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Original Research

Postharvest control of anthracnose lesions and its causative agent, *Colletotrichum musae* by some oils

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Abstract: Anthracnose of banana is incited by *Colletotrichum musae*. It is recognized as one the most destructive diseases of mature and immature banana fruits, resulting in huge economic losses all over the world. Present research deals with screening some oils both *in vitro* and *in vivo* for their antifungal activity against *C.musae.* Clove oil (0.1µl/ml) completely arrested the conidial germination and mycelial growth of *C. musae.* Fenugreek and almond oil exhibited significant inhibition of mycelial growth, 61% and 57% at a concentration of 2µl/ml. However, olive oil was least inhibitory on the test fungi. Clove oil also a showed marked reduction in anthracnose lesions on banana fruits, thereby suggesting disease control. Scanning electron microscopy revealed severely damaged mycelium and conidia. FTIR studies show the presence of important bands representing phenols, terpenes, aldehydes, and ketones. Based on our findings; clove, fenugreek and almond oil demonstrated fungicidal and fungistatic activities against anthracnose pathogen. Hence, these oils can be considered as potential alternatives to chemical treatments.

Key words: Anthracnose; essential oils; clove oil; *Colletotrichum musae.*

Introduction

Banana (Musa spp), a popular tropical fruit is consumed all over the world as a fresh ripe fruit and is also cooked in both raw and ripe form. Apart from being a rich source of potassium and dietary fiber, it also contains vitamin A, C and $B_{6.}$ (1). One of the major global concerns today is to reduce the postharvest losses of fruits and vegetables. Banana is one of the world's most valuable food crops cultivated for its economic importance (2). This fruit is harvested when unripe. Being a climacteric fruit, its ripening is associated with ethylene production and increase in respiration rates. Consequently, it has a short shelf life and is easily perishable (3,4). Huge economic losses of banana fruits occur worldwide due to postharvest decay by fungal pathogens, rough postharvest handling, poor packaging, storage conditions and during transportation (5, 6, 7).

The prime target of postharvest management worldwide is to ensure fresh, safe and quality supply of fruits to the consumers. Banana fruits are attacked by several pathogens during harvest and postharvest conditions. *Colletotrichum musae* is one of the most important pathogens which causes anthracnose and crown rot of banana (8,9). Anthracnose is characterized as a latent infection as the fruits are invaded by the pathogen at the time of development, however, the symptoms appear only during fruit ripening, Symptoms include black-brown sunken lesions which are diamond in shape and may bear acervuli (10,11).

Anthracnose is an aggressive postharvest disease that accounts for huge losses of fruits, if left untreated (12). Therefore, to minimise the microbial spoilage and consequently extend their shelf life various postharvest treatments like edible coatings, controlled ripening and chemical treatments are practiced (10, 13).

One of the common practices adopted to control postharvest decay of fruits is application of synthetic fungicides i.e., Thiabendazole, Benomyl, Imazil and Prochloraz. Treatment of fruits with synthetic fungicides may delay their shelf life whilst preventing anthracnose lesions, but the threat it poses to human and animal health and it's negative impact on the environment cannot be ignored (14,15). Additionally, frequent use of these chemicals has led to development of resistant strains of many pathogenic fungi including *Colletotrichum musae.* Recently, there has been a growing awareness about residual toxicity, carcinogenic impact and environmental pollution due to synthetic fungicides and pesticides. Furthermore, these serious health implications have raised strong public demand for quality fruits that carry few pesticide residues and are safer for consumption (16, 17). Hence, there is a need to replace synthetic fungicides with natural products.

Essential oils are plant secondary metabolites that play a vital role in defense mechanism against phytopathogens. They are a mixture of complex volatile compounds known for their antifungal, antibacterial and medicinal properties (18). Recently, some essential oils derived from various parts of different plants have shown both in-vitro and in-vivo fungal suppression (19, 20). Hence, essential and carrier oils are most apt because they are safe, ecofriendly, possess low toxicity to humans and mammals and are easily biodegradable (21). Present study aims to screen some essential and carrier oils for their role in inhibiting *Colletotrichum musae*- both in vitro and in vivo.

Materials and Methods

Essential oils

All the oils used in the present study are commercial; their purity was ascertained before use. Almond (*Prunus dulcis*), Clove (*Syzygium aromaticum)*, Fenugreek (*Trigonella foenum-graecum)* and olive oil (*Olea europaea*) were purchased from Now foods, U.SA. To ensure uniform distribution of oils they were dispersed in 1.0% Tween 20.Various concentrations in the range of 0.1-2µl/ml(v/v) were used in this study.

Banana fruits

Disease free banana hands (Cavendish cultivars) available in local markets of Riyadh were purchased. Fruits free from wounds, homogenous in color (green), size and shape were chosen and used the same day of purchase for *in vivo* studies and pathogenicity test.

Isolation of *Colletotrichum musae*

Isolation of pathogen was done from typical anthracnose lesions of banana fruits. Fruits were washed with water and small portions (5 × 5-mm^2) were cut from the lesions site. These pieces were then sterilized in 1% sodium hypochlorite solution for 5min and further washed with sterile distilled water thrice. Post treatment, these portions were transferred on to the surface of potato dextrose agar and incubated at 25°C for 7-9 days. Identification was carried out based on their cultural characteristics and micromorphology (22,23). Pure cultures were maintained for further studies at 4°C.

Preparation of conidial suspension

Conidial suspension was prepared from 14 days old colony by flooding the PDA culture plate with sterile distilled water (10ml) and the surface was scrapped with the help of glass rod to dislodge the mycelium and conidia. The suspension was then filtered through muslin cloth and concentration of spore density (1x10^6 spores/ml) adjusted with tween 20.

Pathogenicity test

Healthy green fruits were used to conduct pathogenicity test. Fruits were washed with running tap water, sterilized for 3min with 70% ethanol followed by three rinses with sterile distilled water. Fruits were allowed to air dry after which they were wounded to a depth of 2mm by puncturing them at three places with the help of sterilized needle. Wounds were inoculated by pipetting 6 µl of conidial suspension (10^6 conidia /ml) (24). Control treatment comprised of 6 µl of Tween 20 alone. Inoculated fruits were placed in a tray wrapped with plastic cover and the experimental setup was incubated at 28-30 °C and observed for 10-12 days.

In vitro studies of oils on the growth of *C.musae*.

Poison food technique was employed to determine the inhibitory activity of oils on radial mycelial growth of *Colletotrichum musae.* An Agar plug (5 mm), removed from the periphery of a 14 days old culture plate of *C. musae* was placed in the center of a PDA plate amended with various concentration of oils (0.1-2µl/ml). PDA plates without oil served as control. The experiment was done in triplicates and the plates were incubated at 28- 30°C.Radial growth measurements were taken when the control plates were fully covered by the mycelial growth and percentage mycelial inhibition calculated was as follows: % =DC-DT/DCX100, where DC is mycelial growth in control plate while DT is of treatment.

Conidial germination assay

Effect of oils on spore germination was evaluated by the cavity slide method (25) with slight modification. Conidial suspension10^{6}conidia /ml was prepared as mentioned above. A 50 µl of conidial suspension and essential oils, each of various concentrations were added to the cavity of the slides and mixed well. The slides were placed in a plastic box layered with gauges and saturated with sterile distilled water. This setup was incubated at 25±2ºC for 24 hours in dark. After 24 hours, a drop of lactophenol cotton blue was added to the slide and observed for spore germination under the light microscope. Tween 20 instead of oil served as control. Percent Inhibition of conidial germination was calculated by counting 100 conidia for each treatment and control. Each treatment comprised of three replicates.

In vivo studies (Inhibition of anthracnose lesions on Banana fruit)

Disease-free fruits were washed with water, surface sterilized with 1% sodium hypochlorite for 5 min, cleansed with sterilized distilled water and air dried at 28°C. After drying each fruit was wounded at three places along its length at equidistance. Wounds were made with a sterile borer (6 mm diameter and 2 mm depth). Each wound was loaded with spore suspension (10^6 spores/ml) and 20 µl of essential oils individually. After 1-hour, inoculated fruits were placed on plastic trays, covered with sterile plastic wraps to maintain 80-90% humidity and incubated at 25°C± 2°C (light and dark cycles). Diameter of anthracnose lesion was measured and recorded on7th day and percentage disease inhibition calculated (26). Control fruits were treated with the conidial suspension alone. All the treatments were done in triplicates.

Scanning electron microscopy

Scanning electron microscopy was performed on *Colletotrichum musae* treated with clove oil at concentration of 0.05 µl/ml and 0.1µl/ml, while cultures which were not treated served as control. The method of Alvinda and Natsuaki (27) was adopted with slight modification. Small portions (5x10mm) of the cultures were cut and fixed with 2.5% glutaraldehyde in phosphate buffer at 4°C, pH-7.4. After 48 hours the fungal samples were rinsed in the same buffer thrice, dehydrated in a series of ethanol concentration (60%, 70%, 80%,90%, 100%). Each treatment lasted for 20min; finally, the samples were dried in liquid carbon dioxide (Critical point dryer). Samples were then mounted on SEM stubs, electroplated with thin layer of gold and viewed with Scanning electron microscope (LTD JSM-6060LV (JEOL-Japan) scanned and photographed.

FTIR

Clove oil was subjected to Fourier transform infrared

Table 1. Inhibitory activity of essential oils at different concentrations on growth of *Colletotrichum musae* (in vitro).

Concentration of essential oils (µl/ml)	Percentage mycelial inhibition (%)			
	Clove	Almond	Olive	Fenugreek
0.01	76.29±0.28	NI	NI	24.14±1.15
0.025	84.07±0.64	NI	NI	30.55±0.55
0.05	91.11±1.11	21.10±0.96	NI	34.44±0.96
0.1	100.00±0.00	30.18±1.16	NI	38.14±0.64
0.25	100.00±0.00	43.51±1.15	NI	46.66±0.00
0.5	100.00±0.00	48.69±0.84	NI	49.81±0.32
1	100.00±0.00	55.92±1.59	20.55±1.47	50.00±0.00
2	100.00±0.00	57.77±0.00	25.55±1.92	61.48±0.64

All the values shown in the table are means of three replicates (±SD). NI- indicates not inhibited.

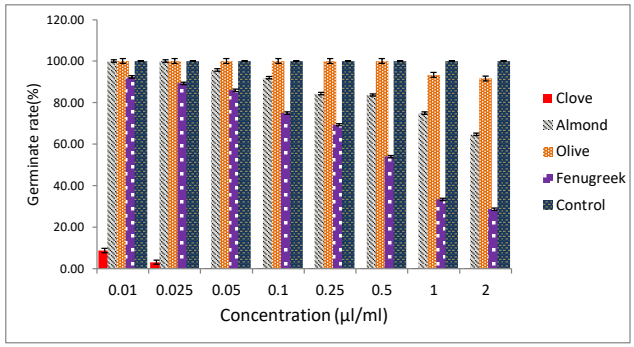

Figure 1. Effect of different concentrations of essential oils on conidial germination of *Colletotrichum musae.* All values presented in standard error bars are means of three independent experimental replicates. Significant difference in means (P≤0.05) were determined by one way ANOVA and Turkey HSD test.

analysis. The instrument used was Nicolet 6700 Spectrometer (Thermo Scientific, USA), it was equipped with a beam splitter and DTGS detector. The Spectrum obtained was analyzed by using a OMNIC software and was in the scan range of 400-4000cm-^{1}.

Statistical Analysis

All the data are means of three replicates. Results from *in vitro* studies on mycelia growth was subjected to Standard Deviation (SD±). Conidial germination, lesion diameter and anthracnose inhibition values were analyzed and significant differences ($P \leq 0.05$) were determined by One Way ANOVA followed by Tukey's HSD test (Vassarstats., U.S.A).

Results

Isolation and Identification

The pathogen isolated, formed white aerial mycelium on potato dextrose agar, later turning orange in color. After 12 days, several orange structures (acervuli) developed abundantly in the media. The pathogen was identified as *C.musae* as the conidial morphology and colony characteristics fitted the description of *C .musae* as mentioned by Mordue and Sutton (22,23).

Pathogenicity test

Artificially inoculated banana fruits developed typical anthracnose lesions after 7 days. Lesions were circular, black, and sunken. The fungus was re-isolated from typical lesions when grown on Potato dextrose agar. The colony characteristics, conidial morphology and lesion characteristics fitted the description of *Colletotrichum musae,* hence confirming the fungus as *C.musae* (28).

Inhibitory activity of oils on radial growth of *C. musae*

In vitro experiments with test oils exhibited varying inhibitory activity (Table.1). Amongst all the oils, clove oil was the most effective, as it caused complete growth inhibition of test pathogen at a very low concentration 0.1µl/ml. However, fenugreek and almond oil were effective at 2µl/ml inhibiting the radial growth of test pathogen by 61% and 57 %. Whereas, olive oil showed negligible inhibition on the growth of *C.musae* even at the highest test concentration.

Efficacy of oils in inhibiting conidial germination of C. *musae*

Except olive oil, all the other oils that were tested exerted antifungal effect on the conidial germination of *C. musae* (Figure 1). Clove oil showed highest inhibition while almond was least inhibitory. Germination of conidia was completely arrested by clove oil at a concentration of 0.05 µl/ml. It was observed that the lowest test concentration of clove oil (0.01µl/ml) was enough to cause more than 90% inhibition of conidial germination. Fenugreek and almond oil at a concentration of 2µl/ml caused significant conidial inhibition while olive oil was least inhibitory even at the highest test concentration (2µl/ml). Control slides showed 100% germination without any inhibition.

Reduction in anthracnose lesions by oils on Banana fruit

The most promising and potent oils (clove, fenugreek) from *in vitro* studies were chosen for the *in vivo* assay. Clove oil demonstrated strong antifungal activity, a lower concentration of 0.1µl/ml inhibited the lesion diameter up to 50% (13.16 mm) in comparison to control (27.50 mm), while a concentration 2µl/ml of clove oil showed marked reduction in diameter of lesions (4.83mm), resulting in 82.42% disease inhibition. Conversely, fenugreek oil at a concentration 2µl/ml showed 48% disease inhibition on fruits. (Figure 2 and 3).

Effect of Clove and fenugreek oil on hyphal and conidial morphology of *Colletotrichum musae*

Scanning electron micrographs of *C. musae* treated with clove oil showed severe damaging and destructive

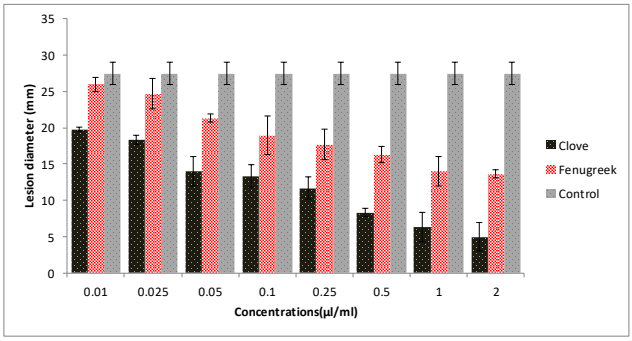

Figure 2. Percentage inhibition of lesion diameter on banana fruit treated with clove and fenugreek oil. All values presented in standard error bars are means of three independent experimental replicates. Significant difference in means (P≤0.05) were determined by one-way ANOVA and Turkey HSD test.

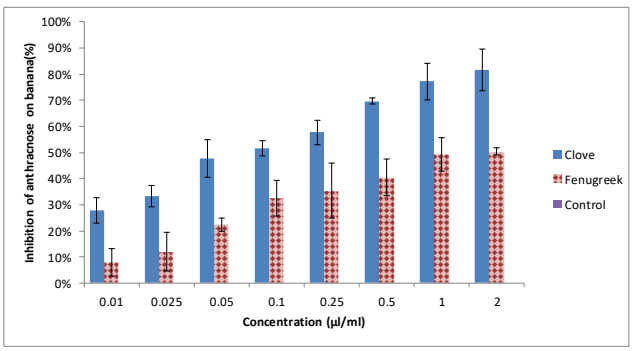

Figure 3. Effect of clove and fenugreek oil on anthracnose disease inhibition on banana fruit. All values presented in standard error bars are means of three independent experimental replicates. Significant difference in means (P≤0.05) were determined by one way ANOVA and Turkey HSD test.

effects on hyphal morphology in comparison to control (Figure 4; B-fenugreek oil treated cells; C-E--clove oil treated cells). Control samples which were not treated exhibited intact mass of mycelium with clear and distinct hyphal and conidial morphology. Hyphae had smooth cell wall and were without any indentation. Micrographs of *C. musae* treated with clove oil at a concentration of 0.05µl/ml and 0.1µl/ml are presented in Figure 4. After treatment the hyphae and conidia were rough and completely covered with small blebs or vesicles. Hyphae and conidia showed prominent alterations in their morphology. There was severe clumping, shriveling and complete distortion in hyphal and conidial morphology.

Identification of Important functional groups of clove oil by FTIR technique

The FTIR analysis of clove oil shows various peaks between 3509 cm^{-1} to 438cm^{-1} . A broad peak at 3509 cm^{-1} represents hydrogen bonded OH stretch due to phenol and alcohols. C-H stretch occurring at 2835cm^{-1} denotes alkanes. The two peaks at 1634cm^{-1} and 1605cm^{-1} are due to conjugation of two phenyl groups. The multiple peaks between 1430-1510 cm^{-1} are due to C=C aromatic stretch and the peak at 1368 cm^{-1} is due to C-H bending due to aromatic stretch. C-O stretching occurs between1032-1264cm^{-1}. Monosubstituted alkene gives 2 peaks near 911 and 993 cm^{-1}. Peaks at 793cm^{-1} and 815cm^{-1} are due to CH_2 bending (Figure. 5).

Discussion

In the present study, commercial oils of clove, fenugreek and almond exhibited significant inhibitory activity against *Colletotrichum musae.* Clove oil was the most potent among all the oils screened. It completely arrested the mycelia growth and suppressed conidial germination as observed in *in vitro* studies. Furthermore, it reduced the anthracnose symptoms on banana fruits and inhibited the disease severity significantly at 2 µl/ml. Our findings are consistent with the findings of Ranasinghe *et al*. (29)., in which cinnamon and clove oil were screened against *Fusarium proliferatum,C. musae and Lasiodiplodia theobromae*. Their findings show that the minimum inhibitory and lethal concentration ranged between 0.03-0.11%. Similarly, Idris *et al.* found basil, rosemary, and cinnamon oil to completely inhibit mycelial growth of *C. musae* at concentration of

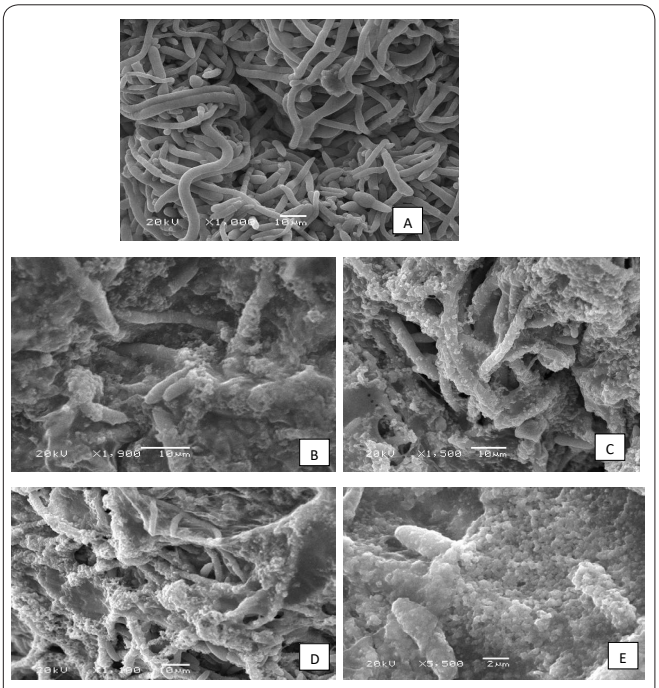

Figure 4. Micrographs of *Colletotrichum musae* treated with clove and fenugreek oil. **A:** Fungi without any treatment (control plate) micrograph shows intact mass of mycelium and conidia. Cell wall is smooth without any invaginations and clumping- **B –C:** *C. musae* treated with clove oil, mycelium shows aggregation and distorted shape, conidial morphology is altered; **D-E:** mycelium shows severe aggregation, small vesicles covering the entire mycelium and conidia structures. The mycelium and conidial structures are completely altered indicating cell lysis.

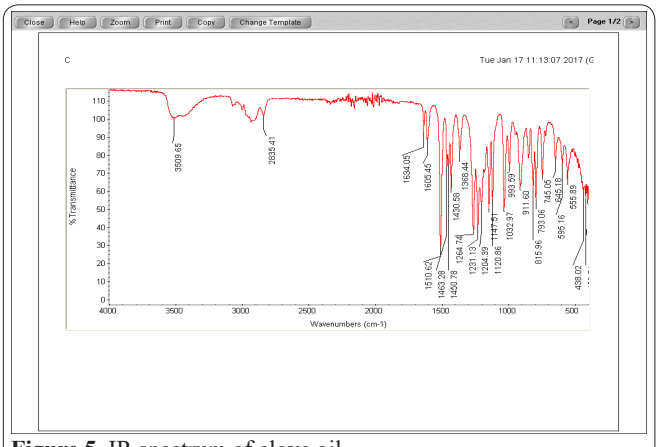

Figure 5. IR spectrum of clove oil.

0.15%, 0.25% and 0.05% (20). Various researchers have shown strong antifungal activity of essential oils and its components on the growth of several phytopathogens, which includes *Alternaria alternata, Aspergillus niger, Fusarium solani*, *Fusarium monoliforme*, *Rhizopus stolonifer, Curvularia lunata and Phoma sorghina* (25,30).

Besides clove oil, in our *in-vitro* study, almond and fenugreek oil exhibited 55% and 50% reduction in mycelia growth at a concentration of 1 µl/ml. Yet, in another study, sweet almond at 1% concentration was able to arrest the growth of *F.semitectum*(crown rot pathogen) by 63%(31). Huiling *et al*., screened 19 phytopathogenic fungal strains against bitter almond essential oil and reported EC_{50} values to vary between 50- 64 µg/mL (32). The highest activity of bitter almond oil was found against *Alternaria brassicae(5*0.2 µg/mL) while the lowest was reported against *Alternaria alternata, Fusarium graminearum* and *Valsa mali* (642.0 µg/mL, 627.5 µg/mL,610.8 µg/mL). Recent studies on methanolic extracts of Fenugreek (aerial parts) have shown inhibitory activity against *Fusarium oxysporum f. sp. radices -lycopersici* (FORL) and *Fusarium oxysporum f. sp. lycopersici* (FOL) at 0.1, 0.3 and 0.6 mg/mL (33). Olive oil did not show significant inhibitory activity against test pathogens in the present study. Geweely (34) screened five pathogenic fungi; *Aspergillus fumigates*, *Epidermophyton floccosum, Microsporum canis, Trichophyton rubrum* and *Candida albicans* against ozonized olive oil. The MIC for all the fungal species ranged between 0.53- 2.0 mg mL^{-1}. These results however contradict our findings.

Micrographs from scanning electron microscopy clearly demonstrate strong fungicidal activity of clove oil. Severe damaging effects on the morphology of mycelium and conidia can be seen in micrograph. Our results are similar to previous studies wherein micromorphology of fungi treated with essential oils were analyzed (35,36). In separate studies, Hua *et al*. and Chen *et al*. (35,36) observed dispersed and altered conidia, impeding conidial germination and absence of conidia. Furthermore, they found hyphae that were shriveled and flattened, appearance of vesicles, swelling and roughness in hyphal wall. The concept of mode of action of essential oil and its components on fungal cell structures is not fully understood. Nevertheless, one possible view is attributed to hydrophobicity of oils which partitions the lipids of cell membrane. This disturbs and damages the integrity of cell membrane and cell wall, and eventually leads to osmotic shock due to imbalance in osmotic pressure, leakage of important ions and cellular structures like ATP, ions, amino acids and nucleic acids (37,38)

The significant antifungal activity of clove oil could be attributed to the presence of major compounds which possess antimicrobial property. Clove oil owes its antifungal activity to Eugenol and Caryophyllene, as these are major compounds which have been identified through GCMS analysis in previous studies (29,39). Whereas, fenugreek and almond oil possess polyphenols, flavonoids, aldehydes, tannins, terpenoids and esters (40,32). FTIR technique was used in our study to determine the functional groups present in clove oil. Various bands on the spectrum correspond to important bioactive compounds of clove oil such as, Phenols, polyphenols, esters, aldehydes, ketones, sesquiterpenes, oils and fats (41,42). Various researchers have attributed the antimicrobial activity of clove oil to phenol(eugenol) and terpenes(Caryophyllene). Eugenol and essential oils being lipophillic in nature can enter the fatty acid chain that constitutes the cell membrane, thereby altering their fluidity and permeability (43,44). This exerts imbalance in cell organization and its function, which may lead to growth inhibition or cell lysis. Recently Tian et al. observed impairment of the ergosterol biosynthesis when *A. flavus* was treated with dill oil (45). Interestingly, yet another study reports morphological changes, cytoplasmic coagulation and vesiculation when *Botrytis cinerea* was treated with eugenol (46).

To accomplish the increasing global demand for fresh produce, various postharvest technologies have been developed. Postharvest treatments are carried out to delay the process of maturation and at the same time minimise the microbial growth. Several approaches like gaseous, chemical and physical treatment are practiced. However, many of these treatments impart quality changes which pose a potential threat to human health (47). Moreover, many countries have laid strict restrictions on the use of certain synthetic fungicides due to their toxicity. This need has prompted the screening of natural products as alternative treatment because they carry broad spectrum antimicrobial properties and yet are not toxic in nature (48).

Essential oils are gaining popularity in post-harvest technology as an effective alternative to synthetic fungicides. Various essential oils have been identified from different parts of plants. They occur naturally as secondary metabolites and exhibit antimicrobial activity (19). Recently, researchers have shown their effectiveness in different application methods such as dips, coatings and sprays (49,50). Consistent with previous reports, we observed that clove and fenugreek were effective in controlling anthracnose of banana (11,51). Ranasinghe, *et al*. reported that when Embul banana was sprayed with Cinnamon oil, the shelf life of banana was increased as it resisted the crown rot disease, besides it did not alter the physio-chemical properties of the fruit (51). In accord with our findings, Duduk *et al*. reported that thyme oil (153µl/ml) completely inhibited the anthracnose symptoms caused by *Colletotrichum acutatum* on Strawberries (52). Similarly, Abd –Alla *et al* (31). reported complete reduction of crown rot of banana with cinnamon and thyme oil. Whereas, Sangeeta *et al*. reported that various oils (*O. sanctum, C. martini, C. nardus and C. citrates)* reduced the severity of crown rot of Cavendish banana cultivar significantly and in addition, increased the shelf life of the fruit (50).

The possible mode of action of essential oils on reduction of lesion diameter and disease symptoms on fruits could be due to the ability of essential oils and its components to diffuse readily through the cell wall and cell membrane of plant tissue. It further disturbs the structure of polysaccharides, phospholipids and fatty acids leading to cell death (53,30). Another view regarding the inhibitory effect of essential oils on sporulation in fungi is attributed to their volatiles, which are emitted on the surface of mycelium during development. Nevertheless, this negative impact decreases the spore load and its proliferation in storage conditions and on fruit

surfaces (54). Yet, other reports show that fruits decay by pathogens was significantly reduced by essential oil treatment. This could be due to an increase in accumulation of pathogenesis related proteins (PR), increase in resistance to pathogen, changes in phenolic and ascorbate concentrations in fruits and increase in heat shock proteins (54,55,56)

Recently, static water toxicity test revealed that components of essential oils are less toxic than the synthetic pesticides (57). Additionally, eugenol was not persistent in fresh water or in soil (58). Based on current information, essential oils are considered safe for human consumption and as an application on various commodities. Hence, considering the effectiveness of essential oils, fruit applications that are oil based will be an excellent postharvest treatment**.**

Based on our results we conclude that clove and fenugreek oil caused strong inhibition of the *C. musae* both *in vitro* and *in vivo.* However, clove oil was highly potent in controlling the mycelia growth, conidial germination and reducing the anthracnose lesions in a remarkable manner. Furthermore, clove and fenugreek oil are considered to be GRAS (Generally Recognized as Safe) product according to FDA. Hence, they can be used as dips, sprays, or coatings to combat anthracnose pathogens and thereby reduce postharvest losses. Despite, their potential as future fungicides and their underlying advantages, much work has to be done to have a better understanding on their mode of action on fruit tissue. Additionally, further research to develop formulations that do not impart any undesirable effects to the fruit's quality and maintain its sensory and physiochemical properties is required.

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