beye, 1978) [6]. The added advantages of such substances are that these are generally biodegradable and non pollutive (Mahadevan, 1982) [26] as well as procurable from the largest of our renewable sources. Higher plants emit volatile substances specially essential oils which keep the air free from pathogens, therefore these oils have traditionally been used for centuries for their antifungal properties (Rios & Recio, 2005) [34]. A number of studies have been carried out to prove the ability of essential oils to control plant pathogens (Soliman and Badeaa, 2002; Valero and Salmeron, 2003) [39, 36]. The essential oil of wintergreen effectively inhibited the spore germination of a powdery mildew pathogen of *Phyllanthus niruri* (Sahayarani, 2003) [35]. Members of the Asteraceae, Lamiaceae, Rutaceae, and Verbenaceae contain essential oils which have toxic effect against several fungi (Lahlou, 2004) [25]. Essential oils consist of a number of compounds that help to create resistance to plant pathogens as (Wink, 2003; Gershenzon & Dudareva, 2007) [43, 18]. The active chemical constituents of essential oils have been evaluated by several workers for antimicrobial properties (Preuss *et al*, 2005; Nostro *et al* 2007; Cavar *et al*, 2008) [32, 29, 10]. Bouzenna and Krichen (2013) [8] reported citronellol as the active antifungal principle of *Pelargonium graveolens* essential oil against *Rhizoctonia solani.* The active antifungal principles of *Corymbia citriodora* essential oil are linalool, α-pinene and β-pinene (Nakahara *et al*, 2003) [28]. In the present project, investigation on the antifungal activity of *Acorus calamus* essential oil was done against the test fungus *viz*, *Fusarium oxysporum* f. sp. *ciceris.* The active antifungal principle of the oil was isolated and identified as α- Asarone (1, 2, 4 - Trimethoxy-5-propenyle benzene) on the basis of IR, NMR and UV spectroscopy analysis.

Table 1: Minimum Inhibitory Concentration (MIC) of *Acorus calamus* oil against *Fusarium oxysporum* f. sp. *ciceris*

Concentration of Oil (µl/l)	Percent Inhibition of Mycelial Growth
1.0 X 10^3	100
0.9 X 10^3	100
0.8 X 10^3	100
0.7 X 10^3	100
0.6 X 10^3	100
0.5 X 10^3	100
0.4 X 10^3	92.0
0.3 X 10^3	74.0
0.2 X 10^3	51.0

Table 2: Minimum Inhibitory Concentration (MIC) of α - Asarone against *Fusarium oxysporum* f. sp. *Ciceris*

Concentration of Oil (µl/l)	Percent Inhibition of Mycelial Growth
1.0 X 10^3	100
0.9 X 10^3	100
0.8 X 10^3	100
0.7 X 10^3	100
0.6 X 10^3	100
0.5 X 10^3	100
0.4 X 10^3	100
0.3 X 10^3	100
0.2 X 10^3	91.0

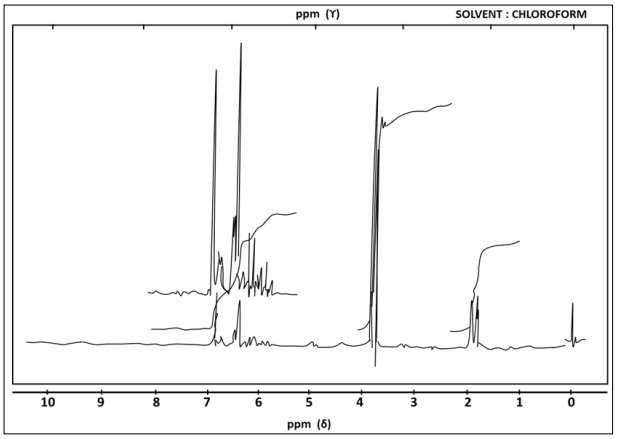

Fig 1: NMR Spectrum of Isolated Compound "X"

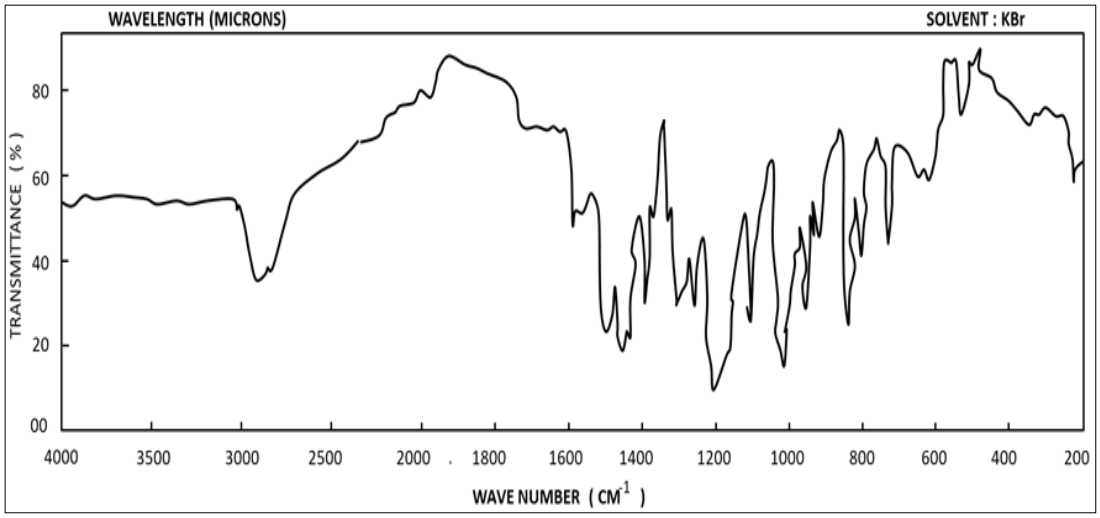

Fig 2: IR Spectrum of Isolated Compound "X"

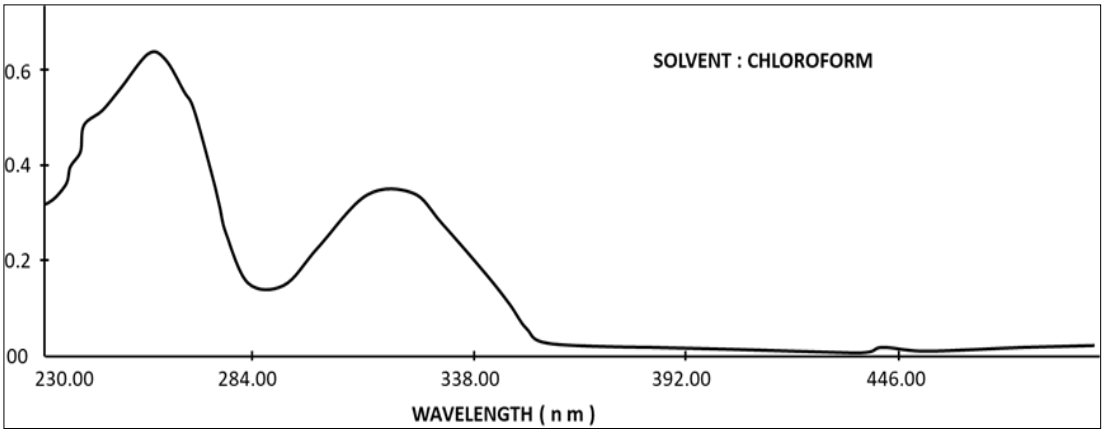

Fig 3: UV Spectrum of Isolated Compound "X"

Conclusion

No doubt, synthetic chemical fungicides are effective in controlling plant diseases, but these have several hazardous impacts like non-biodegradability, carcinogenic nature, high cost, non ecofriendly, pollutive and adverse effect to the health of humans and animals. The use of such synthetic chemical fungicides is being strictly regulated by governments due to their hazardous impacts. Therefore, it is the urgent need to develop new models of fungi-toxicants, which should be easily biodegradable, non-toxic, non-pollutive, ecofriendly and procurable from renewable resources. The natural plant products especially essential oils are considered as the most promising weapon to control fungal diseases of crop plants. These are environmentally safe, biodegradable, and non-toxic to humans & animals and have no adverse effects on crops.

In the present study, the essential oil of *Acorus calamus* showed the most significant antifungal activity against *Fusarium oxysporum* f. sp. *ciceris*. Further, the active principle of the oil was isolated and identified as α- Asarone (1, 2, 4 - Trimethoxy-5-propenyle benzene) on the basis of IR, NMR and UV spectroscopy analysis. The above investigations clearly suggest the significant potential of *Acorus calamus* oil and α- Asarone (the active principle) as antifungal agents against *Fusarium oxysporum* f. sp. *Ciceris* to control wilt of chickpea disease. However, before making any suggestion regarding the replacement of synthetic chemical fungicides with *Acorus calamus* oil and its active principle on a commercial basis, continued research particularly on the mechanisms involved under *in vivo* conditions are required.

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