Antifungal Assay using Plant Essential oils for Controlling Phyto-Pathogenic and Post-harvest Rot causing Fungal Strain Aspergillus niger

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***Abstract*—Fungi are the greatest acquainted groups of plant pathogens and are accountable for variety of symptoms in plants including leaf spots, necrosis, blight, damping off, wilt and also rots and decay of storage produce. Therefore, fungi are the significant destroyers of foodstuffs, checking their nutritive value and render them unfit for eating by creating harmful mycotoxins during their storage and conveyance. Typically, storage fungi are controlled by synthetic chemicals which often have numerous side effects in the forms of carcinogenicity, teratogenicity and residual toxicity. Accordingly, few other biodegradable and eco-friendly control measures need be discovered to replace unsafe synthetic ones. The current investigation is an important step in developing plant-based fungicides against some chief post-harvest fungal pathogen alike *Aspergillus niger*.**

Indiscriminately used synthetic chemical fungicides owing to their non-degradable nature, involve serious problems to human well-being and similarly to the environment. Regulation of fungal pathogens using chemicals, under field condition is not only carcinogenic and hazardous to health but similarly responsible for the serious environmental pollution owing to their non-degradable nature. Furthermore, their undiscriminating practice has resulted into an induced resistance amongst the microbial pathogens. Thus, has imposed the issue of drug-resistant pathogens against traditionally used synthetic chemical fungicides. To overcome this issue; several *in vitro* and *in vivo* research efforts are made on fruits and vegetables like food produce post-harvest storage treatment by means of plant extracts and essential oils to control fungal spoilage and thereby enhancing the shelf-life of fruits and vegetables. Thus, the mission to investigate the effective, bio-safe and bio-degradable substitute fungicide is the major concern in today's time. Diverse plants genera produce an extensive variety of Plant Secondary Metabolites (PSMs) or Phyto-chemicals. In this context; many plants are not been fully explored apart from routine uses for their bioactive properties of secondary metabolites, essential oils and volatile fractions. Accordingly, PSMs which have self-protective part may be exploited for the management of plant diseases in field conditions as well as during transportation and storage due to their edible nature and therapeutic importance. Plant-based antifungal components have a target specific effect and are bio-degradable in nature. Consequently, botanical extracts and essential oils are eco-friendly in nature and assist a greatest alternative to these dangerous synthetic chemicals. Biochemically antifungal principles can interrupt the membrane, producing cell leakage, cytoplasmic evacuation and damage of proton motive force and thus results in the fungal growth inhibition.

Considering the priority; in order to investigate potential botanical antifungals; in the present study, essential oils from four Indian plants namely *Cymbopogon citrates*, *Eucalyptus globules, Gaultheria procumbens* and *Syzygium aromaticum* were used to discover their latent antifungal activity against important crop destroying and post-harvest rot causing fungal strain *Aspergillus niger.* Primary screening experiments in the present investigation in Primary screening *in vitro* assay had revealed that tested essential oils possess an excellent antifungal potential. Further the Secondary screening and dose optimization studies were conducted to find the MIC value for each tested essential oils to inhibit the selected fungal strains using Disc Diffusion Assay. Usually, essential oils of the above stated plants are used for the medicinal purposes, nonetheless here an attempt was made to perceive the antifungal potency and to be used it against the commonly found crop and yield destructing fungal strains by further preparing an appropriate value-added Herbal Fungicide formulation. In the present study, efforts made to test the antifungal potency of four essential oils against Postharvest Rot causing fungi *Aspergillus niger* using Paper disc diffusion assay.

***Index Terms*—Mycotoxins, Synthetic Chemical Fungicides, Post-harvest Fungal Pathogens, Plant Secondary Metabolites (PSMs), antifungal potency, Essential oils, Paper disc diffusion assay**

I. INTRODUCTION

A. Agricultural Scenario and Plant Diseases:

Amongst plant microbial pathogens like bacteria, fungi, viruses etc., fungi are the most significant and predominant pathogens, contaminating an extensive range of host plants and producing economical losses and obliteration of crops in field and harvests throughout storage and transportation. Pre- and Post harvest bio-degradation of innumerable agricultural produce like grains, vegetables and fruits since of microbial attack is a serious problem in their storage and has caused in severe yield losses. Conventionally used synthetic fungicides in agricultural practices and their indiscriminately usage has led to the development of fungicide resistance in pathogens. Furthermore, they are non-degradable in nature, get combined in crop remains, collected in soil, damage valuable soil micro-flora and are accountable for soil and water pollution. Since the development of fungicide resistance and rising consciousness on the hazardous properties of synthetic fungicides to human health and environment, exploring novel safer, eco-friendly, and bio-degradable alternative is the prerequisite today. In this relation, antifungal activity of many plant extracts has been previous stated on newer sources of antifungal plants to control fungal pathogens. Consequently, there is a distinct focus to use botanical extracts and oils to control plant diseases. Spices and other aromatic products derived from plant sources have been in practice for fragrance and flavour of foodstuffs, preservation of foods and for their medicinal value. Making use of essential oils as a fungicide is an attractive and unique method with a great and a potential for controlling post-harvest diseases.

B. Fungal Phyto-pathogens and Fungal Plant-diseases:

Fungal pathogens are the most prevalent pathogens, infecting a wide range of host plants and causing destruction and economical loss of crops and harvested produce. Fruits and vegetables are highly susceptible to pathogenic fungi due to their lower range of pH, higher moisture content and rich nutrient compositions. Also, fungi as a result of fungal colonization and mycotoxins production; turn the food in to inferior in quality and nutrition (Baiyewu *et al*, 2007). As per the suggested reports in developing countries; Fungal diseases accounts pre-harvest yield losses up to 12% or even more, and post-harvest diseases up to 10-30% (Fatima *et al*, 2009). Fungal species like *Aspergillus*, *Fusarium* and *Penicillium* spp. produce mycotoxins and other toxic fungal metabolites which are acknowledged to cause carcinogenicity, genotoxicity, terratogenicity, nephrotoxicity, hepatotoxicity, reproductive disorders and immunosuppression in human and animal body. The shelf-life of post-harvest yield can be amplified by Synthetic chemicals. However, non-target and toxic effects of synthetic chemicals and pollution issues regulatory agencies including World Health Organization (WHO) have banned several control chemicals. Thus, there is an urgent need for alternative approaches without any toxicity problems and having unique mode of action.

C. Post-harvest Fresh Produce deterioration due to Fungi:

Fungi exhibit saprophytic or parasitic mode of nutrition. They predominantly reproduce by the production of huge amount of asexual spores, which is a major source of fungal infestation and rapid proliferation. *Aspergillus, Mucor, Rhizopus, Fusarium, Alternaria, Penicillium* spp. known as storage fungi of important crops are reported to produce mycotoxin/aflatoxins which makes the produce unsafe for consumption. Aflatoxins are biologically active secondary metabolites which are extremely potent carcinogenic, teratogenic, hepatotoxic, allergic sensitizer, immunosuppressive and constrain several metabolic systems (Baiyewu *et al*, 2007; Lokman, 2010). Hasnain *et al* (1998) have reported that, the airborne *Alternaria* can serve a potential allergic sensitizer in vulnerable individuals with bronchial asthma and allergic rhinitis symptoms. In Japan, a new disease of peach *Prunus persica*, caused by fungus *Alternaria alternata* was found to produce phytotoxins in broth culture, which induced necrosis on wounded peach leaves (Inoue and Nasu, 2000).

Plant-based fungal pathogens are responsible to cause severe economic losses to crops as well as harvested products. Fatima *et al* (2009) have reported

that, by and large post-harvest deterioration of fresh fruits, vegetables and other plant products occur during harvesting till consumption due to infection of various fungi viz., *Alternaria alternata* (causes infection in apple, bell pepper, bitter gourd, bottle gourd, papaya, pear, round gourd, sponge gourd, tomato), *Fusarium solani* (infects melon, papaya, egg plant, cucumber, sponge gourd, tomato) *Aspergilllus flavus* and *Aspergillus niger* (infects lemon, mango, round gourd, tomato). A study by Aye *et al* (2009) shows that sheath and stem disease of Rice is caused by *Rhizoctonia* and *Sclerotium* species and harms the Rice production. Infestation by microorganisms like species of *Aspergillus* causes losses in terms of pre- and post-harvest bio-deterioration, spoilage, seed quality and nutritional quality of grains, vegetables, fruits and agricultural produce. Synthetic fungicides are of no use for perishables and stored food and thus there is a need to search for alternative method without any toxicity. Biologically active plant based secondary metabolites, which are defensive in nature and retards the reproduction of undesirable microorganisms can be used here as a more sensible bio-control method for the management of seed borne species of *Aspergillus* (Satish *et al*, 2007). Due to lack of information on the screening/evaluation of diverse plants for their antibacterial activity, many species of higher plants have not been evaluated for presence of biologically active new sources of antifungal constituents and developed in to value added fungicide product. Mohana and Raveesha (2007) have suggested that an aqueous extract of an edible plant *Decalepis hamiltonii* rhizome can be exploited in the management of various seed borne pathogenic fungi of grains*, Aspergillus* spp*., Fusarium* spp*., Penicillium* spp. and *Alternaria alternata* together with the prevention of mycotoxin production during storage. A study by Panda *et al*, 2010 showed inhibition of all the three species of *Aspergillus fumigatus, A. flavus* and *A. niger* using *Cassia fistula* leaves petroleum ether and ethanol extracts.

The fungal strains used for the study was *Aspergillus niger* is a destructive post-harvest pathogens and responsible for yield loss. These fungi can grow at variable temperature and humidity conditions, which makes highly frequent. Thus, there is a need to find safe control measure in order to extend the shelf-life of harvested produce during transportation, without affecting their quality. The Essential/ Volatile oils are volatile in nature and thus give best sterilizing effect when used as a spray. Various mono- and sesqui-terpenoid components present in the VOs (Table 1) have a synergistic effect and affects the fungal physiology. Thus, antifungal potency of *Cymbopogon citrates*, *Eucalyptus globules*, *Gaultheria procumbens* and *Syzygium aromaticum* VOs were tested during the present investigation against selected fungal strains. The outcome of the study can be further used to develop bio-safe botanical fungicide product.

D. Disadvantages with common Synthetic Fungicides:

Chemical fungicides are the primary means of control. Regulation of fungal pathogens with chemicals, under field condition is carcinogenic, non-degradable and also causes serious environmental pollution. They often have non-target effects, get absorbed by the crops, and also impose acute residual toxicity by entering in to a food-chain (Prasad *et al*, 2010). Thus, synthetic fungicides are recently come under special scrutiny by World Health Organization (WHO) (Tripathi *et al*, 2008). Today the indiscriminate usage of the chemical fungicides and development of resistance by fungal pathogen populations has lead to find us the alternative strategies that are eco-friendly and bio-safe (Wang *et al*, 2010).

E. Research Status and Advantages of Botanical Fungicides in Agriculture:

The plant world is a rich storehouse of natural chemicals with the total number of plant chemicals account more than 40,00,000 in number including 10,000 biologically active Plant Secondary Metabolites (PSMs), responsible to play a defensive role in the plants. Variety of higher plants contains rich diversity of bioactive PSMs like Phenols, Flavanoids, Quinones, Tannins, Alkaloids, Saponins, Sterols, Terpenoids, etc responsible to play a defensive role in the plants. Such plant chemicals contribute to diverse biological activities such as antimicrobial, allelopathic, antioxidant and bio-regulatory properties and these natural products thus can certainly substitute harmful synthetic fungicides for plant disease control (Patel and Jasrai, 2009, 2012; Huang *et al*, 2010). Hence plants used in traditional medicines ought to be scientifically investigated as a potential source of novel antimicrobial compounds. These bio-active plant derived compounds can be formulated in to botanical fungicides to combat undesirable

microorganisms and plant disease management (Mohana and Raveesha, 2007). Thus, plants used in traditional medicines should also be scientifically investigated as a potential source of novel antimicrobial compounds (Karbin *et al*, 2009). Fungi predominantly reproduce by the production of asexual spores, which is a major source of fungal infestation and rapid proliferation (Murthy *et al*, 2009).

The fungal inhibition can be due to the limitation of the fungal growth by interfering with the fungal protein production, DNA replication, interference with cellular metabolism, damage to the membrane, following death of the fungal cells. Antifungal activity of secondary metabolites depends on the method and solvent used for extraction, its concentration and composition (Tripathi *et al*, 2008). Kishore *et al,* 2007 demonstrated that Paper disc diffusion assay provides qualitative information on the efficacy of test compounds. This can be used routinely to evaluate antifungal activity of extracts.

Antifungal property of many plants has been studied earlier by many researchers in order to control plant diseases in a bio-safe way. Mostly, fungi gain entrance through natural openings and injuries created during harvesting process, transporting, handling and marketing. This type of storage fungi were isolated from the rotted Sweet Potato *Ipomoea batatas* tubers namely *Aspergillus flavus*, *Aspergillus niger*, *Fusarium solani*, *Fusarium oxysporum* and *Rhizopus stolonifer* by Amienyo and Ataga (2007). They have found an excellent antifungal property of *Zingiber officinale* water extract against all the fungi. Klich (1984), has observed that *Aspergillus flavus* introduced into cotton plants through natural openings before anthesis and thus infect the seed. Many testing methods of Antimicrobial activity of extracts are quite lengthy and difficult to perform routinely. Thus Shafi (1975) has suggested an easy and instant *in vitro* method for MIC (Minimum inhibitory concentration) value of test sample determination called Paper Disc Diffusion Method. It is a simple method of incorporating the antimicrobial drug in agar. Likewise, according to Kishore *et al* (2007), Paper Disc Agar Diffusion Assay method provides qualitative information on the efficacy of test compounds and can be routinely used to evaluate the antifungal activity of extracts.

F. Herbal Fungitoxicants:

Plants are reservoir of biologically active compounds that can affect the metabolic activity of harmful microbes and helps us prevent their unwanted growth. These compounds are also referred as a Plant Secondary Metabolites (PSMs) present in plants can combat with pathogens by different mode of action (Patel and Jasrai, 2013). Plant extracts and oils are non-pollutive, cost effective non-hazardous and do not disturb ecological balance and thus safely be used for controlling plant diseases. Suwitchayanon and Kunasakdakul (2009) have examined that, in soaking method, *Syzygium aromaticum* extract exhibited the MIC 1900 ppm while *Curcuma longa* extract showed the MIC at 7500 ppm against *Alternaria brassicicola*. Methanolic extracts of plants used in traditional Indian medicine *Grewia arborea, Melia azedarach, Peltophorum pterophorus* and *Terminalia chebula* showed significant reduction in growth of *Aspergillus niger* compared to Bavistin (at 5µg/well) in agar well diffusion method on PDA after 36 hr of incubation (Bobbarala *et al*, 2009). The antifungal activity of volatile oils is attributed to its vapour action and presence of phenolic compounds. In another study by Hossain *et al*, 2008, the hydrodistilled essential oils, methanol extract and its fractions of *Orthosiphon stamineus* leaves and stems at 5µl (1000 ppm) concentration, displayed potential antifungal activity in the disc diffusion method against phytopathogenic fungi *Fusarium solani* with MIC 500 µg/ml and *Rhizoctonia solani* with MIC 1000 µg/ml.

G. Active Phyto-chemicals and Therapeutic Importance of Essential oils of Selected Plants:

The essential oils of plants selected for the present study; also comprise many medicinal properties which approves their usage on the fresh produce and safer for human consumption (Table 1).

Table 1. Active Phyto-chemicals and Medicinal Importance of Selected Essential oils

Cymbopogon citrates (DC ex Nees) Stapf
Common name: Lemongrass
Habit: Grass
Useful part: Leaves
Family: Poaceae
Phyto-chemical constituents: α- and β- Citral, linalool, geraniol, citronellol, citronellal,

citronellic acid, cymbopogone, cymbopogonol, neral, nerol, citronelol alcohols, linalool, 1,8-cineole, α-and β-pinene, limonene, β-phellandrene, elemol, β-caryophyllene, β-thujene, myrcene, β-ocimene, terpenolene, α-terpineol menthol, neomenthol, isopulegol, α-camphorene, chlorogenic, caffeic and p-coumaric acids, nerolic and geranic acids, β-sistosterol and steroidal saponin.
Medicinal uses: It cures coryza, influenza, pyrexia, flu, pneumonia, muscle pain, colitis, indigestion, nervous disorders, stomachache, relieves cramping pains, flatulence, diarrhea, vomiting, headaches, leprosy, malaria, ophthalmia and vascular disorders.
Eucalyptus globules Labill
Common name: Eucalyptus
Habit: Tree
Useful part: Leaves
Family: Myrtaceae
Phyto-chemical constituents: 1,8- eucalyptol, α- and β- pinene, α-terpineol, globulol, epiglobulol, alloaromadendrene, limonene, linalool, cymene, phellendrene, terpinene, α-eudesmol, L-pinocarveol, β-sabinene, terpinolene, aromadendrene, citronellal, camphene and fenchene.
Medicinal uses: It relieves cough and cold, blocked nasal passages, sore throats and lung infections, asthma, pulmonary tuberculosis, ulcers, angina, wounds, skin infections, herpes, acne, gingivitis, diptheria, dysentery, dyspepsia, grippe, inflammation, laryngitis, leprosy, malaria, miasma, phthisis, rhinitis, vaginitis, muscular aches and pains, rheumatoid arthritis and sprains.
Gaultheria procumbens L
Common name: Wintergreen
Habit: Tree
Useful part: Twigs
Family: Ericaceae
Phyto-chemical constituents: Arbutin, menthyl salicylate, gautherin, gaultheriline, gaultheric acid, α-pinene, myrcene, γ-3-carene, limonene, 3,7-guaiadiene, γ-cadinene, ericolin, urson and gallic acid.
Medicinal uses: It relieves flatulence, colic, sciatica, myalgia, sprains, muscle and joint discomfort, sore muscles, gout, arthritic and rheumatic pain, backache, headache, neuralgia, catarrh, obesity, edema, poor circulation, heart disease, hypertension, tendentious, cramps, cellulite, eczema, psoriasis, ulcers, broken or bruised bones.
Syzygium aromaticum (L) Merr and Perr
Common name: Clove tree
Habit: Tree
Useful part: Flower buds
Family: Myrtaceae
Phyto-chemical constituents: Eugenol, eugenol acetate and β-caryophyllene, 2-heptanone, acetyleugenol, α-humulene, methyl salicylate, isoeugenol, methyleugenol, stigmasterol, campesterol, phenyl propanoides, dehydrodieugenol, biflorin, quercetin, kaempferol, rhamnocitrin, rhamnetin, eugenitin, myricetin, gallic acid, ellagic acid and oleanolic acid.
Medicinal uses: Used for cureing nausea, dyspepsia, flatulence, ulcers, bruises, colic, chills, impotence, inflamed oral, plaque and gum disease of teeth, toothache, headache, earache, cold, bronchitis, asthma, arthritis, rheumatism and burns.

H. Mode of Action:

Essential oils as Plant Secondary Metabolites are made up of different volatile compounds and grouped as Phenylpropanes, mixture of different Terpenoid compounds and their oxygenated derivatives. Production of essential oils in plants is supposed to be predominantly a defence mechanism and has sole role in plants in terms of providing an antimicrobial activity, allelopathy, attractants, protectants as feeding deterrents, for stress survival and as a phytoalexin. The composition of the oil relatively often differs amid genera and even for species. Researches designate that these oils likewise own an outstanding broad-spectrum antifungal activity against both human and plant pathogens. Fungal growth inhibition by essential oils frequently includes prevention of hyphal growth and sporulation, interrupt nutrient uptake and metabolism, encourage lysis and cytoplasmic evacuation and modify usual physiology of fungi by inducing changes in cell wall composition, plasma membrane disruption, mitochondrial structure disorganization besides interference with respiratory enzymatic reactions of the mitochondrial membrane.

II. MATERIALS AND METHODS

A. Selection of Plant Material:

In the present investigation, essential oils of four plants *Cymbopogon citrates* (DC ex Nees) Stapf, *Eucalyptus globules* Labill*, Gaultheria procumbens* L and *Syzygium aromaticum* (L) Merr & Perr were also used for the study. The ready-made essential oils were purchased from the local market of Bangalore, India (Table 1, 2, Fig 1).

Table 2. Details of Aromatic Plants used in the present study

Plants	Plant Part Used	Family	% Yield*
Cymbopogon citrates	Leaves	Poaceae	0.68
Eucalyptus globules	Leaves	Myrtaceae	1
Gaultheria procumbens	Twigs	Ericaceae	0.5
Syzygium aromaticum	Flower bud	Myrtaceae	15

[*Source: Strong, 1936; Joy *et al,* 2006; Negrelle and Gomes, 2007; Lee *et al,* 2009; Pandey *et al,* 2010]

Cymbopogon citrates

Eucalyptus globules

Gaultheria procumbens

Syzygium aromaticum

Fig 1. Selected Plants for Screening Antifungal Activity

B. Fungal Pathogen Isolation from Infected Plant Material:

For the study, the pathogenic fungi can be isolated in laboratory from the infected material of the host plant, using nutrient media and recognized based on their microscopic characters including spores (Nduagu *et al*, 2008). In the present study, some fungal strains were isolated from the diseased fruits and vegetables on SDA and PDA medium and were additional identified by staining with Cotton Blue stain (Table 3) and studied under microscope. The isolates were used for fungal growth inhibition assays using plant-based essential oils. The infected material was first washed and surface sterilized with 0.1% $HgCl_2$ treatment for 1 min. The treated material was washed (five times) with sterilized water. The infected material (5 × 5 mm) was isolated with sterilized blade and inoculated on PDA media (Table 4) slants under an aseptic conditions (Dube, 1990). The slants were then incubated (27 ±1°C) for 4-6 days. For identification, the fungal growth was observed under microscope. For this, small portion of the hyphal growth was taken under aseptic conditions, mounted on a slide and stained with Cotton blue reagent. The prepared slides were detected

under a microscope for microscopic features of mycelium and sporulation. The recorded data was compared with the characters constructed on the fungal identification keys (Vashistha, 2000).

Table 3. Composition of Cotton Blue stain

Ingredients	Quantity (ml)
Lactic acid	20
Phenol	20
Glycerol	40
Water	20
Aniline Blue (1%)	2

Table 4. Composition of PDA (Potato Dextrose Agar) medium

Ingredients	Quantity (g/l)
Potato	200
Dextrose	20
Agar	20
pH	5.5

Table 5. Composition of SDA (Sabouraud Dextrose Agar) medium

Ingredients	Quantity (g/l)
Dextrose	20
Peptone	10
Agar	20
pH	6.5

Following the process, Fungi *Aspergillus niger* (GUB03) was isolated from the infected plant material Lemon fruits (collected from local markets of Gujarat region) on PDA (Potato Dextrose Agar) media (Table 4) subsequent standardized protocols (Dube, 1990) for the study. Fungal culture was grown and kept on Sabouraud Dextrose Agar (SDA) (Table 5) medium at 28 ± 2°C.

C. Fungal Spore Quantification:

The fungal broth culture was established on SDA media and afterward precise incubation period, spore counting was achieved through Haemocytometer (Table 6).

Table 6. Test-fungi, Incubation Period and Haemocytometer Spore-count

Fungi	Stock code	Incubation period (Days) in broth medium*	Fungal Spore count ($\times 10^6$)
Aspergillus niger	GUB03	5	16.69

D. Determination of In vitro Antifungal Activity Screening Assay of Essential oils:

The present investigation is to screen antifungal potency of *Cymbopogon citrates*, *Eucalyptus globules, Gaultheria procumbens* and *Syzygium aromaticum* essential oils against selected phyto-pathogenic fungal strain *Aspergillus niger* at a designated concentration by Paper disc diffusion assay (GUB03) (Erturk, 2006). For the assay, a fungal broth culture was first established on SDA (Sabouraud Dextrose Agar) broth medium. The spore count of the culture after specific incubation period was performed using Haemocytometer (Table 6). For bioassay, the fungal culture (0.1 ml aliquot) with known spore count was homogeneously seeded with sterilized cotton swab on SDA media (15 ml, ≈ 4 cm thickness) in each petri dish (90 × 90 mm). Then oil loaded Whatman paper discs (6 mm diameter) were placed on the fungal seeded plates with sterile forceps under aseptic conditions. The plates were incubated in upside down position for 72 hr at 28 ± 2°C (Parekh and Chanda, 2007). The experiment was accomplished in triplicates with appropriate untreated controls. The ZI (Zone of Inhibition) was measured by the antibiotic zone reader (Labfine, India). The Primary screening was performed using 10 mg/disc concentration and Secondary screening performed at 0.5, 1, 2.5, 5, 8 and 10 mg/disc concentration of oil to find the MIC value (Minimum Inhibitory Concentration) for each fungi (Huang *et al*, 2010). Detailed method is mentioned as below.

a. *Primary Antifungal Activity Screening:*

In the present study, all four selected plant essential oils were assayed separately against the selected test organisms using Disc diffusion assay. For this, sterilized Whatman filter paper (no 1) discs (6.5 mm) were loaded with 10 mg (10,000 ppm) of oil, under aseptic conditions for the primary screening

experiment to detect their fungal pathogen inhibiting potency. The essential oils were segregated based on the positive and negative antifungal response exhibited in the assay (Prasad *et al*, 2010) and the data was compiled as + (Positive) and – (Negative). For the assay, homogenized fungal broth culture (0.1 ml) (Table 6) was poured on solidified SDA medium (20 ml) in each petriplate using micropipette. The culture was uniformly streaked with sterilized cotton swab under aseptic conditions. During primary screening, the test-oil impregnated paper discs (10 mg/disc) were placed on the medium. The plates were incubated in upside down position for 72 hr at 28 ±1°C (Erturk, 2006; Patel and Jasrai, 2010). The experiment was performed in triplicates. The antifungal effect was observed as formation of clear zone around the essential oil loaded paper disc after incubation, referred as ZI (Zone of inhibition). This indicates a presence of potential antifungal property in tested oil, and marked as (+) signs. The oil sample without any ZI was marked as (–) sign (Table 7).

b. *Secondary Antifungal Activity Screening to find MIC value:*

In the present study, oils indicating potential antifungal activity (+), were further pulled for the secondary screening and dose optimization as per the standard protocols. The selected oil was screened from lower to higher concentrations, using the same Disc diffusion assay to find the minimum effective concentration or the minimal inhibitory concentration (MIC) which is effective to inhibit the fungal growth on seeded SDA medium plate, after the incubation period (Huang *et al*, 2010; Ogbebor and Adekunle, 2008). In other terms for the Secondary Screening; oils were screened at various concentrations against the selected fungal strain. The process of Dose Optimization is thus essential to evade needless wastage of extract/oil in the product. The paper discs loaded with different concentration of oil (0.5, 1, 2.5, 5, 8 and 10 mg/disc) were placed on fungal seeded plates under aseptic conditions. These paper discs loaded with mentioned amount of oil concentration range were placed at an equal distance from each other in an anticlockwise manner. Each plate was replicated thrice. The plates were then incubated upside down for 72 hr and the ZI was recorded by Antibiotic Zone Reader (Labfine, India) (Dawar *et al*, 2008). In case, if the fungal growth is not in a regular circle, then the mean diameter (average of the longest and shortest diameter of the same colony) was calculated.

III. RESULTS AND DISCUSSION

In several parts of world, several researchers have reported the possible antifungal activity from wide-ranging range of plants and diverse solvent extracts; by means of various *in vitro* screening methods/techniques and different artificial nutrient medias. The studies thus supports that botanicals by their varying and unique mode of action can be instigated as a potential alternative against a harmful synthetic chemical fungicide (Patel and Jasrai, 2009; 2010). A relative analysis of plant essential oils and various solvent extracts and their use as an antimicrobial agent is been evaluated by numerous researchers using various techniques. As Kishore *et al,* 2007 demonstrated that Paper Disc Diffusion Assay delivers qualitative evidence on the efficacy of test compounds. This can be used regularly to appraise antifungal activity of extracts, oils etc and also implemented in the present investigation (Table 7, 8).

A. Primary Antifungal Screening Results with Disc Diffusion Assay:

Based on the results (data compiled as + (Positive) and – (Negative)) obtained with Primary Screening (Table 2), the plant extracts were subjected to Secondary screening for Dose Optimization in the selected range (0.5 up to 10 mg/disc) and to find fungitoxic spectrum or the MIC value of the essential oil against test fungi, mentioned as ZI (Zone Of Inhibition) and recorded in mm (millimetre) unit. The Primary Screening (10 mg/disc) for antifungal activity using Paper Disc Diffusion Assay on SDA media (Table 5) revealed excellent results where, all four selected plant essential oils had successfully inhibited the *Aspergillus niger* fungi.

Table 7. Result of Primary screening for Antifungal potential of oils (10 mg/disc) against *Aspergillus niger*

Plant Essential oils	Test fungi
Cymbopogon citrates	+
Eucalyptus globules	+
Gaultheria procumbens	+
Syzygium aromaticum	+

[**Note:** (+) = indicates presence of antifungal activity, (–) = indicates absence of antifungal activity]

Pathogen inhibition at lower MIC value/at lesser extract/oil concentration signifies a very effective inhibitory potential. Therefore, the antifungal oil with well-defined MIC value, can be further utilized for value addition and fungicide development. As Primary Screening experiment revealed that, all four essential oils of aromatic plants own actual antifungal activity at 10 mg/disc tested concentration (Table 7).

Secondary Antifungal Screening Results with Disc Diffusion Assay:

Based on the results found with Primary Screening, the plant Essential oils were subjected to Secondary Screening for Dose Optimization. The fungitoxic spectrum or the MIC value (Minimum Inhibitory Concentration) of the oils against specific test fungi was determined in terms of ZI (Zone of Inhibition). The oils were tested in the selected range of concentration (0.5 to 10 mg/disc) using Paper Disc Diffusion Assay (Table 8, Fig 2) in the Secondary Screening. The assay was carried out on SDA medium with three replicates for each concentration of tested oils. ZI (clear zone showing absence of fungal growth) was recorded as the diameter (mm) of complete growth inhibition. SD (Standard Deviation) for the obtained readings was calculated.

Table 8. Overview of MIC (Minimum Inhibitory Concentration) value obtained with Plant Essential Oils against *Aspergillus niger*

Plants	MIC					
	0.5	1	2.5	5	8	10
Cymbopogon citrates		✓			-	-
Eucalyptus globules		-		✓		
Gaultheria procumbens	-	-	-	✓	-	-
Syzygium aromaticum	✓			-	-	-

Microbial inhibition at a lower MIC value indicates very effective potential. Therefore, deterrence of fungal growth at 0.5, 1 and 2.5 mg/disc conc. is extremely important for preparing fungicide formulation with excellent results. In the existing studies, *Eucalyptus globules* and *Gaultheria procumbens* oil was found to inhibit the selected fungi at MIC value of 5 mg/disc and *Cymbopogon citrates* at 1 mg/disc MIC. At a lowest MIC (0.5 mg/disc), *Syzygium aromaticum* oil inhibited fungi *Aspergillus niger* (Table 8, 9, Fig 2).

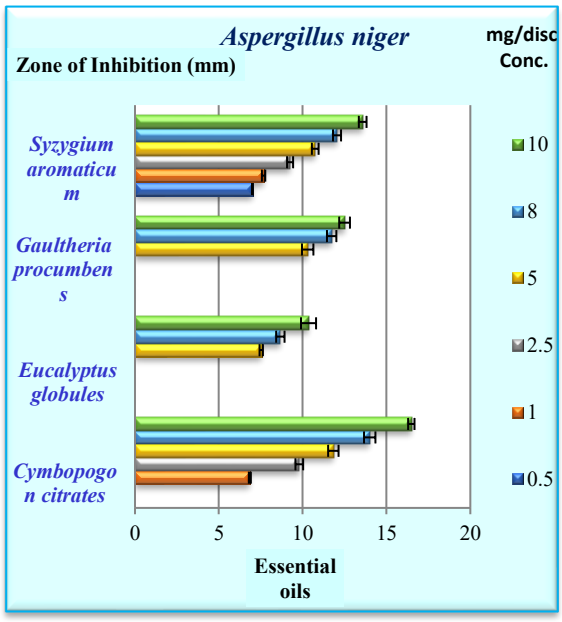


Fig 2. Bar Diagram showing Comparative account of fungal growth inhibition during Secondary screening through essential oil

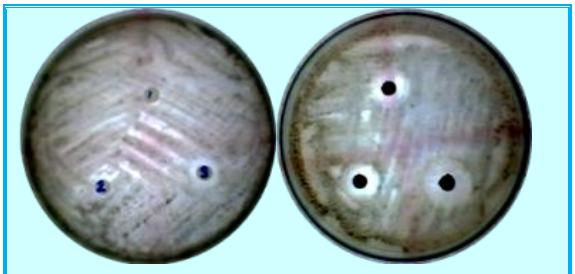
In the current study, all four essential oils exposed brilliant and efficient antifungal activity against the selected pathogens.

Table 9. Secondary Screening and Dose Optimisation study using Paper Disc Diffusion Assay indicating ZI (mm) and Standard Error value

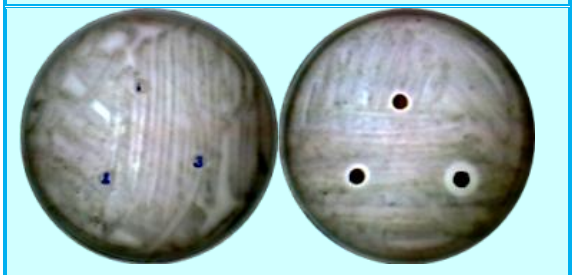
Essential oil	MIC value of oil (mg/disc) and respective ZI (mm)					
	0.5	1	2.5	5	8	10
Cymbopogon citrates	-	6.85 ± 0.06	9.8 ± 0.23	11.83 ± 0.31	14 ± 0.34	16.48 ±0.19
Eucalyptus globules	-	-	-	7.53 ± 0.10	8.67 ± 0.25	10.35 ±0.45

Gaultheria procumbens	-	-	-	10.3 ± 0.34	11.73 ± 0.28	12.5 ± 0.32
Syzygium aromaticum	7 ± 0	7.67 ± 0.10	9.25 ± 0.18	10.75 ± 0.20	12.05 ± 0.24	13.58 ± 0.23

The antifungal activity at a lower essential oil concentration thus indicates lower MIC value inhibition. Consequently, lower the MIC value, better the effectiveness. The lowest MIC value (0.5 mg/disc) was recorded with *Cymbopogon citrates, Gaultheria procumbens* and *Syzygium aromaticum* oils. The inhibition of fungi can be credited to the complex mixture of secondary metabolites, volatile compounds and as Phenylpropanes, Terpenoids and their Oxygenated derivatives. *Cymbopogon citrates* oil in the present study showed highest ZI against *Fusarium oxysporum f.sp. laginariae* followed by *Fusarium oxysporum* (Fig 2, 3).

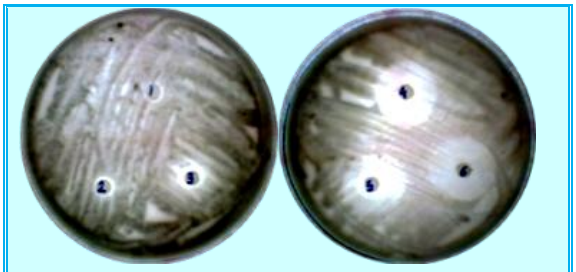


Cymbopogon citrates oil



Eucalyptus globules oil

Gaultheria procumbens oil



Syzygium aromaticum oil

[**Note:** Discs in increasing essential oil concentration from left to right in all figures]

Fig 3. *Aspergillus niger* Inhibition and Dose Optimisation study at 0.5, 1, 2.5, 5, 8 and 10 mg/disc concentration using Plant Essential oils

Primary Screening study had shown positive results indicating that the selected fungal strain *Aspergillus niger* is effectively inhibited by all the four essential oils. Further to detect the MIC (Minimum Inhibitory Concentration) required to inhibit the selected fungal strain; *In Vitro* Secondary Screening and Dose Optimisation experiments were carried out using Paper Disc Diffusion Assay at 0.5, 1, 2.5, 5, 8, 10 mg/disc concentration indicating appropriate dose for effective inhibition of fungal growth (Table 9).

Following the standardised procedure of the assay; the conducted experiment showed an excellent result and the fungi got effectively inhibited at lower MIC value. Pathogen inhibition at a lower MIC value is considered good and represents low antifungal components requirement for the control measures. The detailed results are presented here where *Syzygium aromaticum* oil had exhibited lowest MIC value amongst all oils and showed 7±0 mm ZI at 0.5 mm mg/disc concentration. Next recorded data followed by the *Cymbopogon citrates* oil with inhibition of *Aspergillus niger* at 1 mg/disc concentration MIC with 6.85 ± 0.06 mm ZI. Remaining two oils namely *Eucalyptus globules* and *Gaultheria procumbens* oils exhibited inhibition of *A. niger* at 5 mg/disc concentration with 7.53 ± 0.10 and 10.3 ± 0.34 mm ZI respectively (Fig 3). Highest ZI found for the *Cymbopogon citrates* oil 16.48 ± 0.19 mm followed by *Syzygium aromaticum* oil 13.58 ± 0.23 mm at 10 mg/disc conc. Overall, the Secondary screening results suggests that the selected all four plants essential oils had effectively prevented the rot causing fungal pathogen *A. niger* and can be implemented further for the development of herbal,

Eco-friendly, target specific and no harmful effects on human as well as animal health.

Fungal growth inhibition by essential oils often includes prevention of hyphal growth and sporulation, interruption in nutrient uptake and metabolism, induction of lysis and alternation in fungal physiology by inducing changes in cell-wall composition, plasma membrane disruption, mitochondrial structure disorganization and interference with respiratory enzymatic reactions of the mitochondrial membrane (Kishore *et al*, 2007).According to Suwitchayanon and Kunasakdakul (2009), *Syzygium aromaticum* oil treatment to fungi causes abnormal growths of mycelia, swollen hyphae, septate, pale color, conidial malformation and reducing of conidial number. Nunez *et al* (2001) in their study displayed that, clove oleoresin (0.2- 0.8% concentration) inhibited *Aspergillus niger*. Similarly, *Syzygium aromaticum* oil at 1000 ppm inhibited *Rhizoctonia solani* with 7.5 mm ZI in disc diffusion method on PDA medium after 24 hr of incubation (Sehajpal *et al*, 2009). According to Sridhar *et al* (2003) in their study demonstrated that, *Cymbopogon* essential oil (90 ppm conc.) inhibited *Aspergillus flavus*, *Aspergillus niger*, *Alternaria alternata*, *Fusarium oxysporum* and *Rhizoctonia solani* with 100% radial growth inhibition in Poison Food Technique on Czapek-Dox Agar. In another study by Suleiman *et al*, 2008; in Disc Diffusion Assay on PDA medium, *Cymbopogon citrates* fresh aqueous leaves extract (3.0% concentration) inhibited *Fusarium* sp., (2.14 ±2.35 ZI) after 5 days.

In the present study also, all selected plant oils exhibited significant antifungal activity against *Aspergillus niger*, on SDA media using paper disc method. Insufficient work has been conducted on Botanical controls measures for these highly destructive fungal strains. As these fungi damage the significant crops and affect the yield, the present findings will contribute to control the fungi in an eco-friendly way. In this situation, present work was carried out with aromatic plant oils; the Dose Optimization through Secondary Screening helps to find the effective dose of oil to prevent fungal growth. Accordingly, current study is highly significant in order to demonstrate the use of newer plant sources to control the fast-growing mold.

IV. CONCLUSION

As already proved, plants are the natural reservoir of biologically active compounds that through their unique mode of action, can affect the metabolic activity of destructive microbial pathogens and this way help to combat the pathogen. Moreover, these compounds are biodegradable in nature and thus do not impart any harmful effects to the environment unlike Synthetic Chemical Fungicides. Thus, Plant Secondary Metabolites produced by plants constitute an important source of commercial Microbiocides, Pesticides and many Pharmaceutical drugs. These compounds are worthy of future investigation to prove their efficacy as potential compounds against phyto-pathogens. The research area is huge and vast and there is still further necessity for more and defined research on plant fungal pathogens using botanicals, as this concept is poorly exploited. As the results of the present study are much promising to achieve the above goal to develop bio-safe Botanical Fungicide, the discovery of the present investigation has contributed a significant step towards crop protection strategies. Thus, the results can be further used to prepare a bio-safe herbal formulation for the effective control of fungi in field condition.

The Primary Screening for antifungal activity using Disc Diffusion Assay on SDA media revealed excellent results and thus signifies the existence of antifungal potential amongst the selected plants *Gaultheria procumbens*, *Syzygium aromaticum, Cymbopogon citrates* and *Eucalyptus globules* essential oils. The screening clearly demonstrated the effectiveness of four essential oils as potential antifungal agents. The obtained data signifies the potential use of selected plants as herbal, broad-spectrum and safe bio-protectant for the control of fungal diseases and to increase the shelf-life of harvested produce. This has also clearly signified role of screened plants as potent natural antifungal agent and can be further utilized to develop effective herbal formulation. They can be also used as an eco-friendly fumigant to avert the post-harvest rot of the fruits and vegetables during the storage and transportation and to increase their shelf-life. The results of the current study may similarly form the source for supplementary examination to isolate the active principles, prepare Phyto-chemical profile and elucidate the structures and evaluate them against wider range of drug-

resistance fungal strains. Plant essential oils are often medicinally important and well-studied for antimicrobial activity but poorly explored to screen antifungal potency against various plant disease causing fungal strains.

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