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Mitigating Heart Rot or Black Heart Deterioration of Pomegranates Caused by Storage Fungi by use of Essential Oils

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Abstract: *Pomegranate (Punica granatum) is very important and nutritious fruit crop. India is leading producer and exporter of pomegranate. In India Maharashtra is leading producer of pomegranate. Due to its nutritional and export value its demand is increasing. As the moisture content is very high in freshly harvested fruits about 20% fruits are damaged during transport and storage by fungi. It is observed that inside of the fruit are becoming black and aril are decaying. This disease is known as heart rot or black heart. This rot is due to certain fungi such as Alternaria alternata Keissler, Aspergillus niger van Tieghem, Botrytis cinerea Pers, Penicillium glabrum Wehmer and Pilidiella granati. The infection in pomegranates takes place when flowers are opened and anther dehiscence is going to start. The conidia of Alternaria travel together with pollen grains during pollination. The fungus enters the fruit through the tunnel in style into the interior of the fruit. From tunnels they enter into the locules of the fruits and deteriorate the aril of seeds converting them into black colours. Any strategy to control the black heart disease of pomegranate after harvesting the fruits will not be successful as the pathogen enters the host during flowering stage. Therefore spraying at flowering stage will surely be more beneficial approach. The most effective fungicides against heart rot or black heart of pomegranates are Inspire super, Switch, Inspire XT and Pristine. These fungicides are toxic to environment as well as human being. Therefore the purpose of our study is to search for harmless fungicides of plant origin which can be sprayed at the time of anthesis. Several essential oils isolated from higher plants are reported to have antifungal properties. Two essential oils isolated from lemon grass and eucalyptus were selected for antifungal testing against the four major fungi responsible for heart rot in pomegranate such as Alternaria alternata Keissler, Aspergillus niger van Tieghem, Botrytis cinerea Pers and Penicillium glabrum Wehmer. MIC and MCC of Cymbopogon citratus EO was 4.5µl ml^{-1} and 5.0 µl ml^{-1}, while MIC and MCC of Eucalyptus citriodora EO was 3.0 µl ml^{-1} and 3.0 µl ml^{-1} all the four test fungi. Oil combination test in 1:1 ratio was quite more effective controlling these fungi*

Keywords: Minimum inhibitory concentration, Minimum cidal concentration, Essential oil

I. INTRODUCTION

Pomegranate (*Punica granatum*) is the most important fruit crop grown in tropical and sub-tropical regions of the world. This crop was introduced in India during 15th century from Mediterranean regions. India is the largest producer of pomegranates. In India Maharashtra is the leading producer with an area of about 70.2% of total area in the country with average productivity of 13.24MT (Jain and Desai, 2018). At present China, India, Iran, Turkey and USA are five major producers of pomegranate contributing 76% of total production from all over the world. Among all pomegranate producing countries, India is the biggest exporter of pomegranate. UAE, Netherland, Saudi Arabia and Bangladesh are the major importing countries of pomegranates from India (Jain and Desai, 2018; Quiroz, 2009). Due to its increasing demand and nutritional and export value, it is considered as "Vital Cash Crop" in India. Moisture content is very high in freshly harvested fruits. About 20% fruits are damaged during transportation and storage. It is observed that inside of

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the fruit are becoming black and aril are decaying. This has been the biggest challenge for the producers and packers where this disease has no obvious external symptoms. This disease is known as heart rot or black heart. This rot is due to certain fungi such as *Alternaria alternata* Keissler, *Aspergillus niger* van Tieghem, *Botrytis cinerea* Pers, *Penicillium glabrum* Wehmer and *Pilidiella granati* ((Barkai-Golan, 2001, Michailides *et al*., 2010). From among these *Alternatia* spp was reported to be the major cause of heart rot. According to Michailides, after multiple isolations from pomegranates showing black heart symptoms and confirming Koch's postulates, it became apparent that mainly *Alternaria alternata* and occasionally other *Alternaria* species were the main causal agents of black heart disease of pomegranate. According to Kahramanoglu et al (2014) infection rates varies with different varieties/cultivars such as 20.3% for Acco, 14.9% for Herskovitz, and 9.8% for Wonderful cultivars of pomegranates. Different types of fruit rots are the major limiting factor in term of yield losses both qualitatively and quantitavely. Infected fruits exhibited discoloration of one part of the fruit and emission of hollow sound when knocked in comparison to healthy fruits. Consumers lose their confidence from the product when a healthy looking pomegranate cut open with heart rot symptoms inside. In addition to financial loss, a consumer feels to be cheated. The most important thing is the time and path of infection in pomegranate. The most susceptible stage for *Alternaria alternata* infection to take place is that when flowers are opened and anther dehiscence is going to start. Ezra (2013) suggested that the infection in pomegranates takes place at flowering stage. Michailides (2008) found that inoculation at flowering stage with conidia of *Alternaria* exhibited the more severe infections. The thick leathery rind is resistant barrier for infection in fruit. Although cracking in fruit in late seasons takes place but it does not seems to be the entry point for black heart infection. It is considered that the conidia of *Alternaria* travel together with pollen grains during pollination. *Alternaria alternata* conidia is $1/4^{th}$ of the size of pollen grains of Wonderful variety and pollen grains have the potential to migrate the conidia to the interior of the fruit. Ezra *et al* (2013) reported that fungus causing the disease in pomegranate penetrates the flower leading to disease development in fruit. The fungus enters the fruit through the tunnel in style into the interior of the fruit. From tunnels they enter into the locules of the fruits and deteriorate the aril of seeds converting them into black colours. Any strategy to control the black heart disease of pomegranate after harvesting the fruits will not be successful as the pathogen enters the host during flowering stage. Therefore spraying at flowering stage will surely be more beneficial approach. But big challenge is that flowering in pomegranate is not synchronous. In some flowers fruits are set at the same time blossoms are continuously born on terminal shots. Pomegranate blossoms have dioecious characteristics in that within the same tree there are perfect flowers possessing a pistil and anthers (hermaphrodites) and there are male flowers with anthers and degenerate or nonexistent pistils (Michailides, 2008). Hermaphrodite flowers are characterized by a longer bell shape hypanthium than the more conical male inflorescence. It is the hermaphroditic blossoms that are pollinated, swell towards the stem end, and develop into fruit while the male flowers abscise shortly after bloom and fall to the ground. Levin (2006) reported that the proportion of male to 'bisexual' blossoms may be related to availability of assimilates and freeze damage; when there are insufficient resources for ovary development, development is steered towards male structures. Perhaps the same shortage of resources affecting determination of blossoms affects the ability of tissues within pollinated blossoms to withstand infection. After all, recognition of full blossom or near to full blossom is necessary for application of any fungicide to be effective. The most effective fungicides against *Alternaria* spp. from pomegranates are Inspire super (difenoconazole+cyprodinil), Switch (cyprodinil+fludioxonil), Inspire XT ((difenoconazole+propiconazole), Pristine (pyraclostrobin+boscalid) and also squash & orbit. These fungicides are toxic to environment as well as human being. Therefore the purpose of our study is to search for harmless fungicides of plant origin which can be sprayed at the time of anthesis. Several essential oils isolated from higher plants are reported to have antifungal properties. Two essential oils isolated from lemon grass and eucalyptus were selected for antifungal testing against the four major fungi responsible for heart rot in pomegranate such as *Alternaria alternata* Keissler, *Aspergillus niger* van Tieghem, *Botrytis cinerea* Pers and *Penicillium glabrum* Wehmer.

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II. MATERIAL AND METHOD

Collection of pomegranates from local market and isolation of fungi

Ten samples of pomegranates were collected from local fruit markets in separate pre-sterilized polythene bags. These samples were brought to the laboratory and the temperature, pH and moisture content of the collected samples were studied on the day of collection. For the isolation of fungi, infected fruits were taken out of the polythene bags and then the bags were closed. The fungi from the arils were isolated by standard blotter and agar plate methods as recommended by Neergaard and Saad (1962). The moulds were first isolated from non-disinfected arils and then from surface disinfected arils. 0.1% HgCl2 was used as surface disinfectant of arils.

The blotter technique

In this technique pieces of blotter papers were placed in sterilized Petri dishes (90 mm in diameter) and moistened with sterilized distilled water so that a little amount of surplus water was left on the surface of the paper-wad. Non-disinfected and surface disinfected aril pieces were cut in such a way that each piece contain half healthy and half rotten part and they were placed on three layered water-soaked blotters. Petri plates were then incubated in BOD incubator at a temperature of 28°C±2°C. Tube lights mounted in pairs were placed at a distance of about 41cm above the arils (40cm above the lids of the Petri dishes). Sporulation of fungi was stimulated by alternating cycle of blue light and darkness. The fungi growing on arils were examined after eight days of inoculation. Colonies of different fungi were formed around the arils on the blotter papers. They were then pure cultured and identified on the basis of colony and spore characteristics. Sometimes bacterial colonies develop on blotter and inhibit fungal growth. This is overcome by adding an antibiotic such as streptomycin sulfate to the water used to moisten blotter.

The agar plate method

In agar plate method, the nutrient medium used was potato dextrose agar (PDA) medium. The agar medium was prepared by mixing agar powder with an appropriate quantity of water and nutrient additives (dextrose). This mixture is sterilized in an autoclave for half an hour and cooled to about 50°. This mixture is carefully poured in Petri dishes by lifting the lid only enough to pour in the agar medium, thus avoiding the contamination. Before plating the medium in Petri plates, a little amount of streptomycin was added in the medium to check the bacterial growth. Non-disinfected and surface disinfected arils were placed on the nutrient medium. The surface disinfection eliminates the contamination of the arils by saprophytes, which tend to develop rapidly on the agar and may inhibit or completely obscure the slow growing pathogens. The Petri dishes were incubated for 7-days at 28±2°C and fungi growing in association with each arils were isolated in pure culture and identified with the help of relevant literature. Following are the fungi that were isolated and identified from chosen arils of pomegranates.

Table-1 Fungi isolated from pomegranate aril and epicarp

Fungi isolated	Pomegranate arils	Pomegranate epicarp
Alternaria alternata Keissler	+	-
Aspergillus niger van Tieghem	+	+
Botrytis cinerea Pers.	+	+
Penicillium glabrum Wehmer	+	+
Pilidiella granati Sacc.	-	+
Alternaria solani (Ell. & Mart.) Jones & Grout	+	-
Penicillium italicum Wehmer	+	-
Penicillium expansum Link.	-	+
Rhizopus nigricans Ehrenberg.	-	+

Nine fungi were isolated from pomegranate arils and epicarp. Out of these, four major fungi such as *Alternaria alternata* Keissler, *Aspergillus niger* van Tieghem, *Botrytis cinerea* Pers and *Penicillium glabrum* Wehmer were selected as test pathogen for fungitoxic investigation.

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Collection of plants

Plants were selected on the basis of literature survey from various libraries. It was found that a plant with aromatic characters and with volatile constituents was more effective than those with non-aromatic and non-volatile constituents. The plants were identified with the help of floras (Hooker, 1872-1892; Duthie, 1903-1929; Bailey, 1958; Maheshwari, 1963; Santapau, 1967; Gupta, 1968 and Srivastava, 1976) and the anthentic herbarium specimens lodged in the herbarium of Botanical Survey of India, Central circle, Allahabad, and the Duthie herbarium of the Department of Botany, University of Allahabad, Allahabad, India.

Extraction of essential oil

The essential oil were extracted from the different parts of the plants such as leaves, stems, roots, fruit peels, fruits, woods etc using Clevenger′s apparatus (Clevenger, 1928). This apparatus extract the volatile fraction from the plant parts through hydro-distillation method. Two distinct fractions comprising an upper oily layer and a lower aqueous layer were obtained which were separated by carefully regulating the stopper of the apparatus. The upper oily layer was made anhydrous by treating it with 0.5 grams of anhydrous sodium sulphate in order to obtain the pure essential oil. To isolate oil from the aqueous portion, the oil was extracted with solvent ether in a separating funnel. The ether was removed at reduced pressure, which resulted in an oily residue that was stocked in the oil collected earlier. The oils thus obtained were evaluated at 3000ppm concentration for pesticidal activity against the test fungi viz., *Alternaria alternata* Keissler, *Aspergillus niger* van Tieghem, *Botrytis cinerea* Pers and *Penicillium glabrum* Wehmer.

Antifungal screening of essential oils at 3000 ppm

Antifungal screening of isolated essential oils was carried out following the Poisoned Food Technique of Grover and Moore (1962) with slight modification. The oil samples of 0.3 ml were mixed in acetone (2% of the required quantity of the medium) and then added to 98 ml of pre-sterilized Potato dextrose Agar medium (pH 5.6). In control sets, sterilized water (in place of the oil) and acetone were used in the medium in appropriate amount. Fungal mycelial disc of 5 mm diameter, cuts out from the periphery of 7 days old cultures of storage fungi, were aseptically inoculated upside down on the agar surface of the medium in plates. Inoculated petri plates were incubated at 27±1°C and the observations which were mean value of five replicates in each case, recorded on seventh day. Percentage mycelial growth inhibition (MGI) was calculated as per formula-

$$\textbf{MGI (\%)} = \frac{dc-dt \times 100}{dc}$$

where

dc = Colony diameter in control

dt = Colony diameter in treatment

Table-2 Antifungal screening of the collected plants against the storage fungi at 3000ppm concentration

Plants	Mycelial growth inhibition (%) mean±SD			
	Alternaria altenata	***Aspergillus niger***	***Botrytis cinerea***	***Penicillium glabrum***
Artemisia nilagarica (Clarke) Pamp. (Pt)	94±2.1	84±2.2	64±1.1	91±1.1
Ageratum conyzoides L. (Lf)	90±2.8	70±2.1	60±1.8	80±2.2
Ammomum subulatum Roxb. (Fr)	98±1.0	88±1.1	68±2.0	58±1.9
Apium graveolens L. (Se)	92±1.3	82±1.4	62±2.3	93±1.2
***Cedrus deodara* (Roxb.) Loud. (Wo)**	**100**	**100**	**100**	**100**
Citrus aurantifolia (Christm.et Panz.) Swin. (Lf)	80±3.4	70±3.6	50±3.5	60±1.9
Citrus reticulata Blanco. (Pl)	40±1.9	41±3.5	85±2.1	92±2.8
Citrus sinensis (L.) Osbeck (Pl)	60±3.9	80±3.7	65±3.1	66±3.9
Citrus medica L. (Lf)	82±1.4	72±1.4	52±1.1	85±1.4
Cuminum cyminum L. (Se)	84±0.7	74±0.2	54±3.7	83±0.7
Curcuma longa L. (Rh)	72±1.2	72±1.7	72±2.2	78±1.2

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Curcuma aromatica Salisb. (Rh)	58±1.1	48±1.7	88±2.1	59±1.1
***Cymbopogon citratus* Stapf. (Lf)**	**100**	**100**	**100**	**100**
***Eucalyptus citriodora* Hook. (Lf)**	**100**	**100**	**100**	**100**
Juniperus communis L. (Lf)	45±2.7	44±2.4	85±3.7	45±2.6
Lantana camara Linn. (Pt)	96±0.2	91±0.7	66±3.2	90±0.2
Micromeria biflora (Buch.-Ham ex Don) Ben.(Lf)	76±3.4	72±3.7	56±3.3	76±3.4
Mentha spicata L. (Pt)	90±2.1	80±2.6	60±3.1	90±2.3
Oenanthe javanica (Bl.) DC. (B. Pan Tursi) (Se)	90±1.9	70±1.3	70±1.7	96±1.9
***Ocimum gratissimum* L. (Lf)**	**100**	**100**	**100**	**100**
Piper nigrum L. (Fr)	95±1.7	65±1.5	92±1.6	90±1.7
Pinus sp. (Lf)	84±1.6	74±1.9	64±1.6	74±1.6
Psidium guajava L. (Lf)	75±1.5	65±1.1	72±1.8	70±1.5
***Syzygium aromaticum* (L.) Merr. and Perry (Fb)**	**100**	**100**	**100**	**100**

Lf = leaf, Fr = fruit, Se = seed, Pt = plant, Rh = rhizome, Fb = flower bud, Se = seed, Wo = wood, Pl = peel

During Antifungal screening essential oil from various essential oil bearing plants were tested against dominant storage fungi. The oil of *Cedrus deodara* (Roxb.) Loud. (Wo), *Cymbopogan citratus* (Lf), *Eucalyptus citriodora* (Lf), *Ocimum gratissimum* (Lf) and *Syzygium aromaticum* (Fb) gave 100% inhibition at 3000 ppm concentration inhibiting the mycelial growth completely against all the four test fungi (Table-5). But *Cymbopogan citratus* (Lf), and *Eucalyptus citriodora* (Lf) were selected for the present study because *Cymbopogon* is a grass which can be cultivated easily and *Eucalyptus* leaf is waste product. Fallen leaf can be used for extraction of oil. Extraction of oil from the waste will recycle it. Further the cost benefit ratio will be low.

III. RESULT AND DISCUSSION

Table-3 Minimum inhibitory concentration of *Cymbopogon citratus* leaf oil against dominant fungi isolated from Pomegranate

Concentration of oil (µl ml^{-1})	**Mycelial growth inhibition %**			
	Alternaria altenata	***Aspergillus niger***	***Botytis cineria***	***Penicillium glabrum***
8.0	100	100	100	100
7.5	100	100	100	100
7.0	100	100	100	100
6.5	100	100	100	100
6.0	100	100	100	100
5.5	100	100	100	100
5.0	100	100	100	100
4.5	100	100	100	100
4.0	71	60	100	100
3.5	43	47	100	100
3.0	-	-	100	100
2.5	-	-	63	54
2.0	-	-	48	49
1.5	-	-	-	-
1.0	-	-	-	-
0.5	-	-	-	-

indicates not considered

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Table-4 Minimum inhibitory concentration of *Eucalyptus citriodora* leaf oil against dominant fungi isolated from Pomegranate

Concentration of oil (µl ml^{-1})	Mycelial growth inhibition %			
	Alternaria altenata	*Aspergillus niger*	*Botytis cineria*	*Penicillium glabrum*
8.0	100	100	100	100
7.5	100	100	100	100
7.0	100	100	100	100
6.5	100	100	100	100
6.0	100	100	100	100
5.5	100	100	100	100
5.0	100	100	100	100
4.5	100	100	100	100
4.0	100	100	100	100
3.5	100	100	100	100
3.0	100	100	100	100
2.5	77	65	100	100
2.0	49	41	53	59
1.5	-		44	46
1.0	-	-	-	-
0.5	-	-	-	-

indicates not considered

Results: Minimum inhibitory concentration:

The minimum inhibitory concentrations (MICs) of *Cymbopogon citratus* leaf oil against both the *Alternaria alternata* and *Aspergillus niger* were found to be 4.5 µl ml^{-1} i.e. 4500ppm. MICs of the same oil against *Botrytis cineria* and the *Penicillium glabrum* were 3.0 µl ml^{-1} i.e. 3000 ppm. The oil of *Eucalyptus citriodora* was more effective in inhibiting the growth of the test fungi. It inhibited *Alternaria alternata* and *Aspergillus niger* at 3.0 µl ml^{-1} concentration and *Botrytis cineria* and the *Penicillium glabrum* at 2.5 µl ml^{-1} (Table-3, 4).

Table-5 Minimum cidal concentrations of *Cymbopogon citratus* leaf oil against dominant fungi isolated from Pomegranate

Concentrations of oil (µl ml^{-1})	Mycelial growth inhibition (MGI %)			
	Alternaria altenata	*Aspergillus niger*	*Botytis cineria*	*Penicillium glabrum*
8.0	100^{c}	100^{c}	100^{c}	100^{c}
7.5	100^{c}	100^{c}	100^{c}	100^{c}
7.0	100^{c}	100^{c}	100^{c}	100^{c}
6.5	100^{c}	100^{c}	100^{c}	100^{c}
6.0	100^{c}	100^{c}	100^{c}	100^{c}
5.5	100^{c}	100^{c}	100^{c}	100^{c}
5.0	100^{c}	100^{c}	100^{c}	100^{c}
4.5	100^{s}	100^{s}	100^{c}	100^{c}
4.0	-	-	100^{c}	100^{c}
3.5	-	-	100^{s}	100^{s}
3.0	-	-	100^{s}	100^{s}
2.5	-	-	-	-

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2.0	-	-	-	-
1.5	-	-	-	-
1.0	-	-	-	-
0.5	-	-	-	-

indicates not considered

Table-6 Minimum cidal concentrations of *Eucalyptus citriodora* leaf oil against dominant fungi isolated from Pomegranate

Concentrations of oil (µl ml^{-1})	**Mycelial growth inhibition (MGI %)**			
	Alternaria altenata	***Aspergillus niger***	***Botytis cineria***	***Penicillium glabrum***
8.0	100^{c}	100^{c}	100^{c}	100^{c}
7.5	100^{c}	100^{c}	100^{c}	100^{c}
7.0	100^{c}	100^{c}	100^{c}	100^{c}
6.5	100^{c}	100^{c}	100^{c}	100^{c}
6.0	100^{c}	100^{c}	100^{c}	100^{c}
5.5	100^{c}	100^{c}	100^{c}	100^{c}
5.0	100^{c}	100^{c}	100^{c}	100^{c}
4.5	100^{c}	100^{c}	100^{c}	100^{c}
4.0	100^{c}	100^{c}	100^{c}	100^{c}
3.5	100^{c}	100^{c}	100^{c}	100^{c}
3.0	100^{c}	100^{c}	100^{c}	100^{c}
2.5	-	-	100^{c}	100^{c}
2.0	-	-	-	-
1.5	-	-	-	-
1.0	-	-	-	-
0.5	-	-	-	-

Results: Minimum cidal concentration:

Minimum cidal concentration (MCCs) of *Cymbopogon citratus* leaf oil against both the *Alternaria alternata* and *Aspergillus niger* were found to be 5.0 µl ml^{-1} i.e. 5000ppm. The MCCs of the same oil against *Botrytis cineria* and the *Penicillium glabrum* was 4.0 µl ml^{-1}. The oil of *Eucalyptus citriodora* was fungicidal at its MIC. It was fungicidal to *Alternaria alternata* and *Aspergillus niger* at 3.0 µl ml^{-1} and *Botrytis cineria* and the *Penicillium glabrum* at 2.5 µl ml^{-1}. *Eucalyptus* oil is more effective in inhibiting the growth of the test fungi and also is effective in killing them at lower concentrations (Table-5,6).

Table-7 Minimum Killing Time of *Cymbopogon citratus* leaf oil against dominant fungi isolated from Pomegranate

Minimum Killing Time (MKT)	**Mycelial growth inhibition (MGI %)**							
	Alternaria altenata		***Aspergillus niger***		***Botytis cineria***		***Penicillium glabrum***	
	Pure oil	**MCC**	**Pure Oil**	**MCC**	**Pure Oil**	**MCC**	**Pure Oil**	**MCC**
8hr	100	100	100	100	100	100	100	100
6hr	100	100	100	100	100	100	100	100
4hr	100	100	100	100	100	100	100	100
2hr	100	75	100	65	100	100	100	100
1hr	100	40	100	36	100	78	100	89
45 Min	100	-	100		100	25	100	50
30 Min	100	-	100	-	100	-	100	-

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15 Min	100	-	100	-	100	-	100	-
1 Min	100	-	100	-	100	-	100	-
45 Sec	85	-	89	-	100	-	100	-
30 Sec	24	-	33	-	100	-	45	-
15 Sec	-	-	-	-	65	-	37	-
5 Sec	-	-	-	-	36	-	-	-
1 Sec	-	-	-	-	-	-	-	-

MCC = Minimum Cidal Concentration
indicates not considered

Table-8 Minimum Killing Time of *Eucalyptus citriodora* leaf oil against dominant fungi isolated from Pomegranate

Minimum Killing Time (MKT)	**Mycelial growth inhibition (MGI %)**							
	Alternaria altenata		***Aspergillus niger***		***Botytis cineria***		***Penicillium glabrum***	
	Pure oil	**MCC**	**PureOil**	**MCC**	**Pure Oil**	**MCC**	**Pure Oil**	**MCC**
8hr	100	100	100	100	100	100	100	100
6hr	100	100	100	100	100	100	100	100
4hr	100	100	100	100	100	100	100	100
2hr	100	100	100	100	100	100	100	100
1hr	100	65	100	75	100	100	100	100
45 Min	100	46	100	38	100	100	100	87
30 Min	100	-	100	-	100	68	100	60
15 Min	100	-	100	-	100	29	100	-
1 Min	100	-	100	-	100	-	100	-
45 Sec	100	-	100	-	100	-	100	-
30 Sec	100	-	100	-	100	-	100	-
15 Sec	87	-	85	-	100	-	100	-
5 Sec	34	-	23	-	69	-	55	-
1 Sec	-	-	-	-	56	-	27	-

MCC = Minimum cidal concentration
indicates not considered

Results: Minimum Killing Time:

The minimum killing time of the pure oil of *Cymbopogon citratus* against the *Alternaria alternata* and *Aspergillus niger* by contact method was 1 minute. At MCC this oil took 4 hrs to kill these fungi. The *Botrytis cineria* was killed in 30 seconds by the pure oil. But at MCC this oil killed in 2 hrs. The *Penicillium glabrum* was killed in 45 seconds by pure oil but at MCC it was killed in 2 hrs. The minimum killing times of the pure oil of *Eukalyptus citriodora* against *Alternaria alternata, Aspergillus niger*, *Botrytis cineria* and *Penicillium glabrum* were 30 sec, 30 sec, 15 sec and 15 sec respectively. The minimum killing times of *Eucalyptus citriodora* oil at MCC against the same fungi were 2hrs, 2hrs, 45sec and 1hrs respectively (Table-7, 8).

Table-9: Increased Inoculum density and pesticidal activity of the oil of *Cymbopogon citratus*

Inoculum density at MICs (Disc of fungal pests each of 5mm diameter)	**Mycelial growth inhibition (MGI %)**			
	Alternaria altenata	***Aspergillus niger***	***Botytis cineria***	***Penicillium glabrum***
05	100	100	100	100

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10	100	100	100	100
15	100	100	100	100
20	100	100	100	100
25	100	100	100	100

MICs=Minimum inhibitory concentrations

Table-10: Increased Inoculum density and pesticidal activity of the oil of *Eucalyptus citriodora*

Inoculum density at MICs (Disc of fungal pests each of 5mm diameter)	**Mycelial growth inhibition (MGI %)**			
	Alternaria altenata	***Aspergillus niger***	***Botytis cineria***	***Penicillium glabrum***
05	100	100	100	100
10	100	100	100	100
15	100	100	100	100
20	100	100	100	100
25	100	100	100	100

MICs=Minimum inhibitory concentrations

Results: Increased Inoculum density and pesticidal activity:

It was found that the fungicidal activity of the oil of *Cymbopogon citratus* and *Eucalyptus citriodora* was not reduced as the number of fungal disc was increased. That is the increased inoculums density does not affect the pesticidal activity of the both the oil (Table-9, 10).

Table-11: Pesticidal spectrum of the oil of *Cymbopogon citratus*

Tested fungi	**Mycelial growth inhibition at various concentrations (µl ml^{-1})**		
	3.0 (sub-lethal)	**4.0 (lethal)**	**6.0 (hyper-lethal)**
Aspergillus parasiticus Speare	56	87	100^{c}
Aspergillus fumigatus Fresenius	100^{s}	100^{c}	100^{c}
Aspergillus nidulans (Eidan) Winter	74	100^{s}	100^{c}
Aspergillus terreus Thom.	69	92	100^{c}
Absidia ramosa (Lindt.) Lendn.	100^{s}	100^{s}	100^{s}
Alternaria solani (Ell. & Mart.) Jones & Grout	100^{c}	100^{c}	100^{c}
Cladosporium cladosporioides (Fres.) deVries	78	91	100^{c}
Colletotrichum capsici (Syd.) Butler & Bisby	100^{s}	100^{c}	100^{c}
Colletotrichum falcatum Went.	79	100^{s}	100^{s}
Curvularia lunata (Walker) Boedijin	100^{c}	100^{c}	100^{c}
Cephalosporium sp.	78	100^{c}	100^{c}
Fusarium oxysporum Schlecht.	61	87	100^{s}
Fusarium udum Butler	64	93	100^{s}
Fusarium moniliforme Sheld.	56	89	100^{s}
Fusarium solani (Mart.) Sacc.	66	78	100^{s}
Helminthosporium maydis Nisikado & Miyake	100^{s}	100^{c}	100^{c}
Helminthosporium oryzae Breda de Haan	100^{s}	100^{c}	100^{c}
Macrophomina phaseolina (Tassi) Goid.	69	100^{s}	100^{s}
Mucor sp.	100^{c}	100^{c}	100^{c}
Penicillium expansum Link.	89	100^{c}	100^{c}
Penicillium implecatum Biourge	65	94	100^{s}

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Penicillium minioluteum Dierckx	100^{s}	100^{c}	100^{c}
Pestilotia sp.	100^{s}	100^{c}	100^{c}
Pithium sp.	78	100^{s}	100^{c}
Rhizoctonia solani Kuehn.	61	72	100^{s}
Rhizopus nigricans Ehrenberg.	100^{s}	100^{c}	100^{c}
Trichoderma viride Pers. ex Fr.	79	100^{c}	100^{c}
Trichoderma harzianum Rifai	100^{s}	100^{s}	100^{s}
Trichothecium sp.	100^{s}	100^{c}	100^{c}
Verticillium sp.	100^{s}	100^{c}	100^{c}

S= indicates static activity, C= indicates cidal activity

Table-12 Pesticidal spectrum of the oil of *Eucalyptus citriodora*

Tested fungi	**Mycelial growth inhibition at various concentrations (µl ml^{-1})**		
	2.0 (sub-lethal)	**3.0 (lethal)**	**5.0 (hyper-lethal)**
Aspergillus parasiticus Speare	46	87	100^{c}
Aspergillus fumigatus Fresenius	100^{s}	100^{c}	100^{c}
Aspergillus nidulans (Eidan) Winter	100^{s}	100^{c}	100^{c}
Aspergillus terreus Thom.	54	63	100^{s}
Absidia ramosa (Lindt.) Lendn.	100^{s}	100^{c}	100^{c}
Alternaria solani (Ell.& Mart.) Jones & Grout	100^{c}	100^{c}	100^{c}
Cladosporium cladosporioides (Fres.) deVries	51	72	100^{c}
Colletotrichum capsici (Syd.) Butler & Bisby	100^{s}	100^{c}	100^{c}
Colletotrichum falcatum Went.	100^{s}	100^{c}	100^{c}
Curvularia lunata (Walker) Boedijin	100^{c}	100^{c}	100^{c}
Cephalosporium sp.	100^{s}	100^{s}	100^{c}
Fusarium oxysporum Schlecht.	76	89	100^{s}
Fusarium udum Butler	100^{s}	100^{s}	100^{c}
Fusarium moniliforme Sheld.	87	100^{s}	100^{s}
Fusarium solani (Mart.) Sacc.	71	93	100^{s}
Helminthosporium maydis Nisikado & Miyake	74	100^{c}	100^{c}
Helminthosporium oryzae Breda de Haan	70	100^{c}	100^{c}
Macrophomina phaseolina (Tassi) Goid.	100^{s}	100^{c}	100^{c}
Mucor sp.	63	86	100^{s}
Penicillium expansum Link.	100^{s}	100^{c}	100^{c}
Penicillium implecatum Biourge	100^{s}	100^{c}	100^{c}
Penicillium minioluteum Dierckx	100^{s}	100^{c}	100^{c}
Pestilotia sp.	65	79	100^{c}
Pythium sp.	65	100^{s}	100^{c}
Rhizoctonia solani Kuehn.	76	100^{s}	100^{c}
Rhizopus nigricans Ehrenberg.	100^{s}	100^{c}	100^{c}
Trichoderma viride Pers. ex Fr.	76	100^{s}	100^{c}
Trichoderma harzianum Rifai	79	100^{s}	100^{c}
Trichothecium sp.	100^{s}	100^{s}	100^{c}
Verticillium sp.	100^{s}	100^{s}	100^{c}

S= indicates static activity, C= indicates cidal activity

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Result: Pesticidal spectrum:

The *Cymbopogon citratus* oil exhibited a broad spectrum of fungitoxicity by inhibiting completely at its hyper lethal concentration of 6.0 µl ml^{-1}, the growth of all the 30 fungal species available/isolated; at this concentration it was fungicidal to 20 species. At its lethal concentration i. e. 4.0 µl ml^{-1} it inhibited 21 fungal species and was fungicidal to 15 species. At sub-lethal concentration of 3.0 µl ml^{-1} this oil inhibited 14 fungal species and was fungicidal to 3 fungal species. The *Eucalyptus citriodora* oil also exhibited a broad spectrum of fungitoxicity by inhibiting completely at its hyper lethal concentration of 5.0 µl ml^{-1}, the growth of all the 30 fungal species available/isolated; at this concentration it was fungicidal to 25 species. At its lethal concentration i. e. 3.0 µl ml^{-1} it inhibited 23 fungal species and was fungicidal to 14 species. At sub-lethal concentration of 2.0 µl ml^{-1} this oil inhibited 16 fungal species and was fungicidal to 2 fungal species (Table-11,12).

Table-13: Minimum inhibitory concentration of two oil combinations (*Cymbopogon citratus* + *Eucalyptus citriodora*) in different volume against dominant storage fungi

Oil combinations (in ml) (*Cymbopogon citratus* + *Eucalyptus citriodora*)	**Minimum Effective Concentrations (µl ml^{-1})**			
	Alternaria altenata	***Aspergillus niger***	***Botytis cineria***	***Penicillium glabrum***
0.1 + 0.1	3.5	3.5	2.0	2.2
0.1 + 0.09	3.6	3.6	2.1	2.3
0.1 + 0.08	3.7	3.7	2.2	2.4
0.1 + 0.07	3.8	3.8	2.3	2.5
0.1 + 0.06	3.9	3.9	2.4	2.6
0.1 + 0.05	4.0	4.0	2.5	2.7
0.1 + 0.04	4.1	4.1	2.6	2.8
0.1 + 0.03	4.2	4.2	2.7	2.9
0.1 + 0.02	4.3	4.3	2.8	3.0
0.1 + 0.01	4.4	4.4	2.9	3.5

Results: Minimum inhibitory concentration of oil combinations:

Cymbopogon citratus oil is obtained from the peel which is discarded as waste product of fruit juice industry. Extraction of oil from a waste product and using it as preservative of dried fruits in storage will surely help in recycling of the waste. Dried fruits are costly items and its preservation from a waste product will have very low cost benifit ratio. *Eucalyptus citriodora* oil is obtained from the heart wood of root and stem. *Eucalyptus* oil is comperativily more effective but it is obtained by felling of *Eucalyptus* trees. Therefore, environmentally, it is harmful. To increase the toxicity of *Cymbopogon* oil, less toxic *Cymbopogon* oil and more toxic *Eucalyptus* oils are mixed in different ratio and oil combination testing was done to determine the MICs. By contact method, it was found that the mixing both the oils in 1:1 ratio gives the best result. Reducing the amount of *Eucalyptus* oil, increases the MICs; but increasing the amount of the same oil will not be environmentally beneficial. Thus, the oils can be used more effectively by mixing them in 1:1 ratio (Table-13).

IV. CONCLUSION

Generally the physical and chemical agents are applied to check fungal deterioration of fruits during storage. The physical treatments include heat treatment, low temperature storage and exposure to radiation. However most of physical treatments have their own limitations. Drastic heat treatment considerably reduces the mycoflora but cause changes in the flavour and aroma of the stored commodities. It has also been observed that as soon as the fruits and other commodities stored at low temperature are taken out, there is acceleration in the rate of their decay. Moreover low temperature storage is costly due to the high cost of the electricity. Chemical treatment is considered to be the cheapest

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method to control fungal deterioration of food and feed commodities. Many synthetic fungitoxic chemicals are now being used as fruits preservatives. However, the use of several of these synthetic chemicals has been cautioned due to their carcinogenetic, teratogenicity and residual toxicity. Further these synthetic chemicals have been restricted due to their several other undesirable side effects such as a high and acute toxicity, the long degradation periods, their concentration in food chain, the suspected dangers of chronic poisoning through the continuous intake of small quantities. Besides, due to development of new races of pathogens, many of these fungicides are gradually becoming out of date. Furthermore, the sources of these synthetic fungicides are largely petrochemicals, which are exhaustible. Therefore, haunt for inexhaustible sources of such chemotherapeutants is highly desirable. The antimicrobial properties of green plants have been recognized since prehistoric times. A large number of plants are already reported to possess medicinal properties in Indian Ayurvedic literature dating back to 2500-600BC. However, during recent years, a number of higher plants have been found to exhibit strong toxicity against various phyto-pathogenic fungi. *Trachyspermum ammi* have been found to be particularly effective, often completely inhibiting both fungal growth and toxin production. Many commercially available spices and herbs, turmeric, basil, marjoram, anise, cumin and coriander are able to completely inhibit toxin production.

In present study we have selected *Cymbopogon flexuosus* and *Eucalyptus citriodora* for antifungal study on various parameters such as MIC, MCC, MKT, effect of increased inoculum density and pesticidal activity against the test fungi such as *Alternaria alternata, Aspergillus niger, Botrytis cineria* and *Penicillium glabrum.*. Pesticidal spectrum and oil combination testing was also done. In each case both the oils were very effective against the pomegranate heart rot fungi. Since in pomegranate infection takes place at the time of anthesis, a pesticide developed with these two oils can be sprinkled over pomegranate flowers to prevent the infection.

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