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Antimicrobial activity of eucalyptus (*Eucalyptus globules*), sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*) against Colletotrichum gloeosporioides from Egerton University

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Abstract Mangoes are an important source of vitamins all over the world. However, their production has been endangered by *Colletotrichum gloeosporioides* that causes anthracnose of mangoes. This study was aimed at isolating the fungus from infected mangoes followed by testing the sensitivity of the fungus to essential oils from eucalyptus, sage and rosemary plant. Infected mango fruits were acquired from Njokerio Market near Egerton University. Fungal pathogens were isolated from the infected fruits using potato dextrose agar. Essential oils were extracted from eucalyptus, sage and rosemary plant using distillation method. Sensitivity of *Colletotrichum gloeosporioides* to the extracted essential oils was carried out using agar well technique. The colonies of *Colletotrichum gloeosporioides* had dense whitish mycelium with black and orange color fruiting bodies. The spores were smooth, hyaline and sub cylindrical with round end spores. The conidia were cylindrical in shape, hyaline in colour and the size was 13.4 – 24 x 4.0 – 5.9µm. The appresoria were obovate to clavate in shape, brown in colour and 6.7 – 19.8 x 3.8 – 11.9µm in size.There was a relationship between heating time and yield of essential oils in rosemary (r=0.99) and leaves (r=0.99). Conversely, there was no significant difference in the percentage yield of essential oils from eucalyptus, sage and rosemary (P=0.057). There was no significant difference in the percentage composition of the different compounds in the essential oils extract from eucalyptus, sage and rosemary (F=0.48 P=0.62). The zone of inhibition of essential oils obtained from eucalyptus against *Colletotrichum gloeosporioides* varied from 19±3mm to 21±1mm, sage (18±1mm-19±2mm) and rosemary (17±1mm-19±1mm). The minimum inhibition of essential oils from eucalyptus leaves was120±0.01mg/ml, sage (241±0.00mg/ml) and rosemary (230±0.02mg/ml). The minimum fungicidal concentration of essential oils from eucalyptus was (120±0.01mg/ml), sage (241±0.00) and rosemary (230±0.02). The essential oils from eucalyptus, sage and rosemary have bioactive compounds that have antifungal properties against*Colletotrichum gloeosporioides.*

Keywords *Colletotrichum gloeosporioides*, Essential oils, *Eucalyptus globules,* Mangoes, *Salvia officinalis, Rosmarinus officinalis*

Introduction

Diseases cause a lot of morbidity in both plants and animals in this age. This has worsened with the development of drug resistance being witnessed in nearly all parts of the world [1]. Apparently in some developing countries, plants are the main source of treatment against most diseases. Approximately 20% of the world's plants have been

pharmacologically tested against majority of pathogenic microorganisms [2]. Consequently, there has been introduction of new antibiotics into the drug market. Among the plants that have gained popularity in medicine are the *Eucalyptus globulus*, *Salvia officinalis* and *Rosmarinus officinalis* [3].

Mango business is a strong driver of socio-economic development in many countries by providing labour to many people [4]. Mangoes also attract other players in the value chain addition like fruit processers and suppliers. However, mangoes are however exposed to high temperatures and moisture levels which favour proliferation of anthracnose disease [5]. Anthracnose is the most serious disease of mangoes worldwide especially in humid areas. The disease presents great challenges to people involved in the international commerce of this fruit [6]. Anthracnose disease is caused by two related species of fungi such as *Colletotrichum gloeosporioides* and *Glomerella cingulata* [7].

Currently, fungicides are the most reliable strategy to achieve effective control of anthracnose and safeguard production of mangoes in humid regions. However, they are overused and misused rendering them ineffective against mango anthracnose [8]. Emergence of fungicide resistant *Colletotrichum gloeosporioides* has been reported in several parts of the world [9]. The use of essential oils from plants can be alternative remedy in controlling the fungi [10].

Eucalyptus globulus, native to Australia and Tasmania belong to the myrtaceae family [11].*Eucalyptus* trees are well known for the medicinal properties of the oil contained in their leaves. Essential oils from *Eucalyptus globules* were used in traditional aboriginal medicines to heal wounds and fungal infections [12]. *Salvia officinalis* is a common herbal plant widely cultivated in various parts around the world. However, it is native to the Mediterranean region [13]. Essential oils from *Salvia* species have been shown to possess antimicrobial, antioxidant, anti-inflammatory, anti plasmodial, hypoglycemic and anti-carcinogenic properties. Sage is rich in biologically active compounds, among them phenolics acids and flavonoids [14].

Rosmarinus officinalis originally grew in Southern Europe. The plant is commonly used as spice and flavoring agents in food processing because of its desirable flavor, high antioxidant activity and lately as antimicrobial agent [15]. Harp *et al.* [16] reported that rosemary is a rich source of phenolic compounds with high antimicrobial activity against both bacteria and fungi. This study was aimed at isolating *Colletotrichum gloeosporioides* from infected mango fruits and testing the fungus for sensitivity against essential oils extracted from eucalyptus (*Eucalyptus globules*), sage (*Salvia officinalis*) and rosemary plants (*Rosmarinus officinalis*) [17].

Materials and Methods

The study area

The study was conducted at Egerton University, main campus Njoro in Kenya. Egerton University is located in Njoro Sub County with coordinates as 0^o 23' south, 35^o 35' and altitude of 2000m above sea level. Temperatures range between 17 - 22^{o}C while the average annual rainfall is 1000mm [18].

Collection of infected mango fruits

One hundred and fifty samples of infected mangoes were randomly collected from markets within Egerton University. The samples were weighed before being placed in sterile plastic bags, before storage in a refrigerator until mycological analysis.

Isolation of fungal pathogen

The direct plating technique was used in isolating the fungal pathogen [19]. Four small pieces from the margin of lesion of each sample were directly inoculated on prepared plates of potato dextrose agar which contained (g/L): peeled potato100.0g, glucose 20.0g, agar 15.0g, water 1000.0 ml. The medium was supplemented with chloramphenicol (250mg per liter) as a bacteriostatic agent [20]. The plates were incubated at 28 ± 1^{o}C for 5 to 7 days. Three replicates were prepared for each sample. The resulting fungi isolates were sub-cultured in potato dextrose agar to obtain pure cultures. Identification of the isolates was carried out using macro and micro characteristics and fungal identification keys [21].

Collection of plant materials

Rosemary (*Rosmarinus officinalis*), Eucalyptus (*Eucaluptus agglomerate*) and sage (*Salvia officinalis*) leaves from plants growing wild in Egerton University main campus, Njoro were collected. The leaves were placed in sterile polythene bags and transported to the Department of Biological Sciences laboratories, Egerton University. The samples were stored in a deep freezer at -4^{o}C until processing.

Extraction of essential oils

A sample of 400g of fresh eucalyptus sage and rosemary leaves were separately loaded into 2-Litre round bottom flask containing 1.5 litre of water and placed on a heating mantle having power rating of 450 watt and timed. The samples were boiled with water to release the oil within the leaves. The volatile oils evaporated along with the water into the condenser connected to a flask at 100^{o}C and atmospheric pressure. The condensed steam and oils were collected in a separating funnel after which oil and water were separated. The water was drained off gently and the oils were separately collected in a 10ml measuring cylinder and measured after every 20 min for a period of 3h. The traces of water in the essential oils were removed by adding 1 g of magnesium sulphate in the oil as a drying agent. The yield of essential oils was calculated using the formula provided by Kumari *et al.*[22];

$$Yield\ of\ essential\ oil\ (\%) = \frac{amount\ of\ essential\ oil\ obtained\ (g)}{Amount\ of\ raw\ materials\ used\ (g)} x100$$

Determination of essential oil constituents

The extracted essential oils samples were analyzed using Gas Chromatography Mass Spectrometry (GC-MS) Agilent 6890 gas chromatography instrument coupled to an Agilent 5973 mass spectrometer and an Agilent Chem. The following operating parameters were used for the essential oil sample; capillary GC column HP-5MS 5% phenylmethyl siloxane\(30 x 0.25mm i.d. x 0.25 mm film thickness), a carrier gas Helium (flow rate 1.2mL min^{-1}) and a split-less injection mode. Injector temperature was 250°C; Oven temperature was set initially at 50°C and then raised to 250°C at 10°C min-1 rate till the end of analysis. The eluted analytes were detected using mass selective detector (5973 network) and Electron Impact ionization (EID) at 70 eV.

Sensitivity test of the fungal pathogens to the essential oils

The Fungus from the pure cultures was s picked using an inoculating needle and placed in a Bijo bottle that had sterile distilled water. Using sterile glass rods the fungi was separately crushed inside the Bijo bottle. A fungus inoculum was picked and placed in culture media using a teat pipette. An L shaped glass rod was used to spread the inoculaon the culture media. Paper discs having a diameter of 8mm were made from sterile filter papers. Separately, the discs were dipped into the extracted essential oils, removed and allowed to dry. Aseptically, the discs were placed at the centre of the media having the pure cultures of the fungal pathogens using a sterile pair of forceps. The Petri plates were wrapped using parafilm. Incubation was carried out at 28^{o}C for 7 days. The zones of inhibition were determined in millimeters.

Minimum inhibitory concentration

Two fold serial dilution technique was used in determining minimum inhibitory concentration [8]. One milliliter of sterile Mueller Hinton broth was placed in 11 sterile test tubes. Using a micropipette, 1ml of each essential oil was placed in the second test tube. Serial dilution was carried out up to the 11th test tube. 0.1ml of the standard pathogens were each added from the 1st test tube up to the 10th test tube. The 1st test tube was used as the negative control and the 11thtest tube as the positive control. Incubation was carried out at 28^{o}C for 5 days. Growth was observed by visually using turbidometric techniques.

Data analysis

Data analysis was carried out using Microsoft excel spreadsheet and Statistical Package for Social Sciences (SPSS) version 11.0 software. Pearson's correlation was used to determine the relationship between heating period and yield of essential oils while t-test was used in comparing yield of essential oils in rosemary and eucalyptus.

Results

Morphology of *Colletotrichum gloeosporioides*

Cultural and morphological characteristics of the fungal isolates are presented in table 1. The colonies of *Colletotrichum gloeosporioides* had dense whitish mycelium formed with black and orange color fruiting bodies (Figure 1). The spores were smooth, hyaline and sub cylindrical with round end spores (Table 1). The conidia were cylindrical in shape, hyaline in colour and the size was 13.4 – 24 x 4.0 – 5.9 µm. The appresoria were obovate to clavate in shape, brown in coulour and 6.7 – 19.8 x 3.8 – 11.9 µm in size.

Figure 1: Culture of Colletotrichum gloeosporioides in potato dextrose agar

Yield of essential oils from eucalyptus, sage and rosemary

The yield of essential oils in eucalyptus varied from 0.7% after the samples were heat for 20 minutes to 4.3% after heating for 180 minutes in thyme (Table 2). On the other hand, the percentage yield in sage plant ranged from 0.3% after the samples were heat for 20 minutes to 3.4% after heating for 180 minutes. However, the yield of essential oils from rosemary varied from 0.2% after heating the samples for 20 min to 2.8% after the samples were heat for 180 min. The weights of the plant samples, volume of distilled water and the heating temperature were maintained constant at 400g, 1.5 L, 100^{o}C respectively. There was a relationship between heating time and yield of essential oils in rosemary (r=0.99) and leaves (r=0.99). Conversely, there was no significant difference in the percentage yield of essential oils thyme, sage and rosemary (P=0.057).

Table 1: Morphological characteristics of *Colletotrichum gloeosporioides*

Factor		Observed characteristics
Colonies		Dense Whitish mycelium formed with black and orange color fruiting bodies
Spores		Smooth, hyaline and sub cylindrical with round end spores
Conidia		
i.	Shape	cylindrical
ii.	Colour	hyaline
iii.	Size (µm)	13.4 – 24 x 4.0 – 5.9
Appresoria		
i.	Shape	obovate to clavate
ii.	Colour	brown
iii.	Size (µm)	6.7 – 19.8 x 3.8 – 11.9

Constituents of essential oils from leaves of thyme plant

The percentage composition of Alpha Pinene varied from 10.3% in eucalyptus to 32.7% in rosemary (Table 3). However, the percentage composition of Camphene ranged from 1.6% in eucalyptus to 201.6% in rosemary. The percentage composition Beta Pinene ranged from 7.5% in eucalyptus to12.4% in rosemary. In addition, the variation in percentage composition of Para cymene was 7.6% in rosemary to 10.2% in eucalyptus. The percentage composition of Linalool varied from 5.7% in rosemary to 14.8% in sage, Borneol (6.5% in rosemary-11.2% in sage), Beta Caryophyllene (1.7% in eucalyptus-14.9% in sage), thymol (4.1% in rosemary-24.2% in sage) carvacrol (1.3% in sage-18.9% in eucalyptus) and Alpha Terpinene (2.4% in rosemary-4.6% in eucalyptus). There was no significant difference in the percentage composition of the different compounds in the essential oils extract from eucalyptus, sage and rosemary (F=0.48 P=0.62).

Table 2: Yield of essential oils from eucalyptus, sage and rosemary

Plant	Weight (g)	Distilled H_2O (L)	Heating time (Min)	Temperature (^oC)	Yield (%)
Eucalyptus	400	1.5	20	100	0.7
	400	1.5	40	100	1.0
	400	1.5	60	100	1.5
	400	1.5	80	100	2.0
	400	1.5	100	100	2.7
	400	1.5	120	100	3.0
	400	1.5	140	100	3.2
	400	1.5	160	100	4.2
	400	1.5	180	100	4.3
Sage	400	1.5	20	100	0.3
	400	1.5	40	100	0.5
	400	1.5	60	100	0.7
	400	1.5	80	100	1.1
	400	1.5	100	100	1.5
	400	1.5	120	100	2.0
	400	1.5	140	100	2.6
	400	1.5	160	100	3.0
	400	1.5	180	100	3.4
Rosemary	400	1.5	20	100	0.2
	400	1.5	40	100	0.3
	400	1.5	60	100	0.7
	400	1.5	80	100	1.3
	400	1.5	100	100	1.7
	400	1.5	120	100	1.0
	400	1.5	140	100	2.1
	400	1.5	160	100	2.4
	400	1.5	180	100	2.8

Sensitivity of *Colletotrichum gloeosporioides* to essential oils from eucalyptus, sage and rosemary

The zone of inhibition of essential oils obtained from eucalyptus against *Colletotrichum gloeosporioides* varied from 19±3mm to 21±1mm, sage (18±1mm-19±2mm) and rosemary (17±1mm-19±1mm) (Table 4). There was no significant difference between the zones of inhibition produced by the essential oils from eucalyptus, sage and rosemary (F=0.46 P=0.64).

Table 3: GC-MS analysis of essential oils from thyme leaves

Compound	Composition (%)		
	Eucalyptus	Sage	Rosemary
Alpha Pinene	10.3	22.2	32.7
Camphene	1.6	14.7	20.6
Beta Pinene	7.5	10.2	12.4
Para Cymene	10.2	8.6	7.6
Linalool	14.3	14.8	5.7
Borneol	11.9	11.2	6.5
Beta Caryophyllene	1.7	14.9	4.8
Thymol	12.2	24.2	4.1
Carvacrol	18.9	1.3	3.3
Alpha Terpinene	4.6	3.1	2.4

Table 4: Sensitivity of the fungal pathogens to the extracted essential oils from thyme sage and rosemary

Essential oil	Zone of inhibition (mm)		
	Replicate 1	Replicate 2	Replicate 3
Eucalyptus	21±1	20±2	19±3
Sage	18±2	19±2	18±1
Rosemary	19±1	17±1	18±2

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of essential oils from eucalyptus, sage and rosemary when tested against *Colletotrichum gloeosporioides*

The minimum inhibition of essential oils from eucalyptus leaves was120±0.01mg/ml, sage (241±0.00mg/ml) and rosemary (230±0.02mg/ml) (Table 5). The minimum fungicidal concentration of essential oils from eucalyptus was (120±0.01mg/ml), sage (241±0.00) and rosemary (230±0.02). The minimum inhibition concentration of in essential oils from eucalyptus tree leaves, sage and rosemary were equal to the minimum fungicidal concentration.

Table 5: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of essential oils from thyme, sage and rosemary plant

Essential oil	MIC inhibition (mg/ml)	MFC (mg/ml)
Eucalyptus	120±0.01	120±0.01
Sage	241±0.00	241±0.00
Rosemary	230±0.02	230±0.02

Discussion

Cultural and morphological characteristics of the fungal isolates are presented in table 1.The colonies of *Colletotrichum gloeosporioides* had dense whitish mycelium formed with black and orange color fruiting bodies. The spores were smooth, hyaline and sub cylindrical with round end spores. The conidia were cylindrical in shape, hyaline in colour and the size was 13.4 – 24 x 4.0 – 5.9 µm. The appresoria were obovate to clavate in shape, brown in colour and 6.7 – 19.8 x 3.8 – 11.9 µm in size. These results concurred with those of a previous study carried out in Pakistan [12]. This can be attributed to isolation of the same strain of the fungus [23].

In addition, the percentage yield of essential oils from leaves of eucalyptus plant obtained in the current study concurred with a previous study carried out by Jabbar *et al*. [24]. These may be attributed to the species of

eucalyptus tree from which the essentials oils were extracted. However, the percentage yield of essential oils obtained from sage plant in this study differed with those obtained by Bazie *et al.* [25]. This may be attributed to the nutrient level of the soils in which the plant is growing in. Divya *et al.* [26] explained that the soil nutrient level of a given area influences synthesis of essential oils in sage plant.

The percentage yield of essential oils from rosemary obtained from this study concurred with previous study [27]. This may be attributed to similarity of the species of rosemary that were being studied [28]. Further Fekiya *et al.* [29] explained that the method of extraction of essential oils affects production of essential oils in rosemary sp.

The constituents of essential oils obtained from eucalyptus leaves concurred with a previous study carried out in Parkistan [30]. Gautum [31] asserted that the biochemical pathways used by eucalyptus plant greatly influences the composition of essential oils produced. The compounds obtained from essential oils of sage leaves in the present study agreed with a previous study carried out in India. This may be attributed to similarities in the study areas Lima *et al.* [32].

However, the constituents of essential oils from rosemary slightly differed with previous studies carried out in Brazil [33]. According to Camilletti *et al.* [34], the species of rosemary from which the essential oils were obtained may have contributed to the observed results. On the other hand, the zones of inhibition of the extracted essential oils from thyme, sage and rosemary on *Colletotrichum gloeosporioides* disagreed with a previous study by Bill *et al.* [35]. This may be attributed to the type of solvents used to extract the essential oils [36]. Besides the minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC) obtained in the present study disagreed with a previous study [37]. This may be attributed to variations in the compounds present in the essential oils extracted [38]. In addition, MIC and MFC obtained in this study were equal. This suggested that the essential oils were fungicidal and not fungistatic.

Conclusions

Isolation of *Colletotrichum gloeosporioides* from mangoes was carried out. Essential oils were extracted from thyme, sage and rosemary and their active ingredients determined. In addition, sensitivity test of the extracted essential oils to *Colletotrichum gloeosporioides* was carried out followed by determination of their MIC and MFC.

Recommendations

There is need to test the sensitivity of other fungal and bacterial pathogens to the extracted essential oils. Mass production of essential oils from eucalyptus, sage and rosemary need to be carried out.

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Conflict of interest

We declare no conflict of interest.

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