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Chemical composition and antifungal activity of essential oils of *Tagetes minuta* (Asteraceae) against selected phytopathogenic fungi

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Abstract

Over the years, management of plant pathogenic fungi has primarily relied on the use of synthetic chemical fungicides. However, in the recent past, exploration for biologically active compounds from plants with the aim of discovery and development of novel and eco-friendly biopesticides to combat current and emerging plant pathogenic fungi has received increased interest. This study aimed at extraction and characterization of *Tagetes minuta* essential oils (EOs) as well as evaluation of their antifungal activity against selected phytopathogenic fungi namely: *Fusarium oxysporum, F. solani, Aspergillus flavus*, *A. parasiticus* and *A. niger*. Essential oils were extracted using the steam distillation method in a modified Clevenger-type apparatus. The antifungal activity of the EOs was assessed by disc diffusion method while gas chromatography - mass spectrometry (GC/MS) was used for characterization of the chemical components of the EOs. Twenty compounds corresponding to 96% of the total essential oils and constituting a mixture of monoterpenes (70%) and sesquiterpenes (30%) were identified in the Eos. They included elixene and silphiperfol-6-ene, which are being reported for the first time in essential oils of *Tagetes minuta*. The EOs of *T. minuta* exhibited potent antifungal activity against the studied fungi with the highest growth inhibition observed in *F. oxysporum* and *A. niger* with mean inhibition zones of 28.7mm after five days of incubation. Four out of the five test fungi fell within the category of extremely sensitive (inhibition zone diameters ≥ 20mm) when subjected to the crude EOs. The minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of the EOs against the fungi were in the ranges of 24 - 95mg/mL and 24 - 190mg/mL, respectively. This study thus lays down significant groundwork for a more comprehensive study on the practical feasibility of using *T. minuta* EOs as possible alternative to synthetic fungicides in the management of economically important phytopathogenic fungi.

Keywords: Antifungal activity, essential oils, phytopathogenic fungi, *Tagetes minuta*

1. Introduction

Global agricultural crop losses due to biotic and abiotic factors have been estimated at more than 35% of annual production [1]. In the United States alone, insects, pathogens and weeds destroy about 37% of all potential crops with losses due to insects being 13%, plant pathogens 12% and weeds 12% [2]. Among the biotic factors, plant pathogenic microorganisms such as fungi, bacteria and viruses are the major infectious agents of plants that cause enormous economic losses worldwide by destroying millions of tons of crop yield annually [3]. Among plant pathogens, fungi are responsible for the greatest destruction of plants both in agricultural and natural ecosystems [4, 5]. It is estimated that phytopathogenic fungi are responsible for 40-60% of the total plant pathogenic losses, with losses due to both pre- and post-harvest fungal diseases estimated to be more than 200 billion Euros annually in the United States of America [6].

Among the estimated 1.5 million fungal species on earth, only around 15,000 of them are plant pathogens, majority of which belong to the ascomycetes and basidiomycetes groups [7]. Despite the fact that fewer than 10% of all known fungi can colonize living plants, phytopathogenic fungi are collectively responsible for 70% of all known plant diseases [8, 9]. The position of fungi as a key group of plant pathogens is attributed to a number of factors; many phytopathogenic fungi are capable of infecting any plant tissue during any stage of growth; most have complex life/infection cycles involving multiple (up to five) phases each of Which, may occur on a different plant host and may be characterized by different reproductive strategies [5].

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Furthermore, the presence of both sexual and asexual reproductive stages allow many phytopathogenic fungi to infect plants in different climatic zones from the hot and dry arid zones to the wet zones in the tropic and equatorial regions [10].

Several physical, chemical and biological methods of managing phytopathogenic fungi exist [11]. However, the modern agricultural system of crop protection and management against phytopathogenic fungi has primarily over relied on synthetic chemical fungicides [1]. The use of synthetic pesticides has greatly boosted crop yields and thus increased global food security. However, it has over the years become apparent that over reliance on these chemicals in agriculture is not without serious environmental and health concerns [12]. Some of the drawbacks associated with synthetic pesticides include: development of resistance among targeted pathogens as a result of overuse and persistence of the pesticides in the environment hence higher likelihood of killing beneficial organisms. Besides, several synthetic fungicides remain in plant tissues and products such as fruits and vegetables long after the application thus posing a health risk to humans, animals and the environment [13]. Furthermore, synthetic agricultural chemicals are expensive thus reducing their availability to smallholder farmers especially in developing countries [14, 15]. There is therefore need to focus more on sustainable and cost-effective methods of reducing plant losses occasioned by fungal phytopathogens during production and post-harvest stages. One of the possible approaches is through development of novel plant-derived biopesticides that are both environmentally biocompatible and effective against current and emerging phytopathogens.

Exploration for biologically active compounds from plants aimed at discovery and development of novel antimicrobial agents has received increased interest in the recent past. Essential oils are natural aromatic complexes, formed by certain plants as secondary metabolites [16]. They are isolated from various parts of the plant such as leaves (basil, patchouli, cedar), fruits (citrus), bark (cinnamon), root (ginger), grass (citronella), gum (myrrh and balsam oils), berries (pimenta), fruits (bergamot, orange, lemon, juniper), seed (caraway), flowers (rose and jasmine), twigs (clove stem), wood (amyris), heartwood (cedar), rhizomes (ginger, calamus, curcuma) and saw dust (cedar oil) [17-19].

Essential oils containing mixtures of volatile constituents such as monoterpenes, sesquiterpenes and/or phenylpropanoids, esters, alcohols, and terpenoids among other classes of compounds have been shown to have antifungal, antibacterial, antiviral, nematicidal and insecticidal properties among other biological activities [20-22]. The objective of this study was to: (i) characterize the chemical composition of essential oils of *Tagetes minuta* and (ii) evaluate the antifungal activity of the essential oils against selected phytopathogenic fungi.

2. Materials and Methods
2.1 Collection of plant materials
Aerial parts (leaves, flowers and stems) of *Tagetes minuta* were sampled from four sites within Maseno area (0°0'21.43"S, 34°36'6.23"E, 1524 MASL), Kisumu County, Kenya. The samples were packaged in Kraft bags and transported to the Chemistry laboratory, Maseno University, Kenya. A sub-sample of the plant materials was prepared, packaged and stored according to the herbarium rules and regulations. This sample was later taken to the herbarium at the School of Biological Sciences, University of Nairobi, Kenya for further identification and authentication by a Plant Taxonomist. A voucher specimen (MMG2015/01) was deposited at the University of Nairobi herbarium.

2.2 Extraction of essential oils and determination of yield
Tagetes minuta essential oils were extracted using the steam distillation method in a modified Clevenger-type apparatus [23]. The plant materials were cut into small pieces (≈10cm long), weighed and 4 kg loaded into the still of the tank that was part of the distillation setup. The rid of the distillation tank was tightly secured and the plant material subjected to steam distillation. The collection of EOs started after a heating time of about 40 minutes and continued until no more essential oil was obtained (5-8 hours). After the distillation process was completed, the volatile essential oils were removed from the top of the hydrosol and dried over anhydrous sodium sulphate (Na_2SO_4) [24]. The essential oils were filtered using Whatman filter paper (No. 1), weighed and mean percent yield determined. The oils were then collected into airtight glass vials and stored at -20 °C until when they were required for chemical analysis and antifungal bioassays.

2.3 Fungal test pathogens
Five economically important fungal plant pathogens namely: *Fusarium oxysporum*, *Fusarium solani*, *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus niger* were used as test pathogens. The test pathogens were retrieved from the culture collection center at the Plant Pathology Laboratory, Department of Plant Science and Crop Protection, University of Nairobi. Confirmation of the identity of the pathogens was done based on cultural and morphological characteristics using taxonomic keys [25-27].

2.4 Retrieval of test pathogens and preparation of fungal inocula
Stock culture for each of the test fungal pathogens maintained at -20 °C was retrieved by sub-culturing on Potato Dextrose Agar (PDA). A pure colony was selected from an agar plate culture; a sterile loop was used to pick the top mycelia of a colony which was transferred into a test tube containing 10mL of Potato Dextrose Broth (PDB). The culture tubes were incubated for 5 days at 28 °C to obtain fresh cultures. McFarland standard was used as a reference to adjust the turbidity of fungal suspensions to be within the required range. Exactly 0.5 McFarland equivalent turbidity standards was prepared by mixing 0.05mL of 1% barium chloride dehydrate with 9.95mL of 1% sulfuric acid. Fungal suspensions were prepared in sterile saline solution which was prepared by dissolving 0.85g of NaCl in 100mL of distilled water and autoclaving for 15 minutes at 121 °C and 15 psi pressure. The turbidity of the fungal suspensions was adjusted to 0.5 McFarland, an equivalent of 1 x 10^6 spore/mL.

2.5 Determination of the antifungal activity of *Tagetes minuta* essential oils
Antifungal activity of *T. minuta* essential oils against the test fungi was carried out using the disc diffusion method also known as Kirby-Bauer antimicrobial susceptibility test as described by Souza *et al*. [28]. The basic concept in this method is that, the size of the zone of inhibition can be correlated with the susceptibility of a microorganism to a particular antimicrobial agent. Thus, a larger area of microorganism-free media surrounding the disc implies that the microorganisms are more sensitive to the antimicrobial agent contained in the disc.

One drop of Tween 20 per mL (≈ 0.5 to 1%) was added to a standardized spore suspension to enhance the dispersion of spores [29]. Two hundred microliters of the spore suspension was uniformly spread using a sterile L-shaped glass rod on 9cm Petri plates containing PDA. Sterile Whatman filter paper discs (No. 1, 6mm in diameter) were impregnated with 10μl of undiluted

crude essential oil by holding the discs using a pair of sterile forceps and pipetting the EOs to the paper discs using a micropipette. The discs were then aseptically placed at the center of the inoculated culture plates using a pair of forceps. Dimethyl sulphoxide (DMSO) was used as a negative control while Apron Star® (Thiamethoxam 200g/kg, Mefenoxam 200g/kg and Difenoconazole 20g/kg), a broad-spectrum seed treatment fungicide prepared according to the manufacturer's instruction was used as a positive control. The Petri dishes were then kept in a refrigerator at 4 °C for 2 hours to allow essential oils to diffuse into the agar medium. The plates were finally incubated at room temperature (23 ± 2 °C) and growth of the fungi monitored starting from the 3rd to 14th day. The reading of the diameters of the inhibition zones was done on the 5th and 10th day. This was based on preliminary studies which had shown that on the 3rd day, the fungi were still growing and between the 5th - 7th day, the essential oil activity was at its highest. The tests were conducted in triplicates.

The sensitivity of individual fungi to the essential oil was ranked based on the mean inhibition zone values expressed in millimeters (mm) as follows: not sensitive (-) for total zone diameters of ≤8mm; sensitive (+) for diameters between 8 and 14mm; very sensitive (++) for zone diameters between 15 and 19mm and extremely sensitive (+++) for zone diameters ≥20mm [30, 31].

2.6 Evaluation of antifungal activity of different concentrations of *Tagetes minuta* essential oils

The activity of *T. minuta* essential oils at seven concentration levels was assessed using the disc diffusion method following the procedure described by Clara *et al*. [32] with some modifications. Two hundred microliters of microbial suspension (approximately 1×10^6 CFU/ml) was uniformly spread on PDA Petri plates. Serial dilutions of *T. minuta* essential oil were prepared with DMSO. The essential oils were diluted to the following serial geometric dilutions: 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56% and 0.78%. Sterile Whatman filter paper discs (No. 1, 6mm in diameter) were impregnated with 10µl of different essential oil concentrations and aseptically placed at the center of the inoculated culture plates. The plates were then placed in a refrigerator at 4 °C for 2 hours to allow the essential oils to diffuse into the agar. The plates were then incubated at room temperature (23 ± 2 °C) and the diameters of the inhibition zones measured after 5 days. The tests were conducted in triplicates.

2.7 Assessment of the minimum inhibitory concentrations and minimum fungicidal concentrations of the essential oils on the test fungi

Tube dilution method as described by Caburian and Osi [33] with some modifications was employed in the assessment of minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of the essential oils against the test fungi. Twelve sterile screw-capped falcon tubes (15mL) were numbered #1 to #11 and the last one as #13. One milliliter of potato dextrose broth was introduced into tubes #2 to #11. One milliliter of *T. minuta* essential oil was pipetted into tube #1 and #2, the two tubes were capped and vortexed for 5 seconds; one milliliter was withdrawn from the contents of tube #2 and transferred to tube #3, after capping the tube and mixing by vortexing the contents, one milliliter from the contents of tube #3 was withdrawn and transferred to tube #4 and the process repeated until 1.0mL from tube #9 was added to tube #10. Fifty microliters of standardized fungal inocula (approximately 10^6 spores/mL) was then introduced into tubes #1 to #11 and to tube

#13. To tube #13, 0.5mL of the standard fungicide Apron Star® prepared according to the manufacturer's instructions was added. Tween 20 (0.05%) was added to the essential oil prior to its application into the tubes to improve the oil solubility and also for better spore dispersion [34]. The tubes were then incubated at 28 °C for 72 hours.

After incubation, the tubes were examined for growth by observing for any turbidity. The interest was not on the dilutions that had failed to prevent microbial growth but rather, those that prevented any visible growth. The tube with the lowest concentration (highest dilution) of essential oil at which no visible growth or turbidity was observed was reported as the minimum inhibitory concentration of the oil on the test fungi [35]. The tubes were shaken to homogenize the contents and 0.01mL of the contents of each tube sub-cultured using the spread plate method on PDA plates. The plates were then incubated at room temperature for 72 hours and then observed for any growth of colonies. Minimum fungicidal concentration was determined as the highest dilution (lowest concentration) of essential oil at which no growth occurred following the subculturing onto PDA fungi [35].

All the bioassays were conducted in a biological safety cabinet and in accordance with the protocols of Clinical and Laboratory Standards Institute (CLSI) formerly National Committee for Clinical Laboratory Standards (NCCLS).

2.8 Characterization of chemical composition of essential oils of *Tagetes minuta*

Gas chromatography coupled to mass spectrometry (GC-MS) was used to establish the chemical composition of the EOs. Three replicates (each taken from a different extraction batch) of 1mg of *T. minuta* essential oils were separately weighed and diluted in 1mL of dichloromethane to make a stock solution. From the stock solution, further dilution was made as follows: 100µl of stock topped to 1mL with dichloromethane after which the essential oil was analyzed on an HP-7890A (Agilent Technologies, Wilmington, USA) GC connected to an HP 5975 C (Agilent Technologies, Wilmington, USA) MS. The gas chromatography equipment was fitted with HP-5MS capillary column; 30m × 0.25mm internal diameter; 0.25µm film thickness with 5%-phenyl methyl silicone as the stationary phase (J & W Scientific, Folsom, USA). The operating conditions were as follows: Carrier gas was helium with a flow rate of 1.2 ml/min, constant flow mode; injection mode splitless; oven temperature (35 °C for 5 min to 280 °C at 10 °C/min for 10.5min with a run time of 50 min); injection volume (1µl). The components of the essential oil were identified on the basis of their retention indices (RI) (determined with reference to a homologous series of normal alkanes C_5-C_{31}) and calculated based on a quasi-linear equation for temperature-programmed retention index [36]. Identification of essential oil components was further verified by comparison of their MS fragmentation patterns with those reported in the mass spectra library database (NIST05a and Adams MS HP, USA). Quantitative determination of the constituents was made using the calibration curve of the dose-peak area of a pure compound (1,8-cineole; 99%, Gillingham, Dorset, England), with the relative amount of each individual component expressed as percentage of the peak area relative to the total peak area.

2.9 Data analyses

Data were analyzed using the PROC ANOVA procedure of GENSTAT version 15 and significant differences among means compared using Fisher's Protected LSD at 5% probability level. The inhibitory effects of the essential oils against the test fungi

were expressed as mean ± standard error of the mean inhibition zones diameter (mm). Linear regression analysis was performed to determine correlations between different concentrations of essential oil and their overall antifungal activity assessed as diameters of the inhibition zone. Standard dose-response curves were obtained by plotting essential oil concentrations (mg/mL) against the mean inhibition zone diameters (mm).

3. Results and Discussion
3.1 Percentage yield of *Tagetes minuta* essential oils
The mean percent yield of essential oils from four distillation batches of fresh aerial parts (leaves, stems and flowers) of *T. minuta* was 0.059% w/w (Table 1). The steam distillation method had a relative standard deviation (RSD) of 1.01%. Such a RSD value (specification: precision = maximum of 2% RSD) indicated that the steam distillation method was precise and with minimal wastages [33]. The percent yield of EOs obtained in the current study was higher than that obtained in previous studies from the same plant species sampled from other parts of Kenya but using different extraction methods. For example, essential oil yields of 0.00029% w/w were obtained from fresh aerial parts of *T. minuta* sampled from Bungoma County, western Kenya [37, 38]. In another study, *T. minuta* plant materials obtained from three regions in different agro-ecological zones in Kenya namely: Kasarani (Nairobi County), Bungoma (Bungoma County) and Bondo (Siaya County), produced EO yields of 0.045, 0.039, and 0.035% w/w, respectively [39]. However, higher yields of *T. minuta* EOs than those obtained in the current study have been reported in literature. For instance, EO yields of 1% w/w were reported from leaves of *T. minuta* plants grown in greenhouse conditions in Iran [40], while in another study, essential oil yields of 1.2% w/w were obtained from *T.*

minuta plants grown on experimental farms in the same country [41]. The reported variations in the yields of essential oils of *T. minuta* could be attributed to various interactions between the genetics, ontogenesis and physiological state of the plant with the environment in addition to numerous abiotic factors present in the external environment of the growing plants [38, 42, 43].

Table 1: Percentage yield (% w/w) of *Tagetes minuta* essential oils

Batch No.	Weight of plant material (kg)	Weight of essential oil (g)	Percentage yield (% w/w)
1	4.38	2.588	0.059
2	4.23	2.542	0.060
3	4.60	2.738	0.059
4	4.10	2.412	0.058
			Mean = 0.059 ± 0.0003

3.2 Activity of *Tagetes minuta* essential oils on selected fungal pathogens
Tagetes minuta essential oils had potent antifungal activity against all the test fungal species namely: *F. oxysporum*, *F. solani*, *A. flavus*, *A. parasiticus,* and *A. niger* (Figure 1). There was variation in antifungal activity of crude essential oils among the fungal species with four of the tested species - *F. oxysporum*, *A. flavus*, *A. parasiticus* and *A. niger* - falling within the category of extremely sensitive (diameters of the inhibition zone larger than 20mm) while *F. solani* was ranked as very sensitive (diameters of the inhibition zone between 15 and 19mm) after five days of incubation. These findings concurred with the reported antifungal activity of essential oils, aqueous and organic extracts of *T. minuta* against a wide range of economically important phytopathogens [44-46].
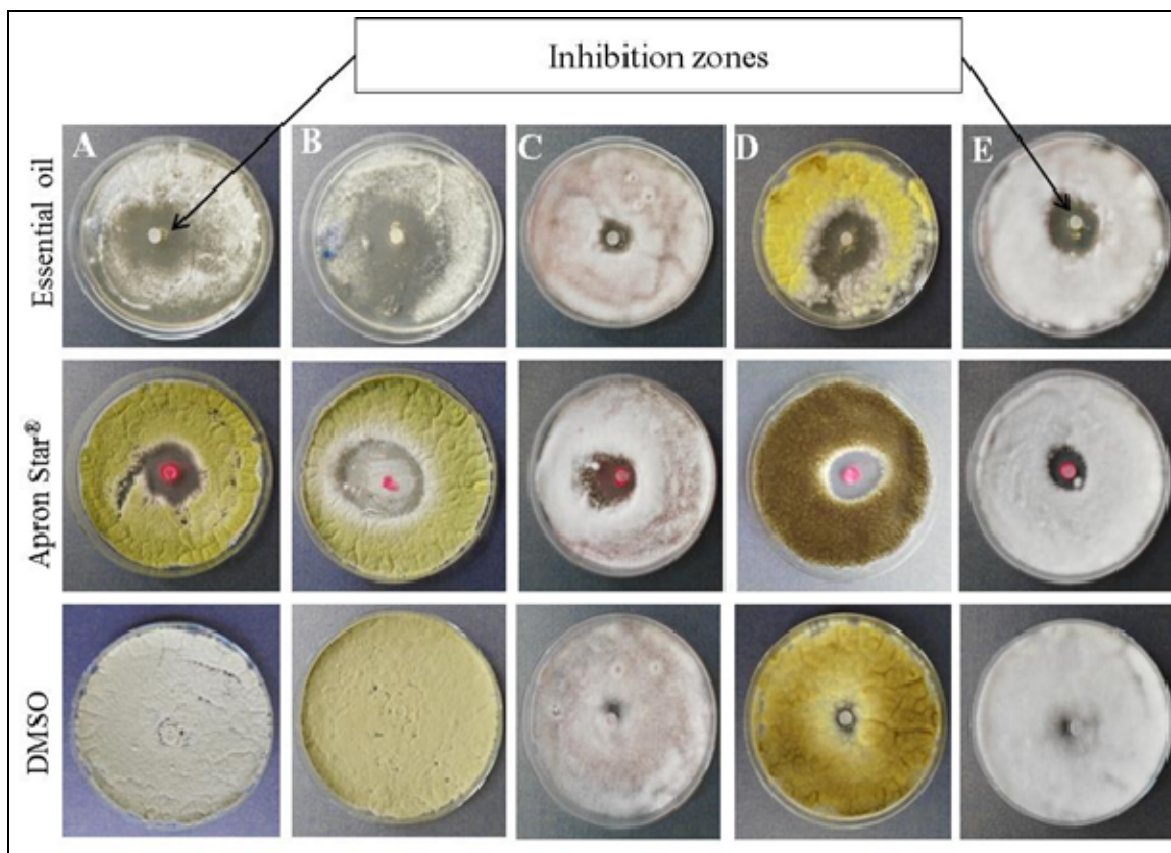

Fig 1: Growth inhibition of *A. flavus* (A), *A. parasiticus* (B), *F. solani* (C), *A. niger* (D) and *F. oxysporum* (E) by essential oils of *Tagetes minuta*, Apron Star® (+ Control) and DMSO (- Control) after 10 days.

In addition to the observed growth inhibition, the essential oils caused a delay in the growth of *A. niger, A. parasiticus* and *A. flavus*. The growth rate of the three fungi after treatment with the EOs was slower when compared to their negative controls. These observations may be attributed to the ability of the essential oils to change the general growth and morphogenesis of fungi as previously reported [47, 48]. In the two studies, microscopic analysis revealed that the essential oils of *Cymbopogon nardus* (L.) Rendle and *Citrus sinensis* (L.) Osbeck produced deleterious ultrastructural modifications in *A. niger* including loss of cytoplasm in fungal hyphae, progressive thinning of hyphal diameter and walls, budding and flattening of hyphal tip, plasma membrane disruption and mitochondrial structure disorganization [47, 48]. The modifications in cytological structures were attributed to the interference of the essential oils with the enzymes responsible for wall synthesis [47]. Considerable changes in mycelial growth and sporulation were also reported in a study that investigated the effects of essential oils of *Foeniculum vulgare* Mill, *Zingiber officinale* Roscoe, *Mentha piperita* L., and *Thymus vulgaris* L. on *A. flavus* and *A. parasiticus*. The authors noted significant inhibitory effects on mycelial growth of *A. parasiticus* by the four EOs with *T. vulgaris* showing the best inhibitory effect on mycelial growth and sporulation of *A. parasiticus* and *A. flavus* [21].

Inhibition of growth of *A. flavus* and *A. parasiticus* by the EOs occurred even in regions of the cultures that were not in direct contact with the oil-impregnated paper discs. This was an indication that the *T. minuta* EOs were able to inhibit the growth

of the two fungi in regions far removed from the primary application sites. The ability of essential oils to cause growth inhibition in fungi without any direct contact has been attributed to the vapour-phase antifungal activities of the essential oil components. There is evidence in literature on the fungistatic and fungicidal activity of volatile vapours of essential oils through inhibition of mycelial growth and sporulation [49, 50]. In one such study, volatile vapours of clove essential oils exhibited potent fungistatic activity whereas direct application of essential oils showed fungicidal activity against dermatophytic fungi *Epidermophyton floccosum*, *Microsporum audouinii*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* [51]. The activity of the EOs against the aforementioned dermatophytes occurred through inhibition of mycelial growth and spore germination.

The results obtained in the current study revealed that on the 5th day, the highest antifungal activity of the EOs was against *F. oxysporum* and *A. niger*, both of which had mean inhibition zone diameters of 28.67mm (Table 2). The activity of the oils against these two fungal species was significantly greater (p ≤ 0.05) than from the standard fungicide Apron Star® within the same period. In general, the activity of the oils against the five test fungal species on the 10th day was significantly lower (p ≤ 0.05) compared to the activity observed on the 5th day. The percentage decrease in the inhibition zones within the two periods was 29%, 21%, 18%, 41% and 14% for *F. solani*, *F. oxysporum, A. flavus, A. niger* and *A. parasiticus*, respectively.

Table 2: Inhibition zones (mm) of *Tagetes minuta* essential oils and Apron star® on five fungi after five and ten days of incubation

Fungi	*Tagetes minuta* essential oil		Apron Star (+Control)		Mean
	5th day	10th day	5th day	10th day	
F. solani	19.83±0.60[ef]	14.17±0.83[h]	21.83±0.44[c-e]	19.17±0.44[fg]	18.75
F. oxysporum	28.67±0.88[a]	22.67±0.88[b-d]	20.33±1.20[d-f]	19.00±0.58[fg]	22.67
A. flavus	23.17±0.44[bc]	19.00±1.15[fg]	22.17±1.30[b-e]	23.33±0.88[bc]	21.92
A. niger	28.67±1.20[a]	16.83±0.73[f]	24.50±0.76[b]	24.00±0.58[bc]	23.50
A. parasiticus	28.23±0.39[a]	24.17±0.60[bc]	28.33±0.88[a]	30.50±1.04[a]	27.81

Values are mean ± standard error of the mean for bioassay conducted in triplicates. Means followed by the same letter(s) are not significantly different (Multivariate analysis, Fisher's protected LSD at p ≤ 0.05).

While *F. oxysporum* was the most susceptible to the growth inhibition effects of the essential oils among the test fungi, *F. solani* was the least sensitive with mean inhibition zones of 19.83 and 14.17mm after 5 and 10 days of incubation,

respectively. A comparison of the antifungal activity of the essential oils and the standard fungicide on the five test fungi after 5 and 10 days of incubation are shown in Figure 2A and Figure 2B, respectively.
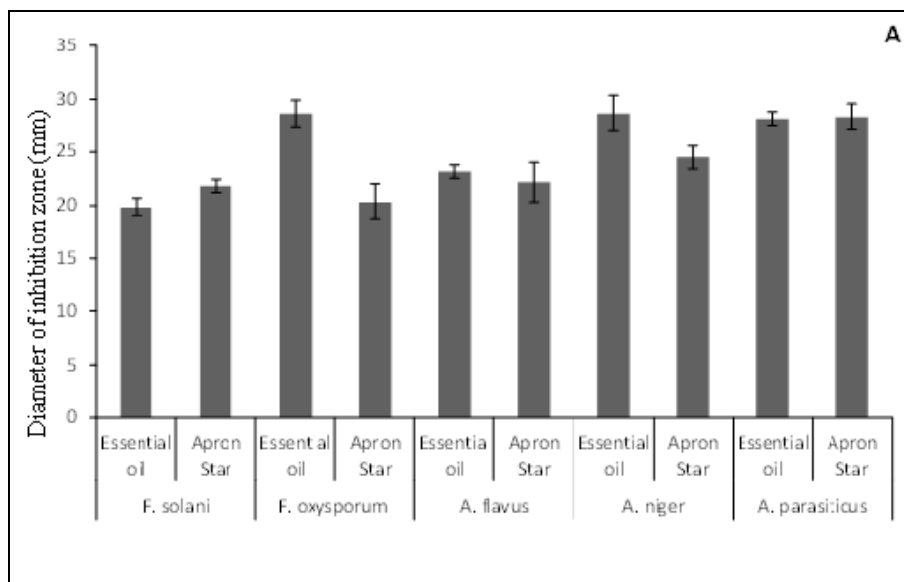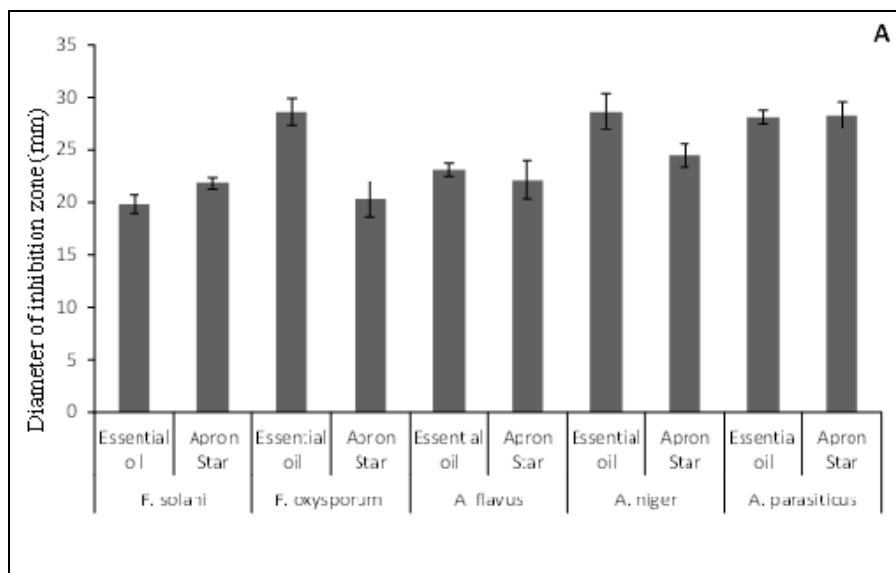
Fig 2: Growth inhibition of the test fungi by *Tagetes minuta* essential oils and Apron Star® after 5 days (A) and 10 days of incubation (B). Error bars represent standard error of mean.

3.3 Activity of different concentrations of *Tagetes minuta* essential oils on the test fungi

There was a concentration-dependent inhibitory activity of *T. minuta* essential oils against the test fungi. In general, increase in concentration of EOs resulted in an increase in the diameters of the inhibition zones and thus, dose-dependent response was clear in most of the test fungi (Table 3). It has previously been reported that antifungal activity and indeed most biological activities of essential oils are dependent on their concentrations [21, 50]. For instance, concentration-dependent antifungal activity of *T. minuta* EOs against *Aspergillus niger* and *Candidas albicans* have been reported [44, 45]. In another study, essential oils of *Azadirachta indica* A. Juss., were found to be efficient in reducing the colony diameter and dry weight in *Fusarium oxysporum* f. sp. *medicagenis* and *F. subglutinans* isolates in a concentration-dependent manner [52]. The concentration-dependent antifungal activities of EOs observed were attributed to changes in concentration of the active principles as the overall EOs concentration changed. Several studies have reported dose-dependent biological activities of various pure compounds isolated from essential oils [53]. Thus, as the concentration of the EOs increases, so does the active component present in the oil and hence an increase in the activity of the EOs in this case represented by larger inhibition zones.

The study nevertheless, found some exceptional instances where essential oils that were more concentrated produced smaller inhibition zones in comparison to the less concentrated/more diluted EOs. Instances where more concentrated EOs produce smaller inhibition zones have been reported in literature and are attributed to the fact that dilute EOs diffused more easily in the agar medium (i.e. aqueous environment) than the undiluted or less dilute EOs [54]. Moreover, polymerization of the undiluted essential oils may also lead to reduced antimicrobial activity and hence smaller inhibition zones [44]. It can therefore be concluded that antifungal activity of essential oils is a function of concentration, solubility, diffusion and evaporation rates of the essential oils in the media among other factors [29, 55]. Commonly used solvents and solubilizing agents such as dimethyl sulphoxide, ethanol and Tween 20/80 also have significant effects on antimicrobial susceptibility testing results [29, 56]. The estimated minimum inhibition concentrations (MICs) based on the activity of different concentrations of *T. minuta* essential oils on the test fungi using the disc diffusion methods were in the range of 6.0 – 24.0 mg/mL.

Table 3: Inhibition zones (mm) on test fungi by different concentrations of *Tagetes minuta* essential oils after five days of incubation

Fungi	Essential oil concentration (mg/mL)*10^2							DMSO (-Control)
	3.8	1.9	0.95	0.48	0.24	0.12	0.06	
F. solani	13.50±0.76	14.83±2.49	9.50±10.76	8.50±0.50	≤8.00	≤8.00	≤8.00	0.00
F. oxysporum	20.83±0.93	18.17±0.60	19.33±0.17	19.33±0.44	12.50±0.87	10.17±0.44	≤8.00	0.00
A. flavus	18.83±0.44	19.50±0.76	13.83±1.09	8.67±0.44	≤8.00	≤8.00	≤8.00	0.00
A. niger	19.33±0.40	19.17±0.60	21.33±0.60	13.83±1.09	12.83±0.73	9.17±0.43	≤8.00	0.00
A. parasiticus	22.83±0.44	20.17±0.93	14.67±0.93	10.33±0.60	≤8.00	≤8.00	≤8.00	0.00

Values are means ± standard error of the mean for bioassays conducted in triplicates.

3.4 Dose-response effects of the essential oils on the growth of fungi

A linear regression model based on essential oils concentration (mg/mL) as the input independent variable is shown in Figure 4. There was a significant correlation ($p \leq 0.05$) between the tested concentrations of the EOs and mean inhibition zones in *A. parasiticus* ($R^2 = 0.87$; $p = 0.002$), *A. flavus* ($R^2 = 0.72$; $p = 0.009$) and *F. solani* ($R^2 = 0.73$; $p = 0.014$). An exception to this pattern was however observed in *A. niger* ($R^2 = 0.36$; $p = 0.092$) and *F. oxysporum* ($R^2 = 0.35$; $p = 0.096$) where no significant correlation ($p \geq 0.05$) was found between the tested essential oil concentrations and the mean inhibition zones. Lack of a linear correlation between the essential oil concentrations and growth inhibition in *A. niger* and *F. oxysporum* could be attributed to the aforementioned factors that result in instances where essential oil that is more concentrated produce smaller inhibition zones in comparison to the less concentrated/more diluted essential oil.
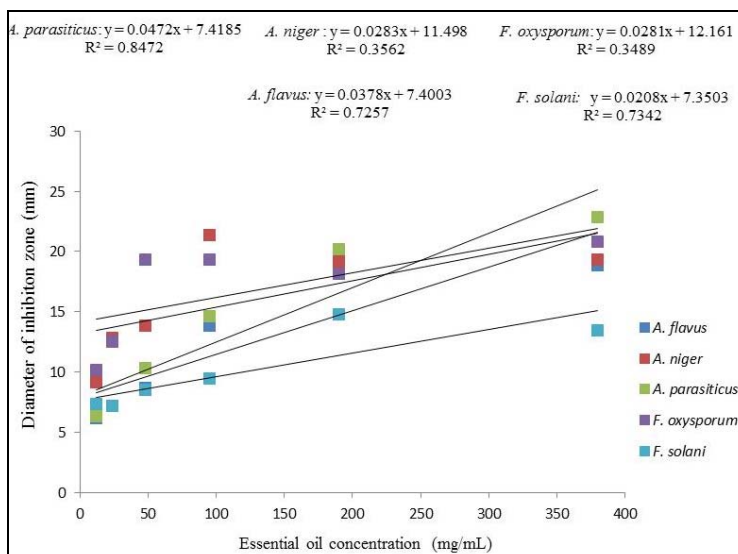
A. parasiticus: $y = 0.0472x + 7.4185$
$R^2 = 0.8472$

A. niger : $y = 0.0283x + 11.498$
$R^2 = 0.3562$

F. oxysporum: $y = 0.0281x + 12.161$
$R^2 = 0.3489$

A. flavus: $y = 0.0378x + 7.4003$
$R^2 = 0.7257$

F. solani: $y = 0.0208x + 7.3503$
$R^2 = 0.7342$

Fig 3: A dose-response curve of inhibition zone diameters (mm) against the concentration of *T. minuta* essential oil (mg/mL) for the five test fungi.

3.5 Minimum inhibitory concentrations and minimum fungicidal concentrations

The results of the minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of *T. minuta* essential oils on the test fungi are shown in Table 4. The MICs of the essential oils of *T. minuta* on the studied fungi ranged between 24 to 95 mg/mL. The MICs values obtained through the tube dilution method were different from those estimated using the disc diffusion method i.e. 6 – 24 mg/mL. The variation in the results from the two methods is indicative of the difficulty of comparing antimicrobial susceptibility testing results that have been conducted using different methodologies, especially in regard to the assessment of minimal inhibition concentrations [57, 58]. In the current study, differences in the solubility, diffusion and evaporation rates of the essential oils in agar and broth media could be among the paramount factors that contributed to the observed differences between the two methods [59]. The minimum fungicidal concentrations (MFCs) were in the range of 24 -190 mg/mL. *Aspergillus niger* and *A. parasiticus* had MICs values of 24 and 48 mg/mL, respectively. The MFCs values for the two fungi were similarly 24 and 48 mg/mL, respectively. For these two fungi therefore, the lowest concentrations of EO at which no visible growth were observed (judged by the naked eye) in the broth tubes were also the lowest concentrations at which the EOs produced biocidal effects on the fungi.

Table 4: Minimum inhibitory concentrations and minimum fungicidal concentrations of the essential oils of *Tagetes minuta* on five fungal species

Tube/Plate No.	EOs concentration (mg/ml)*10^2	Growth of fungi in PDB tubes					Growth of fungi on PDA plates				
		FS	FO	AF	AN	AP	FS	FO	AF	AN	AP
1	Crude EOs	-	-	-	-	-	-	-	-	-	-
2	3.8	-	-	-	-	-	-	-	-	-	-
3	1.9	-	-	-	-	-	-	-	-	-	-
4	0.95	-	-	-	-	-	+	-	-	-	-
5	0.48	+	-	-	-	-	+	-	-	-	-
6	0.24	+	-	-	-	+	+	+	+	-	+
7	0.12	+	+	+	+	+	+	+	+	+	+
8	0.06	+	+	+	+	+	+	+	+	+	+
9	0.03	+	+	+	+	+	+	+	+	+	+
10	0.015	+	+	+	+	+	+	+	+	+	+
11	- ve control	+	+	+	+	+	+	+	+	+	+
13	+ve control	-	-	-	-	-	-	-	-	-	-

[+] Growth and [-] No growth of the fungi; FS - *F. solani*, FS - *F. oxysporum*, AF - *A. flavus*, AN - *A. niger* and AP - *A. parasiticus*

PDA - Potato dextrose agar; PDB - Potato dextrose broth; Tube 1- Fungal inoculum and undiluted crude essential oils; Tube 2 to 10 - PDB, fungal inocula and essential oils of different concentrations ; Tube 11 - Fungal inoculum and PDB (-ve Control); Tube 13 - Fungal inoculum, PDB and the standard fungicide (+ve Control).

3.6 Chemical composition of the essential oils of *Tagetes minuta*

Gas chromatograph - mass spectrometry analyses of *T. minuta* essential oils identified 20 compounds corresponding to 96% of the total oil. Table 5 shows the compounds identified from the essential oil along with the retention indices and concentration percentage. The essential oils comprised of hydrocarbons mainly terpenes; a mixture of monoterpenes (70%) and sesquiterpenes (30%). The most abundant compounds identified in the EOs all of which were monoterpenes were: (E)-tagetone (11.8%), dihydrotagetone (10.7%), allo-ocimene (8.8%), (Z)-β-ocimene (7.0%), limonene (5.3%) and (Z)-tagetone (5.0%). The least abundant monoterpenes were α-phellandrene and (Z)-β-ocimene each representing 3.6% of the total essential oil composition. Sesquiterpenes concentration on the other hand ranged from 3.4 - 3.5% and comprised of (E)-caryophyllene (3.5%), elixene (3.5%), bicyclogermacrene (3.5%), α-humulene (3.5%), silphiperfol-6-ene (3.4%) and one unknown sesquiterpene compound (3.5%). One of the most significant

findings of this study was the identification of two compounds - elixene and silphiperfol-6-ene - two sesquiterpenes that had not been previously reported in essential oils of *T. minuta*. This is therefore the first reported occurrence of the two components in the essential oils of *T. minuta*, representing an important addition to the database of the chemical profile of *T. minuta* essential oils.

The findings of the study were in agreement with numerous earlier studies that have cited monoterpenes and sesquiterpenes as the major subclasses of terpenes that are commonly found in essential oils of *T. minuta*. For example, EOs from *T. minuta* sampled in three regions in Kenya namely; Kasarani (Nairobi County), Bungoma (Bungoma County) and Bondo (Siaya County) were found to contain mostly monoterpenes and sesquiterpenes hydrocarbons in the ratios of 3:7, 2:8 and 4:6, respectively [39]. In another study, 119 compounds were identified in *T. minuta* EOs constituting monoterpenes (47.9%), sesquiterpenes (30.3%), hemiterpenes (15.1%), diterpenes (1.7%) and unknown compounds (5.0%) [38]. Similarly, the two subclasses of terpenes i.e. monoterpenes and sesquiterpenes were reported to constitute the biggest percentage of *T. minuta*

EOs extracted from plants sampled in Zambia, Namibia and Yemen [60-62].

Great variations have been reported in literature on the chemical profiles of essential oils of *T. minuta* sampled from a wide range of geographical locations. Some studies have reported as few as six compounds [61, 63] while others have reported over a hundred compounds in *T. minuta* essential oils [38]. Variations in the chemical composition of *T. minuta* essential oils with respect to harvesting location [63, 64], growth stage [61, 65], plant parts [65, 66], climatic conditions under which the plant is grown [67] and the different chemotypes [68] have all been reported. Despite the reported differences in the actual number of compounds identified in *T. minuta* EOs in different studies, majority of the compounds that have been cited as common constituents of *T. minuta* EOs are terpenes belonging to any of the four subclasses namely; monoterpenes, sesquiterpenes, hemiterpenes and diterpenes [38, 39]. This suggests that the chemical profiles of *T. minuta* EOs from different regions are chemically comparable despite the great variation in the actual number of components present.

Table 5: GC-MS identified constituents in the essential oils of *Tagetes minuta*

No[a]	Rt (min)	Compound Name	Molecular formula	M+g/mol	RI[b]	Base peak	Major peaks	Concentration (%)±SE
1	10.6	Sabinene	$C_{10}H_{16}$	136.2	956	93	136, 121, 91, 79, 77, 69	3.8±0.12
2	11.2	α-Phellandrene	$C_{10}H_{16}$	136.2	983	93	136, 121, 94, 91, 80, 77	3.6±0.06
3	11.7	Limonene	$C_{10}H_{16}$	136.2	1005	68	136, 121, 107, 93, 79, 53	5.3±0.02
4	11.8	(Z)-β- Ocimene	$C_{10}H_{16}$	136.2	1015	93	121, 105, 79, 67, 53, 41	7.0±0.03
5	12.0	(E)-β- Ocimene	$C_{10}H_{16}$	136.2	1027	93	136, 121, 105, 79, 67, 53	3.6±0.01
6	12.1	Dihydrotagetone	$C_{10}H_{18}O$	154.2	1033	85	154, 139, 119, 97, 69, 57, 41	10.7±0.25
7	12.8	Unknown	-	-	1073	71	136, 121, 105, 93, 79, 67	5.4±0.01
8	13.4	Allo-Ocimene	$C_{10}H_{16}$	136.2	1110	121	136, 121, 105, 93, 79, 67	8.8±0.01
9	13.5	Unknown	-	-	1116	91	134, 119, 105, 91, 67, 55	3.9±0.01
10	13.6	Unknown	-	-	1122	43	139, 121, 111, 93, 81, 69	3.7±0.01
11	13.7	(Z)-Tagetone	$C_{10}H_{16}O$	152.2	1126	95	152, 134, 109, 93, 67, 43	5.0±0.06
12	13.8	(E)-Tagetone	$C_{10}H_{16}O$	152.2	1134	95	152, 134, 109, 93, 67, 43	11.8±0.02
13	15.0	(Z)- Ocimenone	$C_{10}H_{14}O$	150.2	1205	93	135, 121, 79, 69, 41	3.8±0.01
14	15.1	(E)- Ocimenone	$C_{10}H_{14}O$	150.2	1213	69	137, 119, 109, 91, 41	3.9±0.01
15	15.6	unknown	-	-	1247	97	126, 83, 70, 55, 43	3.5±0.04
16	16.6	Elixene	$C_{15}H_{24}$	204.35	1310	121	204, 189, 162, 136, 107, 93	3.5±0.02
17	16.7	Silphiperfol-6-ene	$C_{15}H_{24}$	204.35	1319	133	107, 93, 79, 55, 41	3.4±0.01
18	17.7	(E)-Caryophyllene	$C_{15}H_{24}$	204.35	1393	93	133, 107, 105, 91, 79, 77	3.5±0.01
19	18.2	α-Humulene	$C_{15}H_{24}$	204.35	1400	93	147, 121, 107, 80, 67	3.5±0.03
20	18.7	Bicyclogermacrene	$C_{15}H_{24}$	204.35	1401	93	204, 189, 161, 121, 107, 79	3.5±0.03

[b]RI = Retention Index; SE = Standard error of the mean; M+g/mol = Molecular weight

4. Conclusion

Increasing environmental and health concerns arising from the continued use of synthetic antifungal compounds has generated a strong interest in the development of alternative natural fungicides that are effective and environment-friendly. Phytochemicals are recognized as some of the most promising compounds in the development of new biopesticides for the management of plant pathogens. This study revealed promising antifungal activity of the essential oils of *Tagetes minuta* against the test fungi. Four out of five studied fungi were categorized as extremely sensitive with one being ranked very sensitive when subjected to crude essential oil. The study demonstrated *in vitro* antifungal activity of *T. minuta* essential oils against the test fungi and thus shows the potential use of these oils as an alternative to synthetic fungicides. To achieve this however, further studies are required to evaluate the applicability of these oils in the management of plant pathogenic fungi under field conditions.

6. Competing interests

The authors declare that they have no competing interests.

7. References

1. Larrañaga P, Díaz-Dellavalle P, Cabrera A, Alem D, Leoni C, Souza AL *et al*. Activity of naturally derived antimicrobial peptides against filamentous fungi relevant for agriculture. Sustainable Agriculture Research. 2012;

1(2):211-221.

2. Pimentel D. Pest management in agriculture. In D. Pimentel (ed.), Techniques for reducing pesticide use: environmental and economic benefits. John Wiley & Sons, Chichester, 1997, 1-11.

3. Oerke EC. Crop losses to pests. Journal of Agricultural Science. 2006; 144(1):31-43.

4. Von Broembsen SL. Invasion of natural ecosystems by plant pathogens. In H.S. Mooney and J.A. Drake (Eds.), Biological invasion: a global perspective. John Wiley & Sons, Chichester. 1989, 77-80.

5. Fletcher J, Luster D, Bostock R, Burans J, Caldwell K, Gottwald T, McDaniel L, Royer M, Smith K. Emerging Infectious Plant Diseases, In: Scheld WM (Ed.), Emerging Infections 9. ASM Press, Washington DC. 2010, 338-340.

6. Bau HJ, Cheng Y, Yu TA, Yang JS, Yeh SD. Broad-spectrum resistance to different geographic strains of Papaya ringspot virus in coat protein gene transgenic papaya. Phytopathology. 2003; 93(1):112-120.

7. Aiyere T. Molecular characterization of isolates of *Colletotrichum gloeosporioides* from tropical crops and virulence factors of the fungus. MSc. Thesis, London University, United Kingdom. 2004.

8. Knogge W. Fungal infection of plants. The Plant Cell. 1996; 8(10):1711-1722.

9. Carris LM, Little C, Stiles C. Introduction to Fungi, 2012 [http://www.apsnet.org/edcenter/intropp/pathogengroups/pages/introfungi.aspx].Visited on 20 May. 2016.

10. Agrios GN. Plant Pathology (5th Ed.). Elsevier Academic Press, San Diego, California. 2005, 383-390.

11. Patel N, Desai P, Patel N, Jha A, Gautam HK. Agronanotechnology for plant fungal disease management: A review. International Journal of Current Microbiology and Applied Sciences. 2014; 3(10):71-84.

12. Horrigan L, Lawrence RS, Walker P. How sustainable agriculture can address the environmental and human health harms of industrial agriculture. Environmental Health Perspectives. 2002; 110(5):445-456.

13. Al-Samarrai G, Singh H, Syarhabil M. Evaluating eco-friendly botanicals (natural plant extracts) as alternatives to synthetic fungicides. Annals of Agricultural and Environmental Medicine. 2012; 19(4):673-676.

14. Abdallah EM. Plants: An alternative source for antimicrobials. Journal of Applied Pharmaceutical Science. 2011; 1(6):16-20.

15. Katooli N, Maghsodlo R, Razavi SE. Evaluation of eucalyptus essential oil against some plant pathogenic fungi. Journal of Plant Breeding and Crop Science. 2011; 3(2):41-43.

16. Prabuseenivasan S, Jayakumar M, Ignacimuthu S. *In vitro* antibacterial activity of some plant essential oils. BMC Complementary and Alternative Medicine. 2006; 6:39.

17. Burt S. Essential oils: their antimicrobial properties and potential application in foods: a review. International Journal of Food Microbiology. 2004; 94(3): 223-253.

18. Hussain AI, Anwar F, Sherazi ST, Przybylski R. Chemical composition, antioxidant and antimicrobial activities of basil (*Ocimum basilicum*) essential oils depends on seasonal variations. Food Chemistry. 2008; 108(3):986-995.

19. Chi PT. Chemical composition, antioxidant and antimicrobial activities of essential oils extracted from citrus varieties in Vietnam. MSc Thesis, Vietnam National University, Vietnam. 2013.

20. Centeno S, Calvo MA, Adelantado C. Figueroa S. Antifungal activity of *Rosmarinus officinalis* and *Thymus vulgaris* against *Aspergillus flavus* and *A. ochraceus*. Pakistan Journal of Biological Sciences. 2010; 13(9):452-455.

21. Silva FC, Chalfoun CM, Siqueira VM, Botelho DM, Lima N, Batista LR. Evaluation of antifungal activity of essential oils against potentially mycotoxigenic *Aspergillus flavus* and *Aspergillus parasiticus*. Brazilian Journal of Pharmacognosy. 2012; 22(5):1002-1010.

22. Pooja A, Arun N, Maninder K. Screening of plant essential oils for antifungal activity against *Malassezia furfur*, International Journal of Pharmacy and Pharmaceutical Sciences. 2013; 5(2):37-39.

23. Clevenger JF. Apparatus for the determination of volatile oil. Journal of the American Pharmaceutical Association. 1928; 17(4):345-349.

24. Golfakhrabadi F, Khanavi M, Ostad SN, Saeidnia S, Vatandoost H, Abai MR *et al*. Biological activities and composition of *Ferulago carduchorum* essential oil. Journal of Arthropod-Borne Diseases. 2015; 9(1):104-115.

25. Pitt JI, Hocking AD. Fungi and Food Spoilage. Blackie Academic & Professional, London. 1997, 19-143.

26. Klich M. Identification of common Aspergillus species. ASM Press, Washington, DC, 2002, 116.

27. Leslie JF, Summerell BA. The *Fusarium* laboratory manual. Blackwell Publishing Ltd, Ames, Iowa. 2006, 274.

28. Souza EL, Lima EO, Freire KR, Sousa CP. Inhibitory action of some essential oils and phytochemicals on the growth of various moulds isolated from foods. Brazilian Archives of Biology and Technology. 2005; 48(2):245-250.

29. Gomez-Lopez A, Aberkane A, Petrikkou E, Mellado E, Rodriguez-Tudela JL, Cuenca-Estrella M. Analysis of the influence of Tween concentration, inoculum size, assay medium, and reading time on susceptibility testing of *Aspergillus* spp. Journal of Clinical Microbiology. 2005; 43(3):1251-1255.

30. Celikel N, Kavas G. Antimicrobial properties of some essential oils against some pathogenic microorganisms. Czech Journal of Food Sciences. 2008; 26(3):174-181.

31. Babu AJ, Sundari AR, Indumathi J, Srujan RV, Sravanthi M. Study on the antimicrobial activity and minimum inhibitory concentration of essential oils of spices. Veterinary World. 2011; 4(7):311-316.

32. Clara C, Matasyoh JC, Wagara IN, Nakuvuma J. Antifungal activity of *Monanthotaxis littoralis* essential oil against mycotoxigenic fungi isolated from maize. International Journal of Microbiology Research and Reviews. 2013; 2(6):103-109.

33. Caburian AB. Osi MO. Characterization and evaluation of antimicrobial activity of the essential oil from the leaves of *Piper betle* L. International Scientific Research Journal. 2010; 2(1):2-13.

34. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard. NCCLS document M38-A. Wayne, Pennsylvania. 2002.

35. Quinto EA, Santos MG. Microbiology. In B.Q. Guevara (Ed). A guidebook to plant screening: phytochemical and biological, UST Publishing House, Manila. 2005, 67-87.

36. Van Den Dool H, Kratz PD. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. Journal of Chromatography. 1963; 11:463-471.

37. Wanzala W. Ethnobotanicals for management of the brown ear tick *Rhipicephalus appendiculatus* in western Kenya.

PhD Thesis, Wageningen University, The Netherlands. 2009.

38. Wanzala W, Ogoma SB. Chemical composition and mosquito repellency of essential oil of *Tagetes minuta* from the Southern slopes of Mount Elgon in Western Kenya. Journal of Essential Oil Bearing Plants. 2013; 16(2):216-232.

39. Makang'a, OB. Composition and repellency of essential oils of *Tagetes minuta* from different zones in Kenya against brown ear tick (*Rhipicephalus appendiculatus*). MSc. Thesis, Kenyatta University, Kenya. 2012.

40. Karimian P, Kavoosi G, Amirghofran Z. Anti-oxidative and anti-inflammatory effects of *Tagetes minuta* essential oil in activated macrophages. Asian Pacific Journal of Tropical Biomedicine. 2014; 4(3):219-227.

41. Meshkatalsadat MH, Ghomi JS, Moharrmipour S, Nasserui M. Chemical characterization of volatile components of *Tagetes minuta* L. cultivated in South West of Iran by nano scale injection. Digest Journal of Nanomaterials and Biostructures. 2010; 5(1):101-106.

42. Lakušić BS, Ristić MS, Slavkovska VN, Stojanović DL, Lakušić DV. Variations in essential oil yields and compositions of *Salvia officinalis* (Lamiaceae) at different developmental stages. Botanica Serbica. 2013; 37(2):127-139.

43. Lee YL, Phebe D. Production of essential oil in plants: ontogeny, secretory structures and seasonal variations. Pertanika Journal of Scholarly Research Review. 2016; 2(1):1-10.

44. Muyima NY, Nziweni S, Mabinya LV. Antimicrobial and antioxidative activities of *Tagetes minuta, Lippia javanica* and *Foeniculum vulgare* essential oils from the Eastern Cape Province of South Africa. Journal of Essential Oil Bearing Plants. 2004; 7(1):68-78.

45. Shirazi MT, Gholami H, Kavoosi G, Rowshan V, Tafsiry A. Chemical composition, antioxidant, antimicrobial and cytotoxic activities of *Tagetes minuta* and *Ocimum basilicum* essential oils. Food Science and Nutrition. 2014; 2(2):146-155.

46. Uzabakiriho A, Sinayobye OJ, Habimana S. Antibacterial effects *of Tagetes minuta* plant extracts against *Escherichia coli* and *Staphylococcus aureus* in Rwanda. International Journal of Novel Research in Life Sciences. 2015; 2(2):44-48.

47. De Billerbeck VG, Roques CG, Bessière JM, Fonvieille JL, Dargent R. Effects of *Cymbopogon nardus* (L.) W. Watson essential oil on the growth and morphogenesis of *Aspergillus niger*. Canadian Journal of Microbiology. 2001; 47(1):9-17.

48. Sharma N, Tripathi A. Effects of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis of *Aspergillus niger* (L.) Van Tieghem. Microbiological Research. 2008; 163(3):337-344.

49. Chee HY, Lee EH. Antifungal activity and inhibitory modes of volatile vapours of essential oils. Mycobiology. 2004; 32(2):102-104.

50. Aguiar RW, Ootani MA, Ascencio SD, Ferreira TS, Santos MM, Santos GR. Fumigant antifungal activity of *Corymbia citriodora* and *Cymbopogon nardus* essential oils and citronellal against three fungal species. Scientific World Journal. 2014; 1-8.

51. Chee HY, Lee MH. Antifungal activity of clove essential oil and its volatile vapour against dermatophytic fungi. Mycobiology. 2007; 35(4):241-243.

52. Geraldo MR, Arroteia CC, Kemmelmeir C. The effects of

53. Tabanca N, Ma G, Pasco DS, Bedir E, Kirimer N, Baser K *et al*. Effect of essential oils and isolated compounds from *Pimpinella* species on NF-$_k$B: a target for anti-inflammatory therapy. Phytotherapy Research. 2007; 21(8):741-745.

54. Hood JR, Wilkinson JM, Cavanagh HM. Evaluation of common antibacterial screening methods utilized in essential oil research. Journal of Essential Oil Research. 2003; 15(6):428-433.

55. Lalitha MK. Manual on Antimicrobial Susceptibility Testing, 2005. [http://www.ijmm.org/documents/Antimicrobial.doc]. Visited on 20 May, 2016,

56. Jović J, Mihajilov-Krstev T, Žabar A, Stojanović-Radić Z. Influence of solvent on antimicrobial activity of *Carlinae radix* essential oil and decoct. Biologica Nyssana. 2012; 3(2):61-67.

57. Hili P, Evans CS, Veness RG. Antimicrobial action of essential oils: the effect of dimethylsulphoxide on the activity of cinnamon oil. Letters in Applied Microbiology. 1997; 24(4):269-275.

58. Suhr KI, Nielsen PV. Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. Journal of Applied Microbiology. 2003; 94(4):665-674.

59. Saidana D, Mahjoub MA, Boussaada O, Chriaa J, Cheraif I, Daami M *et al*. Chemical composition and antimicrobial activity of volatile compounds of *Tamarix boveana* (Tamaricaceae). Microbiological Research. 2008; 163(4):445-455.

60. Chisowa EH, Hall DR, Farman DI. Chemical composition of the essential oil of *Tagetes minuta* L. from Zambia. Journal of Essential Oil Research. 1998; 10(2):183-184.

61. Moghaddam M, Omidbiagi R, Sefidkon F. Changes in content and chemical composition of *Tagetes minuta* oil at various harvest times. Journal of Essential Oil Research. 2007; 19(1):18-20.

62. Ali NA, Sharopov FS, Al-kaf AG, Hill GM, Arnold N, Al-Sokaria SS *et al*. Composition of essential oil from *Tagetes minuta* and its cytotoxic, antioxidant and antimicrobial activities. Natural Product Communications. 2014; 9(2):265-268.

63. Chamorro ER, Ballerini G, Sequeira AF, Velasco GA, Zalazar MF. Chemical composition of essential oil from *Tagetes minuta* L. leaves and flowers. Journal of the Argentine Chemical Society. 2008; 96(1-2):80-86.

64. Senatore F, Napolitano F, Mohamed M, Harris PJ, Mnkeni PN, Henderson J. Antibacterial activity of *Tagetes minuta* L. (Asteraceae) essential oil with different chemical composition. Flavour and Fragrance Journal. 2004; 19(6):574-578.

65. Chalchat JC, Garry RP, Muhayimana A. Essential oil of *Tagetes* from Rwanda and France: chemical composition according to harvesting, location, growth stage and part of plant extracted. Journal of Essential Oil Research. 1995; 7(4):375-386.

66. Weaver DK, Wells CD, Dankel FV, Bertsch W, Sing SE, Sirharan S. Insecticidal activity of floral, foliar and root extracts of *Tagetes minuta* (Asterales: Asteraceae) against adult Mexican bean weevils (Coleoptera: Bruchidae). Journal of Economic Entomology. 1994; 87:1718-1725.

67. Mohamed M, Harris P, Henderson J, Senatore F,

neem [*Azadirachta indica* A. Juss (meliaceae)] oil on *Fusarium oxysporum* f. sp. *medicagenis* and *Fusarium subglutinans* and the production of fusaric acid toxin. Advances in Bioscience and Biotechnology. 2010; 1:1-6.

Napolitano F. Effect of drought stress on the yield and composition of volatile oils of drought-tolerant and non-drought-tolerant clones of *Tagetes minuta*. Planta Medica. 2002; 68(5):472-474.

68. Gil A, Ghersa M, Leicach S. Essential oil yield and composition of *Tagetes minuta* accessions from Argentina. Biochemical Systematics and Ecology. 2000; 28(3):261-274.