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Assessing essential oil components as plant-based preservatives against fungi that deteriorate herbal raw materials

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ABSTRACT

This study assesses the antifungal efficacy of 14 essential oil (EO) components and some of their combinations as inhibitory to the growth of the aflatoxigenic fungus *Aspergillus flavus* $LHPA_9$ isolated from biodeteriorating *Asparagus racemosus* herbal raw materials. The aim was to determine whether they could be recommended as plant-based preservatives for enhancement of the shelf life of herbal raw materials. Thymol, eugenol, menthol, and their combinations were highly efficacious as their minimum inhibitory concentration (MIC) for inhibition of fungal growth as well as aflatoxin B_1 secretion was less than 1.0 μl ml^{-1}. Geranyl acetate, linalool, β-asarone, 1, 8-cineol, and E-citral were moderately antifungal as their MIC ranged between 1.0 and 5.0 μl ml^{-1}. During antioxidant activity 2, 2-diphenyl-1-picrylhydrazyl assay, thymol, eugenol, and β-caryophyllene showed strong radical scavenging activity, whereas β-asarone and p-cymene showed moderate activity. Some combinations of EO components showed synergism while others exhibited an additive or antagonism effect in their activity. The findings point to a recommendation that EO components are good alternatives to synthetic preservatives to prevent deterioration of stored herbal raw materials by fungal and aflatoxin contamination and free-radical oxidation.

1. Introduction

Post-harvest fungal deterioration of herbal raw materials used as pharmaceuticals and nutraceuticals is a serious concern, particularly in tropical and subtropical countries where the hot and humid environment and the unscientific practices of harvesting, collection, transportation, and storage favor the proliferation of molds and mycotoxin accumulation on herbal raw materials (Dubey et al., 2008). Deterioration of stored herbal raw materials by molds and mycotoxins has been reported to affect the chemical composition of herbal raw materials, thereby decreasing their medicinal value (Efuntoye, 1996). In addition to microbial deterioration, the shelf life of herbal raw materials is also shortened because of the generation of reactive oxygen species. To enhance the shelf life of raw materials there needs to be control measures that can effectively inhibit fungal growth, mycotoxin secretions, as well as generation of free radicals. Although application of synthetic chemicals as preservatives has greatly contributed to enhancing shelf life of food items by preventing microbial contamination, such measures are not appropriate for treatment of herbal raw materials destined for drug preparation due to their side effects on human health (Brul and Coote, 1999). Moreover, some of the synthetic preservatives used as antioxidants have been reported to enhance the mycotoxin-secreting potency of associated fungi (Kumar et al., 2007; Dorea, 2008; Prakash et al., 2010). In view of such undesirable side effects, pharmaceutical firms are in search of alternative preservatives for treatment of herbal raw materials during their post-harvest processing (Ames, 1983; Madhavi and Salunkhe, 1995).

The best way to avoid and eradicate fungal and mycotoxin-caused biodeterioration of herbal raw materials and protect the material from free-radical oxidation is the use of antifungal and antioxidant compounds of botanical origin, which are generally considered safe for human health and the environment (Burt, 2004). Some essential-oil-(EO) based preservatives, such as "DMC Base Natural" (50% EO from rosemary, sage, and citrus and 50% glycerol) and carvone, a monoterpene of the essential oil of *Carum carvi*, have already been commercially available and used on large scale (Prakash et al., 2012). Plant essential oils are mixtures of different major and minor components and their biological activity is generally determined by their major components or synergism/

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antagonism among different components (Burt, 2004; Bakkali et al., 2008; Prakash et al., 2010; Mishra et al., 2012; Shukla et al., 2012). The chemical profile of a particular essential oil differs because of varied seasons, geographical conditions, plant parts used, time of harvesting, and method of isolation (Rakotonirainy and Lavedrine, 2005; Prakash et al., 2012). Essential oil components, being pure compounds, are more stable than essential oil mixtures, and their biological activity will always be the same against microorganisms. Moreover, essential oil compounds can be chemically and biotechnologically synthesized (Carvalho and Fonseca, 2006) and sometimes they are more economical to use than their parent essential oil. Because of their long history of safe use in drug formulation and as flavoring agents, most of these compounds are expected to be safe without posing any significant risk to human health, particularly at low doses (Rakotonirainy and Lavedrine, 2005; Smith et al., 2005). Hence, it would be more judicious to assess the efficacy of pure essential oil components than the essential oil mixtures. Although some essential oil components have previously been tested for their fungitoxicity against some phytopathogenic and wood-decaying fungi (Mahmoud, 1994; Voda et al., 2003; Lee, 2007; Chang et al., 2008; Yen and Chang, 2008; Kordali et al., 2008; Combrinck et al., 2011; Prakash et al., 2012), only a few reports are available on EO components and their combinations regarding fungitoxic and aflatoxin inhibitory activity against fungi deteriorating herbal raw materials. Among different mycotoxins, aflatoxin B_1 produced as secondary metabolites by *Aspergillus flavus*, *Aspergillus parasiticus*, and a few strains of *Aspergillus nomius* (Bhatnagar and Garcia, 2001) attracts more attention due to its severe toxic effects on the liver, kidney, nervous system, muscular system, digestive system, genital system, and respiratory organs (Efuntoye, 1996; WHO, 2007; Dubey et al., 2008; Prakash et al., 2011; Mishra et al., 2012). Furthermore, it is classified as a group 1 human carcinogen by the International Agency for Research on Cancer (WHO-IARC, 1993; Razzaghi-Abyaneh et al., 2008; Shukla et al., 2008; Prakash et al., 2010; Tian et al., 2011).

In the present study, 14 EO components and their combinations (1:1 v/v) were assessed for their ability to control growth and aflatoxin secretion in the aflatoxigenic strain *A. flavus* $LHPA_9$ procured from biodeteriorating raw material of *Asparagus racemosus*; their free-radical-scavenging activity was assessed as well. The aim was to determine the possibility of their recommendation as plant-based preservatives to enhance the shelf life of herbal raw materials during post-harvest processing.

2. Materials and methods

2.1. Chemicals and equipment

Chemicals and solvents, viz., chloroform, methanol, toluene, isoamylalcohol, tween-20, tween-80, sodium sulfate, and constituents of media preparations, were purchased (high-purity, analytical-grade) from Sisco Research Laboratories Pvt. Ltd., Mumbai (India). Purchase of 2, 2-diphenyl-1-picrylhydrazyl radical was from Hi-Media Laboratories Pvt. Ltd., Mumbai. Essential oil components were purchased from Ozone International, Mumbai (purity of the EO components was >99%); and the Centrifuge and UV transiluminator were from Zenith Engineers, Agra (India).

2.2. Test organism

2.2.1. Selection of toxigenic A. flavus

The aflatoxin B_1 producing strain *A. flavus* $LHPA_9$ was selected as the test fungus for this study. This strain has previously been isolated from raw material of *A. racemosus* during mycological analysis of some herbal drug raw materials in our laboratory (Mishra et al., 2012).

2.2.2. Preparation of fungal spore suspension

For spore suspensions, 20 ml of 0.1% tween 80 (in sterile distilled water) was poured onto a 7-day-old culture of toxigenic *A. flavus* grown on PDA medium. The fungal spores were scraped off with a sterilized spatula and filtered with sterilized muslin cloth. The spore density (10^6 spores ml^{-1}) was counted with the help of a hemocytometer.

2.3. Antifungal assay of essential oil components and their combinations

2.3.1. Determination of MIC and MLC of essential oil components and their combinations

The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of the EO components and their combinations against the toxigenic strain *A. flavus* $LHPA_9$ were recorded according to the method described by Shukla et al. (2008). Requisite amounts (1–70 μl) of the 15 EO components and mixtures (1:1 v/v) of four components, viz., thymol, eugenol, menthol, and geranyl acetate, were dissolved separately in 0.5 ml acetone and 9.5 ml SMKY broth medium (sucrose, 200 g; potassium nitrate, 0.3 g; magnesium sulfate, 0.5 g; and yeast extract, 7 g; in 1 l of distilled water) in culture tubes so as to procure different concentrations (0.1 μl ml^{-1} to 7 μl ml^{-1}). Culture tubes containing only acetone (0.5 ml) and SMKY medium (9.5 ml) served as controls. The tubes were inoculated with 10 μl of spore suspension (approx.10^6 spores ml^{-1}) of the toxigenic strain *A. flavus* $LHPA_9$ and incubated at 28 ± 2 °C for 10 days.

The lowest concentration of EO component/their combination that did not permit any visible growth of the toxigenic strain of the fungus in culture tubes during 10 days of incubation was considered as the MIC. After 10 days, the inoculated fungal cells from the tubes of treated sets, where there was no visible growth, were subcultured onto fresh Czapek Dox agar (CDA) plates, and revival of their growth was observed. The lowest concentration of EO component/their combination at which no fungal growth reversal occurred on the fresh CDA plates during 10 days of incubation was considered as the minimum lethal concentration (MLC).

2.4. Antiaflatoxigenic assay of essential oil components and their combinations

2.4.1. Determination of minimum aflatoxin B_1 inhibitory concentration (MAIC) of EO components and their combinations

The minimum aflatoxin B_1 inhibitory concentrations (MAIC) of essential oil components or their combinations were recorded against the toxigenic strain *A. flavus* $LHPA_9$ following Shukla et al. (2008). Requisite amounts (1–70 μl) of the 15 EO components or their combinations (1:1 v/v) were dissolved separately in 0.5 ml acetone and 24.5 ml SMKY broth medium in 100-ml conical flasks so as to procure different concentrations (0.1 μl ml^{-1} to 7 μl ml^{-1}). Conical flasks containing only acetone (0.5 ml) and SMKY medium (24.5 ml) served as controls. Thereafter flasks were inoculated with 50 μl of spore suspension (approx.10^6 spores ml^{-1}) of the toxigenic strain *A. flavus* $LHPA_9$ and incubated at 28 ± 2 °C for 10 days. After incubation, the contents of each flask were filtered (Whatman No. 1) and the biomass of filtered mycelium was dried at 80 °C (for 12 h) and weighed. The filtrates were extracted with 20 ml of chloroform in a separating funnel. The extracts were evaporated to dryness on a water bath at 70 °C and the residues were re-dissolved in 1 ml of chloroform. The detection of AFB_1 produced was done by the TLC technique. Fifty microliters of the chloroform extracts were spotted on TLC plates along with the standard of AFB_1, and the TLC plates were run in toluene: isoamyl alcohol: methanol (90 : 32 : 2 v/v/v). The developed plates were air-

dried and AFB_1 was observed in an ultraviolet fluorescence analysis cabinet at the wavelength of 360 nm. Initial detection of the intensity of AFB_1 in the control set was made on a visual basis by comparing the color and intensity of fluorescence of the samples and standard spot. The quantitative estimation of AFB_1 was done by a spectrophotometer. For quantitative estimation, spots of AFB_1 on TLC plate were scraped out and dissolved in 5 ml of cold methanol, shaken, and centrifuged at 3000 rpm for 5 min. The optical densities of supernatants were recorded at the wavelength of 360 nm and the amount of AFB_1 present in the sample was calculated according to the following formula (Soares and Rodriguez-Amaya, 1989):

$$AFB_1 \text{ content } (\mu g/l) = \frac{D \times M}{E \times l} \times 1000$$

Where D = absorbance, M = molecular weight (312), E = molar extinction coefficient of AFB_1 (21,800), and l = path length (1 cm).

2.5. Antioxidant activity of essential oil components and their mixtures

2.5.1. DPPH radical scavenging screening assay on TLC

Five microliters (1:10 dilution in methanol) of each EO component or combination was spotted separately on TLC plates and developed in solvent system ethyl acetate and methanol (1:1). Thereafter plates were sprayed with 0.2% 2, 2-diphenyl-picrylhydrazyl (DPPH) solution in methanol and left for 30 min at room temperature (27 ± 2 °C). The conversion of purple-colored DPPH into a pale yellow color at developed spots was considered as indicating positive antioxidant activity of EO components (Tepe et al., 2005).

2.5.2. DPPH radical scavenging assay of EO components and their combinations by spectrophotometer

Free-radical-scavenging activity of EO components and their combinations was measured through the bleaching of the purple-colored methanol solution of DPPH into a pale yellow color due to conversion of DPPH (a stable free radical) into 1,1-diphenyl-2-picrylhydrazine (Burtis and Bucar, 2000). This spectrophotometric assay uses the stable radical DPPH as a reagent. Various concentrations of the EO components/combinations were added to 5 ml of a 0.004% methanol solution of DPPH. After 30 min incubation at room temperature (27 ± 2 °C), the absorbance was read against a blank at 517 nm. Inhibition of free radicals by DPPH as a percentage (IC %) was calculated following Tepe et al. (2005) in the following way:

$$IC\% = \left(A_{blank} - A_{sample}/A_{blank}\right) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound.

2.6. Statistical analysis

Antifungal, antiaflatoxigenic, and antioxidant experiments were performed in triplicate and data were analyzed with mean ± standard error, which was calculated using SPSS software. The statistical level of significance was fixed at $P < 0.05$.

3. Results

Among 14 essential oil components, thymol, eugenol, and menthol were highly efficacious against the toxigenic strain *A. flavus* $LHPA_9$ as their minimum inhibitory concentrations (MIC) were recorded at 0.2 μl ml^{-1}, 0.5 μl ml^{-1}, and 0.9 μl ml^{-1}, respectively. Thymol and eugenol were found to be fungicidal, as the inhibited fungal spores could not revive their growth on fresh medium. Thymol showed a fungicidal nature even at its MIC (0.2 μl ml^{-1}) while the MLC of eugenol was 0.6 μl ml^{-1}, slightly higher than its MIC (0.5 μl ml^{-1}). However, menthol showed only a fungistatic nature as its MLC was not recorded even at 7.0 μl ml^{-1} (Table 1). The EO components such as geranyl acetate, linalool, β-asarone, 1, 8-cineol, and E-citral showed moderate antifungal activity as shown by their comparatively higher range of MIC (1.6 μl ml^{-1} to 5.0 μl ml^{-1}) and were found to be fungistatic in nature, having MLC levels of more than 7.0 μl ml^{-1} (Table 1). The remaining EO components showed poor antifungal activity, with MICs measured at more than 7.0 μl ml^{-1}. Thymol, eugenol, and menthol also showed high aflatoxin inhibitory efficacy. The aflatoxin inhibitory concentration of each essential oil component except menthol was lower than their fungal mycelial growth inhibitory concentration. Minimum concentrations for AFB_1 inhibition (MAIC) of thymol (0.1 μl ml^{-1}) and eugenol (0.3 μl ml^{-1}) were lower than their MIC values for growth inhibition of toxigenic strain *A. flavus* $LHPA_9$. However, the MAIC of menthol was

Table 1
Antifungal and antiaflatoxigenic efficacy of essential oil components/combinations (1:1 v/v) against the toxigenic strain *A. flavus* $LHPA_9$.

S. No.	Essential oil components and their mixture	Minimum inhibitory concentration (μl ml^{-1})	Minimum lethal concentration (μl ml^{-1})	Minimum aflatoxin B_1 inhibitory concentration (μl ml^{-1})
1	Thymol	0.2	0.2	0.1
2	Eugenol	0.5	0.6	0.3
3	Menthol	0.9	>7.0	0.9
4	Geranyl acetate	1.6	>7.0	1.2
5	Linalool	2.4	>7.0	2.0
6	β-asarone	3.0	>7.0	1.2
7	1, 8-Cineol	4.0	>7.0	3.0
8	E-citral	5.0	>7.0	4.0
9	β-caryophyllene	>7.0	>7.0	6.0
10	α-pinene	>7.0	>7.0	>7.0
11	Carvone	>7.0	>7.0	>7.0
12	P-cymene	>7.0	>7.0	>7.0
13	Carvacrol	>7.0	>7.0	>7.0
14	Ocimene	>7.0	>7.0	>7.0
15	Thymol + Eugenol	0.3	0.4	0.2
16	Thymol + Menthol	0.3	0.5	0.2
17	Eugenol + Menthol	0.6	1.2	0.4
18	Thymol + Eugenol + Menthol	0.3	0.5	0.2
19	Menthol + Geranyl acetate	0.7	1.6	0.6

0.9 μl ml^{-1}, which was exactly same as its MIC. Essential oil components, viz., geranyl acetate, linalool, β-asarone, 1, 8-cineol, and E-citral, showed moderate aflatoxin inhibitory activity as their MAIC ranged between 1.2 μl ml^{-1} and 4.0 μl ml^{-1} (Table 1). Out of six remaining EO components, β-caryophyllene, which could not inhibit fungal growth even at 7.0 μl ml^{-1}, showed aflatoxin inhibitory activity at 6.0 μl ml^{-1} and five EO components that could not inhibit fungal growth—α-pinene, carvone, p-cymene, carvacrol, and ocimene—also showed poor aflatoxin inhibitory activity as their MIC and MAIC were recorded at more than 7.0 μl ml^{-1}.

Among five different combinations of EO components three combinations, viz., thymol + eugenol, thymol + menthol, and thymol + eugenol + menthol, showed strong antifungal activity, exhibiting complete inhibition of growth of toxigenic strain *A. flavus* LHPA$_9$ at their MIC of 0.3 μl ml^{-1}. The combinations eugenol + menthol and menthol + geranyl acetate were moderately antifungal, exhibiting their MIC at 0.6 μl ml^{-1} and 0.7 μl ml^{-1}, respectively. However, menthol + geranyl acetate had a MIC value significantly lower than the components' individual MIC values, showing a strong synergistic effect, and the rest of the combinations, namely thymol + menthol, thymol + eugenol + menthol (both combinations showed MIC values slightly lower than the intermediate value of individual components), thymol + eugenol, and menthol + eugenol, showed MIC values in between the MIC value of individual components, showing an additive effect. All the combinations tested were fungistatic at their MIC but turned fungicidal at higher concentrations (Table 1).

Regarding aflatoxin inhibitory activity, all the five combinations of EO components checked aflatoxin B$_1$ production by the toxigenic strain *A. flavus* LHPA$_9$ at concentrations lower than their MIC for fungal growth inhibition (Table 1). The combination menthol + geranyl acetate also exhibited synergism in inhibition of aflatoxin. However, other combinations showed an additive effect in inhibition of aflatoxin similar to fungal growth inhibition.

Eleven EO components exhibited positive antioxidant activity. The strongest free radical scavenging activity was shown by eugenol (IC$_{50}$ = 0.0067 μl ml^{-1}), followed by β-caryophyllene (IC$_{50}$ = 0.091 μl ml^{-1}), thymol (IC$_{50}$ = 0.109 μl ml^{-1}), and β-asarone (IC$_{50}$ = 0.53 μl ml^{-1}) (Table 2). P-cymene, geranyl acetate, and 1, 8-cineol showed moderate radical scavenging capacity, with IC$_{50}$ values of 19.6 μl ml^{-1}, 38.0 μl ml^{-1}, and 80.0 μl ml^{-1}, respectively. The rest of the EO components showed poor radical scavenging activity, as their IC$_{50}$ values were more than 100 μl ml^{-1}.

Among combinations of EO components, thymol + eugenol (IC$_{50}$ = 0.0075), menthol + eugenol (IC$_{50}$ = 0.0114), thymol + menthol + eugenol (IC$_{50}$ = 0.0131), β-caryophyllene + β-asarone (IC$_{50}$ = 0.082), and thymol + menthol (IC$_{50}$ = 0.238) showed strong radical scavenging activity, while the combinations geranyl acetate + p-cymene (IC$_{50}$ = 16.8), geranyl acetate + 1, 8-cineol (IC$_{50}$ = 86.5), and α-pinene + ocimene (IC$_{50}$ = 88) showed moderate activity (Table 3).

The combination α-pinene + ocimene showed marked synergism as a radical scavenger as individually these components showed poor antioxidant activity (IC$_{50}$ = >200) whereas the IC$_{50}$ value of the combination (IC$_{50}$ = 88) was lower than that of individual IC$_{50}$ values. Similarly, the combinations β-caryophyllene + β-asarone and geranyl acetate + p-cymene also showed synergistic radical scavenging activity. The combination thymol + eugenol showed an additive effect for radical scavenging activity as the IC$_{50}$ value of the mixture was in between the individual IC$_{50}$ values of the components. However, the combination geranyl acetate + 1, 8-cineol showed antagonism for free radical scavenging capacity as the IC$_{50}$ value of the mixture was more than that of the IC$_{50}$ values of individual components (Table 3).

Table 2
Free radical scavenging capacity of some essential oil components in term of their IC$_{50}$ values.

S. No.	EO components	IC$_{50}$ values μl ml^{-1}
1	Thymol	0.109
2	Eugenol	0.0067
3	Menthol	>200
4	Geranyl acetate	38.0
5	Linalool	>200
6	1,8-Cineol	80.0
7	E-citral	176.0
8	β-asarone	0.53
9	α-pinene	>200
10	β-caryophyllene	0.091
11	Carvone	180.0
12	P-cymene	19.6
13	Carvacrol	136.0
14	Ocimene	>200

4. Discussion

In this investigation some EO components and their combinations were assessed against the growth and aflatoxin secretion by of the aflatoxigenic strain *A. flavus* LHPA$_9$ and also their free radical scavenging activity. Individual EO components, as opposed to the essential oils themselves, have some significant advantages over essential oils. The components because they are pure compounds, and chemically stable, have less chance of alterations to their biological properties than the essential oils themselves. This is in contrast to the essential oils, which, being mixtures of a large number of compounds, can have a chemical profile that varies over time (Prakash et al., 2012). The changing chemical profile of essential oils does not provide stable and uniform biological properties. Another confounding factor is that the biological activity of the essential oil may be due to its major components or due to interaction between its different components.

Among the 14 EO individual components and the five combinations tested for fungitoxicity and AFB$_1$ inhibitory capacity, thymol and eugenol were found to have strong efficacy as fungal growth inhibitors and aflatoxin suppressors. The free hydroxyl group attached to the aromatic ring may be responsible for their strong activity. Moreover, thymol is a monoterpene phenol derivative and eugenol is a phenylpropene, and their lipophilic nature would damage membrane integrity of the toxigenic strain of the *A. flavus*, as has been reported (Lambert et al., 2001; Chao et al., 2005). Moreover, thymol and eugenol have been reported to inhibit the H$^+$ ATPase system, leading to intracellular acidification and cell death (Ahmad et al., 2010). Thymol and eugenol have long been used as natural preservatives to prevent fungal contamination of cheese (Vazquez et al., 2001; Venturini et al., 2002; Valero et al., 2006; Undeger et al., 2009) and also as medicines and tonics due to their antiseptic, antispasmodic, and carminative properties (Didry et al., 1994). Hence, these EO components may be exempted from toxicity data requirements and may be recommended for

Table 3
Free radical scavenging capacity of some combinations of EO components in 1:1v/v combination in terms of their IC$_{50}$ values.

S. No.	Mixtures of EO components	IC$_{50}$ values (μl ml^{-1})
1	Thymol + Eugenol	0.0075
2	Eugenol + Menthol	0.0114
3	Thymol + Menthol	0.238
4	Thymol + Eugenol + Menthol	0.0131
5	β-caryophyllene + β-asarone	0.082
6	Geranyl acetate + 1,8-Cineol	86.5
7	Geranyl acetate + P-cymene	16.8
8	α-pinene + Ocimene	88.0

treatment of herbal raw materials based on the findings of the present investigation.

Menthol, the cyclic terpene alcohol, was found to be the third most active EO component in terms of being fungitoxic and AFB_1 inhibitory. Menthol may also be recommended as a safe preservative for herbal raw materials as it is widely used in flavoring for toothpaste, oral hygiene products, and chewing gum (Galeotti et al., 2002).

Five EO components, viz., geranyl acetate, linalool, β-asarone, 1, 8-cineol, and E-citral, showed moderate antifungal and antiaflatoxigenic activity in our study. The lower fungitoxic efficacy of these components compared to thymol, eugenol, and menthol may be explained by their simple aliphatic chain form and cyclic form, and the absence of hydroxyl group in the cyclic form.

Most of the EO components inhibited aflatoxin production at concentrations lower than their fungitoxic concentration, suggesting two different modes of action on test fungi for aflatoxin inhibition and growth inhibition, as has been reported in the case of some essential oils and their major components (Shukla et al., 2009; Prakash et al., 2010). The components may be causing inhibition of carbohydrate catabolism in *A. flavus* by acting on some key enzymes, thereby reducing the ability to produce aflatoxins, as has been reported by Tian et al. (2011).

Eugenol was also found to be the strongest radical scavenger among all tested components in this study, followed by β-caryophyllene, thymol, and β-asarone. A perusal of the literature shows that it is a better radical scavenger than butylated hydroxytoluene (BHT), a synthetic antioxidant whose radical scavenging activity has been studied by Prakash et al. (2010). The phenol derivative nature of thymol and eugenol may be responsible for their strong free radical scavenging nature, as has been reported by Rice-Evans et al. (1997), Ferguson (2001), Wang et al. (2003), Lima et al. (2006), and Pripdeevech and Chukeatirote (2010). However, the reason for the strong radical scavenging activity of β-caryophyllene and β-asarone has not been fully understood and further investigation is warranted to correlate their radical scavenging activity with their chemical structure.

Scardavi (1966) has defined three types of interactions between drugs: additive, synergistic, and antagonistic. Additive interaction means the effect of two combined drugs have an effect that is equal to the sum of their individual actions. Antagonistic interaction means that the effect of two combined drugs is actually less than the effect of the two drugs taken independently. Synergistic interaction means that the effect of two drugs taken together is greater than their separate effects at the same doses. When synergism occurs it is possible to use a lower combined dose of each drug to achieve the desired effect, that would be possible if they were used separately (Tallarida, 2001). In the present investigation some of the EO components, such as menthol and geranyl acetate, when combined together showed synergistic antifungal and antiaflatoxigenic effects; i.e., the activity of the combination was significantly greater than the sum of the activities of their individual components. Similarly, the combinations α-pinene + ocimene, β-caryophyllene + β-asarone, and geranyl acetate + p-cymene showed marked synergism as radical scavengers; however, individually both of the components were ineffective or less effective as free radical scavengers. On the other hand, other combinations showed additive or antagonistic antifungal, antiaflatoxigenic, and antioxidant activity. The synergistic/additive/antagonistic activity may be because of reactions between the components, causing changes in their chemical structures. The synergism/additive action may be due to positive interaction between components. However, in case of antagonistic action, one component may be masking the efficacy of another one. Different plant-based preservatives used in the food industry are mixtures of different essential oils and their components (Burt, 2004). Based on the findings of this investigation, care should be taken when mixing different components during formulation of plant-based preservatives.

The EO components/combinations showing antioxidant, antifungal, and aflatoxin-inhibitory efficacy would be ideal plant-based preservatives against free radical oxidation as well as microbial contamination of herbal raw materials. Based on these findings, the efficacious EO components or combinations of components should be subjected to large-scale in-vivo trials so as to see if they should be recommend as plant-based preservatives to control deterioration of herbal raw materials by fungi, aflatoxins, and free radicals. Being volatile in nature, the oil components may be used as fumigants during post-harvest processing of herbal raw materials. The MIC of two synthetic preservatives, viz., Nystatin and Wettasul-80, tested earlier in our laboratory (Prakash et al., 2010) against the toxigenic strain of *A. flavus* was found at 1.85 μl ml^{-1} and 2.78 mg ml^{-1}, respectively, which was more than that for thymol, eugenol, menthol, and geranyl acetate and the five different combinations of EO components tested in the present study. Hence, because of better efficacy, comparatively low doses of EO components/combinations would be required during their formulation as plant-based preservatives. The components are major and common ingredients of many essential oils of higher plants. Hence, these may be safely and economically used as plant-based preservatives. However, future experiments on LD_{50} values on mammalian systems are required before formulation of the components as plant-based preservatives.

5. Conclusion

In view of their strong antifungal and aflatoxin inhibitory efficacy and antioxidant potency, the EO components thymol, eugenol, and menthol, and some possible combinations may be recommended in formulations of plant-based preservatives against microbial and aflatoxin deterioration of herbal raw materials as well as in enhancement of their shelf.

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