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ASSAY OF BOTANICAL OILS AGAINST IMPORTANT PHYTOPATHOGENIC FUNGI

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ABSTRACT

In vitro antifungal activity of six essential oils of *Oscimum tenuiflorum* (Purple leaves/ Krishna Tulsi) *Cymbopogon citrates* (Nimbu ghas), *Origanum majorina* (Sweet majoram), *Ocimum citriodorum* (Nimbu tulsi) *Oscimum bascillicum* (Gulal tulsi) and *Ocimum sanctum* (Green leaves /Shree tulsi) were investigated against three economically important phytopathogenic fungi, *Pythium aphanidermatum*, *Fusarium solani* and *Fusarium moniliformae* isolated from infected ginger rhizomes. The experiment was carried out by Whatman disc method using Whatman paper No.3 on Potato Dextrose Agar with three replicates. Six concentrations of essential oils i.e., 15, 30, 45, 60, 75 and 90% were assayed against all the three test fungi respectively. The experiment was carried out at 27°C and mycelial growth was measured after every third day, upto 15 days. The result showed that all the six types of essential oils suppressed the mycelial growth of all three test fungi upto various level. Among the three tested fungi *Pythium aphanidermatum* was found to be the most sensitive and showed 100% growth inhibition at all concentrations by all the six essential oils used. It is an evident from this study that all the oils used in this study, may have potential to control plant pathogenic fungi and these could be considered for developing new fungicides.

KEYWORDS: Antifungal activity, Essential oils, *Pythium aphanidermatum*, *Fusarium solani*, *Fusarium moniliforme.*

INTRODUCTION

Fungal diseases cause a heavy yield loss in various types of crops including vegetable and other cash crops. Synthetic or chemical fungicides are most commonly as well as widely used control measure against fungal diseases. Although these chemical or synthetic fungicides are much effective but they have many drawbacks, which includes the resistance development in targeted/specific microorganism/(s) as well as their ill effects on human health and on environmental conditions are also reported (Avis 2007). Moreover inappropriate and extraordinary use of the chemical and synthetic fungicides not only results negative impacts on ecosystems, but it has also caused carcinogenic as well as toxicological problems (Cameron and Julian 1984; Osman and Al-Rehiayam 2003; Masuduzzaman *et.al.*, 2008; Siva *et.al.*,2008; Gurjar *et.al.*, 2012). Besides this, certain pathogens become resistance against fungicides and hence the fungicides become ineffective (Zhonghua and Michailides, 2005). Hence this is very important to search an environmentally safe and economically viable alternative for the control of plant fungal diseases so that the dependence on the chemical and synthetic fungicides can be reduced. Now days, not only interest but also the need has generated for the development of biodegradable, safer and effective antifungal agents by using natural substances such as plant products as well as essential oils from different plant parts against fungal diseases (Chuang *et. al.*, 2007; Prasad *et. al.*, 2004).

Active biological compounds i.e. secondary metabolites present in plants are more safer, acceptable and adaptable, than synthetic/chemical compounds and also shows a rich source of permanent control agent against plant pathogens (Tripathi *et al.* 2008). In recent years, the focus has shifted to the plant origin based fungicides all over the world. Dahanukar *et.al.*, (2000) has reviewed the study and research about antifungal components which are plant based from 1994-1998, as a innovative scientific tool and as a scientific approach.

Essential oils extracted from plants are the concentrated liquid, containing volatile aromatic compounds these are hydrophobic in nature and are much effective to inhibit fungal pathogens as well as having significant potential to use in IPM (integrated pest management) programs (Soylu *et.al.*, 2006). Essential oils extracted from several plants having significant antifungal activities and hence they could be used as effective antifungal agents (Adam *et.al.*, 1998; Sokmen, 1999). Essential oils are not phytotoxic as well as they are also significant inhibitor of several fungal pathogens (Pandey *et.al.*, 1982; Chuang *et.al.*, 2007). Bowers and

Locke in 2000 have also reported that essential oils from plants have significant inhibitory impact against soil-born fungal pathogens.

Hence essential oils from plants seems to be an effective alternative to synthetic/chemical fungicides against phytopathogenic fungi, which is because of presence of biological active sesquiterpenes, aldehydes,, monoterpenes, phenols, carbohydrates, ethers, alkaloids, quinons, flavonoids, ketones, alcohols, sterols, tannins and saponins etc. (Kalemba & Kunicka, 2003; Burt, 2004). Sumonrat *et.al.*, (2008) reported phenolics as significant antimicrobial compounds in the essential oils extracted from plants. Other researchers also reported the presence of bioactive compounds in the essential oils (Isman, 2000; Soliman and Badeaa, 2002; Daferera, Ziogas, & Polissiou, 2003; Kalemba & Kunicka, 2003).

The action mode of essential oils studied and observed by researchers suggested that antimicrobial activity of essential oil is due to the interactions initiated by terpenes in the enzymatic machinery which is related with the production of energy as well as in the formation of components of microbial cells (Omidbeygi *et.al*., 2009). Besides this, terpenes affects the permeability as well as different functions of microbial cell membranes, the active antimicrobial components of the essential oils interact with the intracellular sites of microbial cells after crossing the cell membranes and penetrating into the same (Lucini *et.al.*, 2006; Cristani *et.al*.,2007; Tatsadjieu *et.al*., 2009). The bioactive components of essential oils produces a flux which induces the protons towards the exterior of the microbial cell and causes certain changes and ultimately death of microbial cell (Lucini *et.al.*, 2006; Cristani *et.al*.,2007; Tatsadjieu *et.al*., 2009). Daferera *et. al.* (2000) reported the antifungal activity of oils extracted from plants may be because of formation of hydrogen bonds in between the active sites of target enzymes and hydroxyl group of oil components.

Many of the plant species are known to be potential source of antimicrobial compounds but only some of them have been studied scientifically (Wilkins and Board, 1989, Paster *et. al*., 1990). Many researchers reported that the bioactive components from plant essential oils are able to inhibit plant pathogens or at least it may used as a model for the construction of new pesticidal compounds (Lentz *et al.* 1998, Hernandez *et al*.1999, Amadioha 2000, Rasooli and Mirmustsfa 2002).The compounds present in plants were reported to have biological activities such as antibacterial (Dorman *et al.*, 2000), insecticidal (Isman 2000) and nematicidal (Pandey *et al.*2000). In recent years the results of research finding related with the pesticidal properties of essential oils extracted from plants were used by certain

commercial companies and launched pesticidal components in the market like Cinnamite TM, Valero TM and Acaricide as fungicide for use on crops such as citrus, nuts and grapes these fungicidal components are introduced in the market by the company named as Mycotech (Liu *et.al.*, 2001).

However, there are only limited data available on the inhibitory activity of oils extracted from plants against plant pathogenic fungi (Nuzhat and Vidyasagar 2013).

In the present study both of the test genus *Fusarium* and *Pythium* are widespread/ ubiquitous in nature, they exists in different niches such as parasites (considered to be major plant pathogens causes several diseases in many plants including crop plants, vegetables and bedding crops), saprophytes (found on dead and decayed plant tissues) and antagonists (against several fungi) (Plaats- Niterink, 1967; 1968; Nelson 1994; Shirley, 2007). *Pythium* and *Fusarium* species have wide range of hosts and they are the most important economically damaging soil borne pathogens which affects a large number of vegetable crops also (Porter *et. al*., 2007).

According to an estimation the diseases caused by *Pythium* species in various crops are responsible for losses of multibillion dollars worldwide (van West et al., 2003). Among the various species of *Pythium*, *P. aphanidermatum* is the most common parasitic phyto-pathogen which affects various crop plants. *P. aphanidermatum* causes infections on a broad range of plants, belongs to different families viz., *Amaryllidaceae, Amaranthaceae, Araceae, Bromeliaceae, Basellaceae, Chenopodiaceae, Cactaceae, Coniferae, Compositae, Cruciferae, Convolvulaceae, Euphorbiaceae, Cucurbitaceae, Gramineae, Leguminosae, Malvaceae, Linaceae, Passifloraceae, Moraceae, Solanaceae, Rosaceae, Violaceae, Umbelliferae, Zingiberaceae, Vitaceae* etc (Waterhouse & Waterston ,1964). There are different diseases varies according to host plants caused by the *P. aphanidermatum* it may be the causal agent of damping-off (pre and post), rots, cottony blight, cottony-leak and stalk rot etc.

The genus *Fusarium* also has a broad range of economically important host plants. *Fusarium* species are also economically important phytopathogens and causes severe damages in the field of agricultural productions at global level (Saremi, 2000; Bentley *et. al*., 2006; Bockus *et. al*., 2007) and resulted in loss of billions of dollars (Rehman *et. al.*, 2013). *Fusarium* species produces mycotoxins, particularly *Fusarium moniliforme* secretes fumonisins, 12,13-

epoxytrichothecene mycotoxins (trichothecolone, diacetoxyscirpenol (DAS), and T-2 toxin), the palmitoyl esters of trichothecolone, T-2 tetraol (T-2 TOL), scirpenetriol, along with free and palmitic acid conjugates zearalenone (ZON) (Jime´Nez *et.al*.,1997). Centeno and Calvo (2002) reported that *Fusarium solani* also secretes mycotoxins.

Fusarium moniliforme has a wide range of host plants and it is cosmopolitan in nature (Booth, 1971). The well-known diseases caused by this fungus are: bakanae and foot rot or stunting of rice, pokkah boeng of sugarcane, corn ear rot, stalk rot, leaf blight, stalk rot of sorghum, endosepsis/brown rot/internal rot of fig (Booth 1971), and crown rot of asparagus (Endo and Burkholder, 1971), it also causes wilting, seedling blight as well as rot diseases in many crop plants (Rangaswami, 1975).

The species *solani* of the genus *Fusarium* is also an important phytopathogen of a wide range of host plants. The predominant hosts for *F. solani* are potato, pea, bean and members of the cucurbit family such as melon, cucumber, pumpkin etc. Diseases, such as root, fruit, surface rot, stem rot, sudden death syndrome of soybean, foot rot of bean and dry rot of potato as well as damping-off.

The objectives of this study is to test the inhibitory effect of six essential oils derived from *Oscimum tenuiflorum* (Purple leaved/Shyam Tulsi), *Cymbopogon citrates* (Nimbu ghas), *Origanum majorina* (Sweet majoram), *Ocimum citriodorum* (Nimbu tulsi), *Oscimum bascillicum* (Gulal tulsi) and *Ocimum sanctum* (Green leaved/Shri tulsi) against three economically important soil born fungi *Pythium aphanidermatum, Fusarium solani* and *Fusarium moniliforme* isolated from infected rhizomes of ginger.

The table below shows references regarding the antimicrobial properties, medicinal value as well as secondary metabolites present in the selected plants.

Table No.-1: Showing References for antimicrobial activity and Medicinal uses of Test plants.

S.No.	Botanical name	Local name	Family	Medicinal uses	Secondary metabolites	Reference
1.	*Ocimum bascillicum*	Gulal Tulsi	Lamiaceae	Bactericidal, Anti-Gonorrhoea, Dysentery, Antiviral, Antimicrobial, Treating cancer, Diabetes, Asthama	Linalool, Methyl Chavicol, 1,8-Cineole, Alpha Terpinene, Ethyl-2-methyl-butyrate	Damir *et.al*.,2015; Chiang *et.al*., 2005; De-Almeida *et.al*.,2007; Tohti *et.al*.,2006; Mansosroi *et.al*., 2006; Dube 1989;
2.	*Ocimum citriodorum*	Nimbu tulsi	Lamiaceae	Antimicrobial, Antibacterial, Antioxident	Eugenol, β-Caryophyllene Linalool, Methyl chavicol, Eugenol, 1,8-cineole, Geranial , Neral Limonene 1,8-Cineol Citronellal Alfa-pinen	Carovi et.al., 2010 Kashyap et.al., 2011 Abhay et. al., 2014; Hakkim et.al., 2008 Martin et.al., 2014
3	*Ocimum sanctum* (Green leaved Tulsi)	Green/ Shree Tulsi	Lamiaceae	Bronchitis, Malaria, Diarrhea, Dysentery, skin disease, arthritis, Eye diseases, Insect bites, Anticancer, Antidiabetic, Antifungal, Antimicrobial, Cardioprotective,	Eugenol, urosolic acid, limatrol, caryophyllene , methyl carvicol , Linalool	Priyabrata *et.al*., 2010; Shishodia *et.al*., 2003
4.	*Cymbopogon citrate*	Nimbu Ghas	Poaceae	Antibacterial, Antifungal, Analgesic, Anti-inflammatory	Alpha-pinene, Cis-sabinene hydrate, 1-8 cineole, Geranyl acetate, Geraniole, Terpinolene, Linalool, Limonene, 3-myrcene, Neral, Geranial	Inouye *et al.*, 2001, Inouye *et. al*., 2006; Bansod and Rai, 2008; Revathi *et. al*., 2012), Negrelle and Gomes, 2007; Sulaiman 2013
5.	*Origanum majorina*	Sweet majoram	Lamiaceae	Antibacterial Anthelmintic, Alexipharmic, Heart diseases,	Terpin-4-ol, Linalool, Gamma-terpinen, Trans-sabinen	Ben *et. al*., 2001; Kirtikar and Basu 1985; Farooqi and

				Fevers,Leucoderma Sudorific, Asthma, Hysteria and Paralysis	hydrat, Alfa-terpinen, Sabinen, Alfa-terpineol Alfa-terpinolen, Para-cymen, Cis-sabinen hydrat, Linalool acetat, Beta-caryophyllen, Beta-phellandren, Eremophilen, Menth-2-en-1-ol 1.3; Limonene	Sreeramu, 2004).
6.	*Ocimum tenuiflorum* (Purple leaved)	Shyam/Krishna Tulsi	Lamiaceae	Anticancer Radioprotective, Anticarcinogenic Antioxidant Chemo-preventive Immuno-therapeutic, Antimicrobial,	α-Pinene Camphor, Citral, Geraniol, β-Pinene Citronellal Eugenol,Vanillin, Linalool	Soumen *et.al.*,2013 Awasthi & Dixit, 2007 Khan *et. al.*, 2010 Kathiresan *et.al.*, 1999; Devi, 2001, Joshi, 2013; Prashar *et.al.*, 1994; Mukherjee *et.al.*,2005 Singh *et.al.*,2005; Joshi, 2013; Godhwani, & Vyas, 1987; Singh & Majumdar, 1997; Godhwani et al., 1987

MATERIALS AND METHOD

Plant Material and Extraction of essential oils

Leaves of all the six selected plants *Ocimum tenuiflorum, Cymbopogon citrates, Origanum majorina, Ocimum citriodorum, Oscimum bascillicum* and *Ocimum sanctum* were collected from fields where they are growing in their natural habitats. Plant materials were identified from Botanical Survey of India (BSI), Jodhpur, Rajasthan. Plant materials were washed twice with distilled water and subjected to Clevenger's hydro-distillation apparatus to extract their essential oils according to the method established by Montes *et al*., (2001). Anhydrous sodium sulfate was used to remove water after extraction. The resulting oils were placed in sealed plastic tubes (Negahban *et al*. 2006) and were stored in refrigerator at 4 °C for use throughout the experimental work.

Isolation an Identification of Test Fungi

Test fungi were isolated from infected ginger rhizomes. Small pieces of infected ginger rhizomes were surface sterilised by washing with running tap water followed by 0.5% NaClO (sodium hypchlorite), then they are blotted dry on sterile filter paper. The blocks of 3-5mm were placed on different mediums i.e. PPP agar (0.05 g penicillin, 0.10 g of pimaricin and 0.05 g of polymyxin per liter in corn meal agar), PARP agar (0.25 mg of ampicillin, 0.005 g of pimaricin, 0.01 g of rifampicin, and 0.10 g of pentachloronitrobenzene per liter in corn meal agar) and water agar (WA) according to Jeffers and Martin (2010) to isolate *Pythium* species. To isolate *Fusarium* species from infected ginger the selective medium as described by Nash & Snyder (1962) was used containing 0.1% pentachloronitrobenzene (PCNB). Plates containing infected ginger blocks on selective media were incubated for 2 days in the dark at 25±1°C then they were observed for the presence of test fungi respectively.

On the basis of colony characteristics of test fungi the single hyphae of all the three test fungi were transferred separately to fresh PDA medium and microscopically identified by using Olympus microscope (Olympus, Japan, Model No. BX51) on the basis of standard keys as suggested by Van der Plaats-Niterink (1981) and Waterhouse (1967, 1968) for *Pythium aphanidermatum* and by Wang *et.al.* (1996) and Alexopolous &Mims (1993) for *Fusarium solani* and *Fusarium moniliforme* respectively**.**

Isolated test pathogens were again identified and confirmed by Dr. Anila Doshi (Head Dept of Plant Pathology) from Rajasthan College of Agriculture (RCA, Udaipur, Rajasthan, India).

In-vitro antifungal activity test

The antifungal assay was carried out in Petri dishes (90 mm in diameter) by Whatman disc method using Whatman paper No.3.on Potato Dextrose Agar (PDA) media. Three replicates were used for each experiment. A disc of 6 mm dia is taken from the periphery of 7 days old cultures of test pathogens. Then they were placed inverted in the centre of each plates, the perti plates were containing discs of 6 mm diameter loaded with 10 µl of various concentrations (15%, 30%, 45%, 60%, 75%, 90%) of different essential oils. Plates were incubated in the dark at 27°C. Mycelial growth was measured after every fifth day, upto 15 days. Antifungal activity of taken essential oils were assayed against all the three test fungi respectively. Control sets were also run simultaneously without essential oils. Three replications of each concentration were assayed. The mean radial mycelial growth was measured with a Verniercaliper by measuring the diameter of the colony in two directions at

right angles. The diameter of colony was measured and the inhibition percentage of mycelium was calculated according to the method as described by Deans and Svoboda (1990).

Inhibition (%) = [(C-T)/C] x 100

Where: C is diameter of fungal colony in control plate (mm) and T is the fungal colony diameter in treatment plate (mm).

Statistical analysis

Experiments were performed in three replicates and data were analyzed are the average mean of three replicates.

RESULTS

Antifungal activity of plant essential oils were tested against three phytopathogenic fungi *Pythium aphanidermatum, Fusarium solani* and *Fusarium moniliforme*. All the six essential oils (at all concentrations) inhibited the growth of test fungi completely (100%) up till six days.

Among 6 essential oils, all the used concentrations from 15% to 90% were giving inhibitory activity against all of the three test fungi among which *Pythium aphanidermatum* was found to be the most sensitive as all the six essential oils at all the taken concentrations were completely (100%) inhibited the growth of *Pythium* up till the 15th day (when final results were taken).

In case of *F. moniliforme* essential oil of *Cymbopogon citrates* at 90% concentration completely inhibited the growth while the same concentration of *O. citridorum, O. sanctum, O. majorina, O. tenuiflorum* and *O. basillicum* showed 90%, 80%, 70%, 66% and 65% growth inhibition of the same respectively.

F. solani was found to be most sensitive against *Cymbopogon citrates* and showed 100% inhibition at 90% concentration. Remaining oils i.e. *O. citridorum, O. sanctum, O. tenuiflorum, O. majorina* and *O. basillicum* showed 90%, 80%, 72%,70%, and 65.5% growth inhibition at the same concentration respectively.

Result images and details of pathogen inhibition (in %) are given in the fig. no.1 (a, b, c) and Table no.2 respectively.

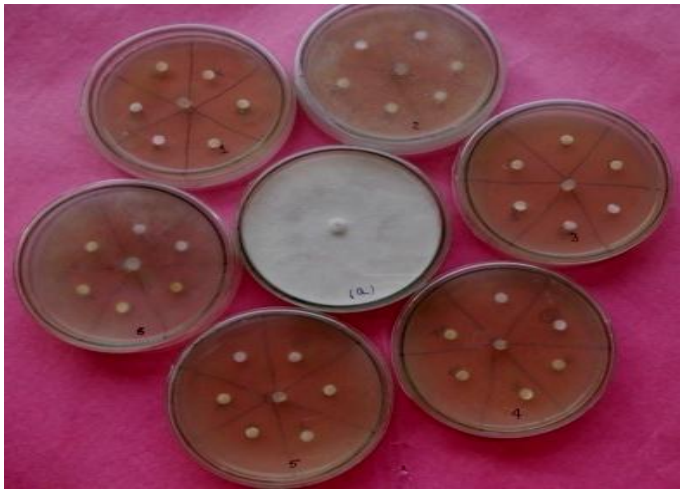

Fig. 1- *P. aphanidermatum.*

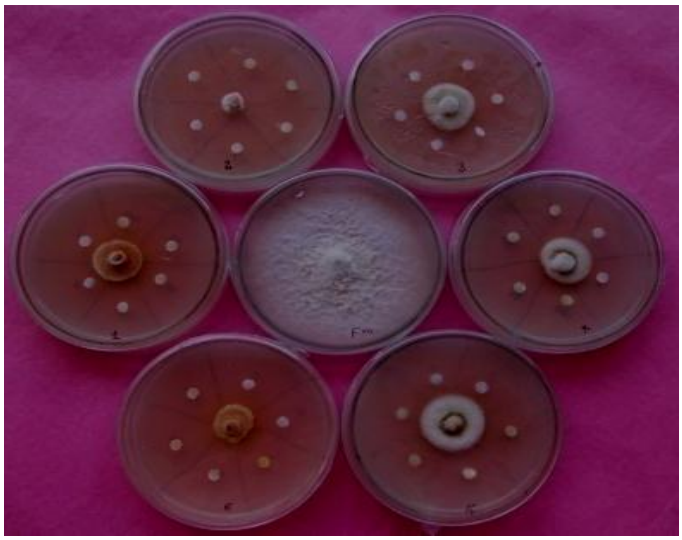

Fig. 2- *F. moniliforme.*

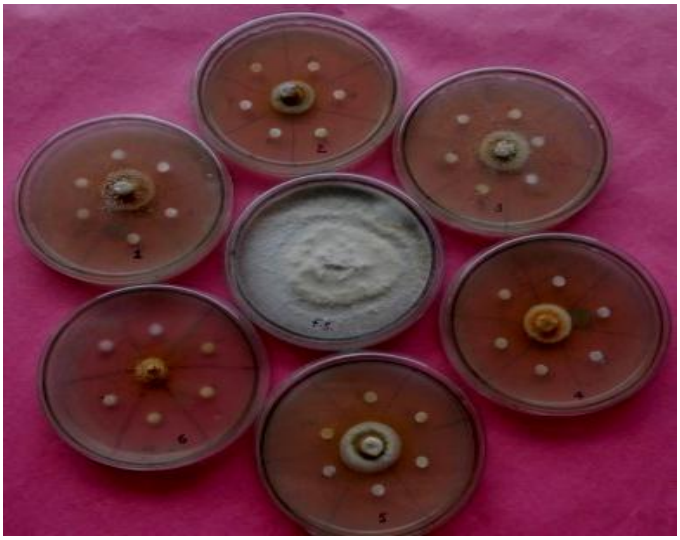

Fig. 3- *F. solani*

Table No. 2: ***In vitro Antimicrobial Activity of Essential oils on mycelial growth % inhibition on 15th day of P. aphanidermatum, F. moniliforme and F. solani.***

Name of Plant	Oil Conc. (in %)	*P. aphanidermatum* % inhibition	*F. moniliforme* % inhibition	*F. solani* % inhibition
O. tenuiflorum	15	100	65	70
	30	100	65	70
	45	100	65	72
	60	100	66	72
	75	100	66	72
	90	100	66	72
C. citrates	15	100	90	90
	30	100	90	90
	45	100	90	90
	60	100	90	90
	75	100	95	95
	90	100	100	100
O. majorina	15	100	70	70
	30	100	70	70
	45	100	70	70
	60	100	70	70
	75	100	70	70
	90	100	70	70
O. citridorum	15	100	75.5	75.5
	30	100	75.5	75.5
	45	100	75.5	75.5
	60	100	75.5	75.5
	75	100	85.5	85.5
	90	100	90	90
O. bacillicum	15	100	60	60
	30	100	60	60
	45	100	60	60
	60	100	60	60
	75	100	60	60
	90	100	65	65
O. sanctum	15		78.8	78.8
	30		78.8	78.8
	45		78.8	78.8
	60		78.8	78.8
	75		78.8	78.8
	90		80	80

DISCUSSION

A broad range of essential oils are presently being investigated and launched at global level as novel alternative inhibitory agents against phytopathogens. Many researchers have reported the antifungal and antibacterial activity of plants essential oils against the micro-organisms including *Pythium* and *Fusarium* (Barrera-Necha *et al.* 2009). Essential oils may provide potential alternatives to control chemicals presently used to control phytopathogens, it is due to the presence of various bioactive agents in it (Isman, 2000) as well as they tend to have less environmental effects, low mammalian toxicity, and wide public acceptance.

Plants essential oils are made up of about 60 compounds (Bakkali *et. al*. 2008). They are concentrated, hydrophobic liquid having 2 or 3 compounds as main component found at fairly higher in concentrations (20–70%) and other compounds in relatively lower amounts (Bakkali *et. al.* 2008). The inhibitory activity of plants essential oils is related to terpenoids and phenolics compounds such as eugenol, carvacrol, p-cymene and thymol these compounds showed a high antimicrobial as well as inhibitory activity when tested in their pure form (Zambonelli *et. al*,. 1996; Vukovic *et. al*., 2007; Tullio *et. al*., 2007). Essential oil bearing plants constitute a rich source of bioactive chemicals, which have been reported to be antifungal in nature (Daferera, Ziogas, & Polissiou, 2003; Kalemba & Kunicka, 2003).

Results in the present study also supports and resembles with the results of many researches as follows: Manohar *et.al*., (2001) reported that oil extracted from *oregano* had significant antifungal activity. Marin *et.al.,* (2004) reported antifungal activity of *Oregano* and *Cymbopogon* oils against *Fusarium spp.* Velluti *et. al*., (2004) also reported the same that essential oils of *Oregano* and *Cymbopogon* were significant against *Fusarium*. Christian, (2007) reported the similar results that *Oregano* oil completely controlled the *Pythium* and have significant results against *Fusarium.* The antibacterial and antifungal activities of *Oregano* oil inhibit a number of phytopathogens including *Fusarium* has also been reported by many workers (Ravid, & Juven, 1995; Daouk, Dagher, & Sattout, 1995; Daferera *et al*., 2003; Soylu, 2006; Soylu, & Kurt, 2006). The sensitivity of *B. cinerea* to the essential oil of *Origanum vulgare* L.(Daferera *et. al*., 2003) and *Origanum syriacum* L. (E. Soylu, Kurt, & Soylu, 2010), has also been reported. Khan *et. al*., (2010) reported the antifungal activity of *Ocimum sanctum* he also reported that the activity is due to the presence of eugenol and methyl chavicol. Sethi *et.al*., (2012) reported that *Ocimum sanctum* exhibited strong

antifungal activity. Oyeboade *et. al*., (2013), also reported the antifungal activity of essential oil of *Oregano* against *Botrytis cinere*a.

A commercial formulation Gloves Off®', contains thymol and carvacrol as its active ingredients (these are also present in the test oils as reported by many workers, shown in the table no.1) which inhibits mycelium growth and spore germination of *Botrytis cinere*a. Tanovic *et al*., (2007), showed that the essential oils of scots pine, eucalyptus, juniper, orange, rosemary, and thyme, showed antifungal activity against *Pythium*, *Fusarium* and *Rhizoctonia*. Sun *et.al*., (2007) also reported antifungal activity of 39 essential oils against soil borne pathogenic fungi, *Fusarium* and *Pythium.*

CONCLUSION

Essential oils are naturally occurring phytochemicals which have various applications and have long been known and used throughout the world for various purposes such as flavoring agents, antifungal, antibacterial as well as in treatment of various diseases. Natural products such as essential oil and extracts may tend to have less deleterious side effects than corresponding synthetic drugs. Here in the present study all of the six essential oils were giving significant results against all the three economically important phytopathogenic fungi. It can be concluded that these selected oils could lead to development of a new environmental friendly antifungal agent, which may be used as a major constituent in the biopesticides against many plants diseases.

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