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Safety assessment of *Zanthoxylum alatum* Roxb. essential oil, its antifungal, antiaflatoxin, antioxidant activity and efficacy as antimicrobial in preservation of *Piper nigrum* L. fruits

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ABSTRACT

The investigation deals with antifungal, antiaflatoxin and antioxidant efficacy of *Zanthoxylum alatum* Roxb. essential oil (EO), its two major constituents and their comparison with five commonly used organic acid preservatives. The chemical profile of EO, characterized through GC and GC–MS analysis, revealed linalool (56.10%) and methyl cinnamate (19.73%) as major components. The EO, linalool and methyl cinnamate completely inhibited the growth of a toxigenic strain of *A. flavus* (LHP-10) as well as aflatoxin B_1 secretion at different concentrations. Methyl cinnamate was found to be more efficacious than EO, linalool and five organic acid preservatives, showing antifungal and antiaflatoxigenic efficacy at a low concentration (0.6 μl/ml) and the nature of its toxicity was fungicidal. However, EO showed strong antioxidant activity with an IC_{50} value at 5.6 μl/ml. Moreover, EO was found to have negligible mammalian toxicity as its LD_{50} value, determined through oral administration on mice, was calculated to be 6124 μl/kg body weight during safety profile assessment. During in vivo investigation on fruit systems, the *Zanthoxylum* EO, when tested as fumigant, provided 66.27% and 86.33% protection respectively at 1.25 μl/ml and 2.5 μl/ml against fungi infesting *Piper nigrum* L. fruits demonstrating its practical efficacy as a plant based antimicrobial for post harvest application.

1. Introduction

In spite of the use of all available means of food protection, pest infestation and spoilage of foods is still a major problem in different parts of the world. Protection of agricultural food products is an urgent task for researchers/scientists/agri-industries to satisfy the hunger of the world's >6 billion population (Anonymous, 2010). Food commodities are frequently colonized by toxigenic strains of molds during post harvest storage and processing with accompanying losses in quality, quantity, nutrient content and monetary value (Bata and Lasztity, 1999). The Food and Agriculture Organization (FAO) has estimated that 25% of the world's food crops are affected by mycotoxins such as aflatoxins, ochratoxins, trichothecenes, zearalenone and fumonisins. Nearly 10% of agricultural food commodities in the world are severely spoiled by molds to the extent that they are not fit for consumption by humans and livestock (Set and Erkmen, 2010). Of the mycotoxins, aflatoxins, which are polyketide secondary metabolites of *Aspergillus flavus*, *A. parasiticus* and *A. nomius* have been intensively studied because of their carcinogenic potential, and their thermostability and bioaccumulative nature (Dubey et al., 2008; Galvano et al., 2005). Because of their toxic properties aflatoxins have been listed by the International Agency for Research on Cancer (IARC) as Group 1 human carcinogens (International Agency for Research on Cancer, 1993). Hence aflatoxin contamination represents a serious threat to food contamination in both developing and developed countries (Wagacha and Muthomi, 2008).

Aspergilli, are cosmopolitan fungi able to contaminate food commodities at any stage of food processing and storage. Consumption of aflatoxin contaminated foods causes aflatoxicosis in humans and animals which sometimes may be fatal because of the acute toxicity and carcinogenicity of aflatoxins (Wagacha and Muthomi, 2008). In plants, aflatoxins inhibit seed germination, seedling growth, root elongation, and adversely affect synthesis of photosynthetic pigments (chlorophyll, carotenoids), proteins, nucleic acids and different enzymes (Shukla et al., 2009). In addition, AFB_1-8,9-exoepoxides, the metabolic product of aflatoxin B_1 are reported to be responsible for the stimulation of lipid peroxidation which leads to enhancement of highly reactive molecules (ROS) in the affected organs (Choy, 1993). The oxidation of lipid during post harvest processing of food commodities causes rancidity, thereby reducing shelf life.

Hence, an ideal preservative for enhancement of shelf life of food grains and spices should be efficacious as an antimicrobial, inhibit aflatoxin formation as well as being an antioxidant. Different synthetic

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antimicrobials/antioxidants have been used to control storage molds in food commodities. However undesirable side effects of some of these compounds on mammalian systems and the environment, the development of resistance in microorganisms and residual toxicity have necessitated the search for safer alternatives (Brul and Coote, 1999). In addition some synthetic preservatives also generate molecules of toxic reactive oxygen species (ROS) causing oxidative stress by damaging proteins, lipids and DNA, and some are also reported to stimulate aflatoxin biosynthesis (Jayashree and Subramanyam, 2000).

In view of these such negative effects food industries and regulatory agencies are interested in finding natural products exhibiting significant antimicrobial, antiaflatoxin and antioxidant properties. Essential oils of some aromatic plants are recognized additives for food preservation to prolong shelf life and improve the quality of stored food products (Bluma and Etcheverry, 2008; Gutierrez et al., 2009; Holley and Patel, 2005). Some essential oil-based preservatives are already commercially available. 'DMC Base Natural' comprising 50% EO from rosemary, sage and citrus and 50% glycerol and carvone, a monoterpene of the essential oil of *Carum carvi* (Burt, 2004) is widely used as a safe food additive. Many of the commercial plant based essential oils are included on the Generally Recognised As Safe (GRAS) list fully approved by the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) in the USA for addition to food and beverages. Western society is trending towards 'green consumerism,' desiring fewer synthetic food additives and products with a smaller impact on the environment (Burt, 2004).

Zanthoxylum alatum Roxb. (Rutaceae) is a common shrub or a small tree of the Himalayan regions in India. The ethnomedicinal importance of its seeds has been well known for a long time in the Indian medical system as a stomachic, carminative, disinfectant, antiseptic, and in the treatment of fever, dyspepsia, cholera and general debility (Jain et al., 2001; Tiwary et al., 2007).

The aim of the present investigation was to evaluate the fungitoxicant, antioxidant and anti-aflatoxigenic activities of the essential oil of *Z. alatum* and to assess its toxicity to mammalian systems. This would enable its recommendation as a non-toxic plant based food additive for protection of food commodities from both molds and aflatoxin contamination. Its antioxidant activities could enhance the shelf life of food products and prevent lipid peroxidation.

2. Materials and methods

2.1. Chemicals and equipment

All chemicals were procured from HiMedia Laboratories Pty Ltd., Mumbai, Qualigens Fine Chemicals Pty Ltd., Mumbai and Genuine Company Mumbai, India. The major items of equipment used were hydro-distillation apparatus, (Merck Specialities Pvt. Ltd., Mumbai, India), centrifuge, UV transilluminator (Zenith Engineers, Agra, India) and spectrophotometer (Systronics India Ltd., Mumbai, India).

2.2. Mold strains

The toxigenic strain of *A. flavus* (LHP-10) was chosen as the test fungus in the present study. Some other molds (*Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus candidus*, *Aspergillus sydowi*, *Aspergillus terreus*, *Alternaria alternata*, *Curvularia lunata*, *Cladosporium cladosporioides*, *Fusarium nivale*, *Penicillium italicum* and *Trichoderma viride*) were used during fungitoxic spectrum studies. The fungal strains had been isolated earlier in our laboratory during a mycological survey of spices and legume seeds (Prakash et al., 2010; Shukla et al., 2009). Cultures were maintained on Potato Dextrose Agar (PDA) slants at 4 °C.

2.3. Plant material and extraction of the essential oil

Seeds of *Z. alatum* Roxb. were procured from the Varanasi herbal market, India and authenticated by Prof. N. K. Dubey at the Banaras Hindu University (BHU), Varanasi, India with the help of relevant taxonomic literature (Duthie, 1960). A voucher specimen (Rut./Zan-143/2009) has been deposited in the herbarium of the Laboratory of Herbal Pesticides, Department of Botany, BHU, Varanasi. Prior to hydrodistillation, the seeds were thoroughly washed twice with distilled water, crushed with a mortar and pestle and then subjected to hydrodistillation (4 h) in Clevenger's apparatus (Prakash et al., 2010). The EO was separated and collected in a sterilized glass vial. Water traces from the EO were removed by adding anhydrous sodium sulfate. The EO was stored at 4 °C in the dark until use.

2.4. Chemical characterization of the essential oil of Z. alatum

Zanthoxylum EO was subjected to gas chromatography (Perkin Elmer Auto XL GC, MA, USA) equipped with a flame ionization detector. The GC condition were: EQUITY-5 column (60 m × 0.32 mm × 0.25 μm); H_2 was the carrier gas; column head pressure 10 psi; oven temperature program isotherm 2 min at 70 °C, 3 °C/min gradient to 250 °C, isotherm 10 min; injection temperature, 250 °C; detector temperature 280 °C.

GC-MS analysis was performed using a Perkin Elmer Turbomass GC–MS. The GC column was EQUITY-5 (60 m × 0.32 mm × 0.25 μm) fused silica capillary column. The GC conditions were: injection temperature, 250 °C; column temperature, isothermal at 70 °C for 2 min, then programmed to 250 °C at 3 °C/min and held at this temperature for 10 min; ion source temperature, 250 °C. Helium was the carrier gas. The effluent of the GC column was introduced directly into the source of the MS and spectra obtained in the EI mode with 70 ev ionization energy. The sector mass analyzer was set to scan from 40 to 500 amu for 2 s. The identification of individual compounds was based on their retention times relative to those of authentic samples and matching spectral peaks available with Wiley, NIST and NBS mass spectral libraries or with the published data (Adams, 2007).

2.5. Determination of total phenolic content of Zanthoxylum EO

Total phenolic content of the *Zanthoxylum* EO was determined spectrophotometrically using the Folin–Ciocalteu reagent according to the method of Gholivand et al. (2010). A solution (0.1 ml) containing the 1000 μg oil was prepared and mixed with 46 ml of distilled water in a volumetric flask. 1 ml Folin–Ciocalteu reagent was added and the mixture was thoroughly shaken using an electronic shaker and left for 3 min to allow complete reaction. Three milliliters aqueous solution of 2% Na_2CO_3 was then added. The solution was incubated for 4 h at room temperature (25 ± 2 °C). At the end of the incubation period, the absorbance of each mixture was measured at 760 nm. The same procedure was also applied to standard solutions of gallic acid (0–1000 μg/0.1 ml) and a standard curve was obtained.

Total phenolic content of the oil was obtained by comparing the absorbance value of oil at 760 nm with the standard curve and expressed as μg gallic acid equivalent/mg of oil.

$$\text{Absorbance} = 0.0011 \times \text{gallic acid } (\mu g) + 0.13$$

2.6. Fungitoxic evaluation of Zanthoxylum oil, linalool and methyl cinnamate and organic acid preservatives against toxigenic strain of A. flavus

The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the EO of *Z. alatum*, its major constituents linalool and methyl cinnamate and five organic acid preservatives against the toxigenic strain of *A. flavus* (LHP-10) were measured by the broth dilution method of Shukla et al. (2009). Requisite amounts of the EO,

its major constituents and five preservatives (benzoic acid, propionic acid, formic acid, acetic acid and sorbic acid) were dissolved separately in tubes containing 0.5 ml DMSO and 9.5 ml PDB (potato dextrose broth) so as obtain different concentrations (0.25 to 5.0 μl/ml) and inoculated with *A. flavus*, 10^6 spores/ml. PDB tubes containing DMSO (0.5 ml) and 9.5 ml PDB (potato dextrose broth) inoculated with 10^6 spores/ml served as control. Streptomycin (300 mg/L) was added to the medium for controlling bacterial growth in both treatment and control sets. The tubes were incubated at 30 ± 2 °C for one week. The lowest concentration of the essential oil resulting in no growth of the toxigenic strain of *A. flavus* (LHP-10) was taken as the MIC. For determination of minimum fungicidal concentration (MFC) of the essential oil, 500 μl of the medium from tubes showing no fungal growth were reinoculated onto additive-free PDA plates and monitored for fungal growth. The lowest concentration preventing revival of fungal growth was taken as the MFC.

2.7. Z. alatum EO, linalool and methyl cinnamate and organic acid preservatives in suppression of aflatoxin B_1 production by the toxigenic strain of A. flavus

Requisite amounts of *Z. alatum* EO, its major constituents linalool and methyl cinnamate, and the five organic acid preservatives were dissolved separately in 0.5 ml DMSO and added to 24.5 ml SMKY to achieve concentrations ranging from 0.20 to 2.0 μl/ml. Streptomycin (300 mg/L) was added to the medium for controlling bacterial growth. Each medium was then inoculated with *A. flavus* LHP-10 (10^6 spores/ml) and was incubated for ten days at (27 ± 2 °C). After incubation, the contents of each flask were filtered (Whatman No. 1) and the biomass of filtered mycelium was dried at 80 °C (12 h) and weighed. The contents of each flask were filtered and extracted with 20 ml chloroform. The extract was evaporated to dryness in water bath and redissolved in 1 ml chloroform. Fifty microliters of chloroform extract was spotted on TLC plates and developed in toluene:isoamylalcohol:methanol (90:32:2; v/v/v). The plate was air dried and AFB_1 was observed under a UV trans-illuminator (360 nm). The blue spots were removed from the TLC plate, dissolved in methanol (5 ml) and centrifuged at 3000 rpm (5 min). Absorbance of the supernatant was recorded at 360 nm and AFB_1 was calculated following Kumar et al. (2007).

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

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

2.8. Fungitoxic spectrum of Z. alatum EO

The fungitoxic spectrum of *Z. alatum* essential oil was evaluated against twelve common storage molds (*A. flavus*, *A. niger*, *A. fumigatus*, *A. candidus*, *A. sydowi*, *A. terreus*, *Alternaria alternata*, *Curvularia lunata*, *Cladosporium cladosporioides*, *Fusarium nivale*, *Penicillium italicum* and *Trichoderma viride*) using the poisoned food technique (Prakash et al., 2010). Requisite amounts of essential oil were added separately to plates containing 0.5 ml DMSO and 9.5 ml molten PDA then mixed to obtain the final concentrations of 1.25 μl/ml and 2.5 μl/ml. A 5 mm disk from a seven day old colony of each fungus was separately placed upside down on the center of the plates. Plates containing only DMSO (0.5 ml) and 9.5 ml PDA inoculated with the test fungi served as control. The plates of both treatment and control sets were incubated at 30 ± 2 °C for ten days. The percent inhibition of fungal growth was calculated by the following formula:

$$\% \text{ mycelial inhibition} = \frac{dc - dt}{dc} \times 100$$

dc average diameter of fungal colony in control sets
dt average diameter of fungal colony in treatment sets

2.9. Antioxidant activity of Z. alatum EO

2.9.1. Free radical scavenging activity of EO, linalool and methyl cinnamate and organic acid preservatives

Free radical scavenging activity of the *Z. alatum* EO, linalool, methyl cinnamate and organic acid preservatives was measured by recording the extent of bleaching of a DPPH solution from purple to yellow following Prakash et al. (2011). During the DPPH radical assay, DPPH solution is mixed with a substance that has the capacity to donate a hydrogen atom giving rise to the reduced form 1,1 diphenyl-2-picrylhydrazine (non radical). Different concentrations (2.0 to 700 μl/ml) of the essential oil, linalool and methyl cinnamate and organic acid preservatives (100 to 500 μl/ml) were added to 0.004% DPPH solution in methanol (5 ml). After 30 min incubation at room temperature (25 ± 2 °C), the absorbance was measured against a blank at 517 nm using spectrophotometer. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (2.0 to 10 μg/ml) were used as positive controls. Scavenging of DPPH free radical with reduction in absorbance of the sample was taken as a measure of the antioxidant activity. The IC_{50} of the test compounds, which represented the concentration that caused 50% neutralization of DPPH radicals, was calculated from the graph plotting percentage inhibition against concentration.

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

Where, A_{blank} is the absorbance of the control (without test compound), and A_{sample} is the absorbance of the test compound.

2.9.2. β-carotene/linoleic acid assay of EO

The *Z. alatum* EO, BHT and BHA were subjected to a β-carotene/linoleic acid bleaching test as described by Ebrahimabadi et al. (2010) to further ascertain their antioxidant activity. A stock solution of β-carotene and linoleic acid was prepared by dissolving 0.5 mg of β-carotene in 1 ml of chloroform, 25 μl of linoleic acid and 200 μl Tween 40. The chloroform was completely evaporated under vacuum in a rotary evaporator at 40 °C; then 100 ml of distilled water were added and the resulting mixture was vigorously stirred. The *Z. alatum* EO, BHT and BHA were dissolved separately in DMSO (2 g/l) and 350 μl of each solution was added separately to 2.5 ml of the above mixture in test tubes. The test tubes were incubated in a hot water bath at 50 °C for 2 h, together with blanks and BHT and BHA as positive controls. The absorbance of each sample was measured at 470 nm on an ultraviolet spectrophotometer. The test tube with BHT and BHA maintained the yellow color during the incubation period. Antioxidant activities (inhibition percentage, I%) of the oil, BHT and BHA were calculated using the following equation:

$$I\% = \left(A_{\beta\text{–Carotene after 2 h assay}} / A_{\text{initial }\beta\text{–Carotene}}\right) \times 100$$

where $A_{\beta\text{-Carotene after 2 h assay}}$ is the absorbance of β-carotene after 2 h assay remaining in the samples and $A_{\text{initial }\beta\text{-Carotene}}$ is the absorbance of β-carotene at the beginning of the experiments. All tests were carried out in triplicate and inhibition percentages were reported as means ± SD of triplicates.

2.10. Safety profile of the Z. alatum oil

An LD_{50} value represents the lethal dose of a chemical per unit weight which kills 50% of a population of test animals. The safety

limit of the essential oil was determined by acute oral toxicity recording LD_{50} value on mice (*Mus musculus* L.) with an average weight 30 g and age 3 months (Prakash et al., 2011). Mice were procured from the Institute of Medical Sciences, Banaras Hindu University, Varanasi, and were provided normal animal diet. They were kept in the laboratory under controlled environmental conditions (30 ± 2 °C) for a week for proper acclimatization before performing the LD_{50} experiments. A stock solution of Tween-80 and distilled water (1:1) was prepared. Different doses of essential oil (0.05 to 0.5 ml) along with 0.5 ml stock solution were orally administered separately through a syringe with a catheter to each treatment set containing 10 mice. The animals were fed only once with the oil doses. In control sets equal doses of Tween-80 and distilled water (1:1) was given to mice. The mortality of the test animals was observed from 4 to 24 h and LD_{50} of EO was calculated by Probit analysis (Finney, 1971).

2.11. Efficacy of *Z. alatum* essential oil as an antimicrobial in a food system

To determine the in vivo antifungal efficacy of *Z. alatum* EO during storage, *Piper nigrum* fruits were procured from different retail outlets located in adjacent area of Varanasi city, India. The collected samples were stored in sterilized polythene bags to prevent further contamination. Fruit samples of *P. nigrum* were surface-sterilized with 1% solution of sodium hypochlorite and rinsed (3–5 times) with sterile distilled water under aseptic laboratory conditions to ensure the removal of surface mold contaminants. A seven-day-old culture of *A. flavus* LHP-10 was used to inoculate the fruit samples. A spore suspension was prepared in 0.1% Tween-80, and the spore density was adjusted to ~10^6 spores/ml. Fruit samples (1 kg) were placed separately in glass containers (2 L volume) and were inoculated with 2 ml of spore suspension by uniform spraying. The fruits in these containers were fumigated with *Z. alatum*. Requisite amounts of the essential oil were soaked separately onto a cotton swab (2 cm) to obtain 1.25 μl/ml (MIC) and 2.5 μl/ml (MFC) according to the aerial volume of container and placed inside the lid of the glass container. Samples inoculated with *A. flavus* but not treated with the EO served as control sets. After 6 months storage (28 ± 2 °C; 70% RH), the samples of control and treated sets were subjected to mycobiota analysis by the serial dilution method of Aziz et al. (1998) (Kumar et al., 2011). Fungal species were identified by cultural and morphological characteristics following Raper and Fennell (1977) for the genus *Aspergillus*, Pitt (1979) for the genus *Penicillium*, and Domsch et al. (1981) for other molds. The percent protection of fruit samples in treatments was calculated based on number of fungal isolates in treatment and control sets.

$$\text{Percent protection of fruit samples} = \frac{Dc - Dt}{Dc} \times 100$$

Where; Dc = total fungal isolates in fruit samples of control sets, Dt = total fungal isolates of samples in treatment sets.

2.11.1. Statistical analysis

All the experiments were performed in triplicate and data analysis was done on mean ± SE subjected to one way ANOVA. Means are separated by the Tukey's multiple range test when ANOVA was significant ($p<0.05$) (SPSS 10.0; Chicago, IL, USA).

3. Results

The EO of *Z. alatum* was light yellow in color and its yield was 1.8% (v/w) during hydrodistillation. Its GC and GC–MS profile showed the presence of 20 different components. Their retention time and area percentage are summarized in Table 1 and Fig. 1. Linalool (56.10%) and methyl cinnamate (19.73%) were the major components of the *Z. alatum* EO accounting for 95.54% of the total oil composition. The total phenolic content (TPC) of the essential oil obtained by comparing the absorbance value of oil at 760 nm to the standard curve (Fig. 2) was found to be 21.68 ± 0.91 μg/mg. The MIC and MFC of *Z. alatum* oil and its major components linalool and methyl cinnamate, and the five organic acid preservatives against *A. flavus* LHP-10 are summarized in Table 2. The MIC of methyl cinnamate, the major component of the *Zanthoxylum* oil was 0.6 μl/ml which was more effective than the *Z. alatum* oil (MIC, 1.25 μl/ml) and the five organic acid preservatives, benzoic acid, propionic acid, formic acid, acetic acid and sorbic acid, with MICs of 1.25, 1.50, 2.00, 2.00 and 1.00 μl/ml respectively. However, the MIC of linalool, the other major component of the *Zanthoxylum* oil, was 2.0 μl/ml which was higher than that of the oil alone against *A. flavus* LHP-10. The MIC and MFC concentrations of methyl cinnamate against the test fungus were same (0.6 μl/ml) thereby showing its activity was fungicidal even at its MIC. The MFC of *Zanthoxylum* oil, linalool, and the organic acid preservatives was higher than their MIC, hence their toxicity was fungistatic rather than fungicidal at their MIC.

Table 1
Chemical composition identified by the GC–MS analysis of *Zanthoxylum alatum* oil.

S.N.	Compound	Rt (min)	%
1	Styrene	8.7	0.14
2	1,4-methano-1H-cyclopropa(D) pyridazine	9.0	0.22
3	Methyl heptenone	11.0	0.05
4	β-myrcene	11.2	2.55
5	β-phellandrene	11.9	0.23
6	α-terpinene	12.3	0.70
7	Benzene	12.4	0.21
8	DL-limonene	12.88	9.8
9	γ-terpinene	14.0	0.68
10	β-terpinene	14.5	0.48
11	Limonene oxide	15.5	0.43
12	Linalool	15.98	56.10
13	Cyclohexene	19.5	0.79
14	Trans-isolimonene	20.0	0.74
15	β-pinene	22.5	0.38
16	Piperitone	23.0	0.11
17	Neolyratol	23.5	0.12
18	Phellandral	24.0	0.77
19	Methyl cinnamate	29.0	19.73
20	Trans-caryophyllene	31.0	0.31
	Total		94.54

Rt, retention time.

The antiaflatoxigenic efficacy of *Z. alatum* oil, its major components linalool and methyl cinnamate, and five organic acid preservatives against *A. flavus* LHP-10 is summarized in Tables 3 and 4. Methyl cinnamate, *Zanthoxylum* oil, benzoic acid, sorbic acid and propionic acid checked aflatoxin secretion by *A. flavus* LHP-10 at their MIC for fungal growth. However, linalool, formic acid and acetic acid showed inhibition of aflatoxin B_1 secretion by *A. flavus* at a concentration lower than their MIC for inhibition of growth. Methyl cinnamate was recorded to be more effective in preventing aflatoxin production than *Zanthoxylum* oil, linalool and the five organic acid preservatives. DMSO did not exhibit any significant inhibitory effect on mycelial biomass or aflatoxin B_1 elaboration up to 5.0 μl/ml.

In the antifungal assay, the essential oil of *Z. alatum* exhibited a broad range of antifungal activity inhibiting all the molds tested at 1.25 μl/ml except *A. niger*, *A. sydowi*, *A. alternata*, *C. cladosporoides* and *C. lunata*. However, at 2.5 μl/ml it completely inhibited growth of all tested molds (Table 5).

The free radical scavenging investigations measured by recording the extent of bleaching of DPPH from purple to yellow, showed that

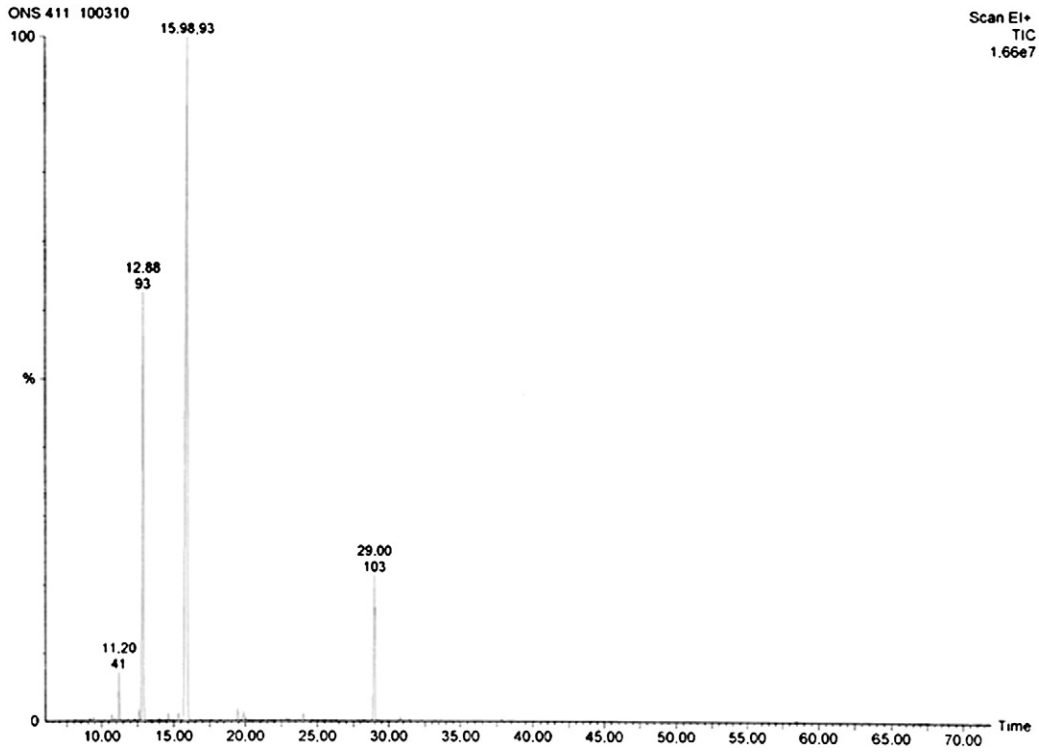

Fig. 1. GC–MS chromatogram of *Zanthoxylum alatum* essential oil.

the *Zanthoxylum* EO had strong antioxidant activity with an IC_{50} value at 5.6 μl/ml. Comparative IC_{50} values of synthetic antioxidants used as positive controls was 7.8 μg/ml for BHT and 5.03 μg/ml for BHA. Methyl cinnamate, linalool and the five organic acid preservatives did not show significant free radical scavenging activity as their IC_{50} values were very high. During further confirmation of antioxidant activity of *Z. alatum* EO, BHT and BHA, by β-carotene/linoleic acid bleaching, the inhibition of oxidation of linoleic acid was 29.08% by the *Z. alatum* EO compared with 80.16% for BHA and 65.35% for BHT (Table 6).

During safety profile trials on mice, the LD_{50} of *Z. alatum* oil, determined through oral administration, was calculated to be 6124 μl/kg body weight.

During storage trials (Table 7) a total of 6 mold species with 344 isolates were isolated from the fruit samples of *P. nigrum* in the control set after 6 months storage. However, 5 fungal species (with 116 isolates) and 3 species (with 47 isolates) were isolated from the fruit samples fumigated with *Z. alatum* EO at 1.25 μl/ml and 2.5 μl/ml respectively. Hence, *Z. alatum* EO showed 66.3% protection at 1.25 μl/ml and 86.3% at 2.5 μl/ml compared with the control sets.

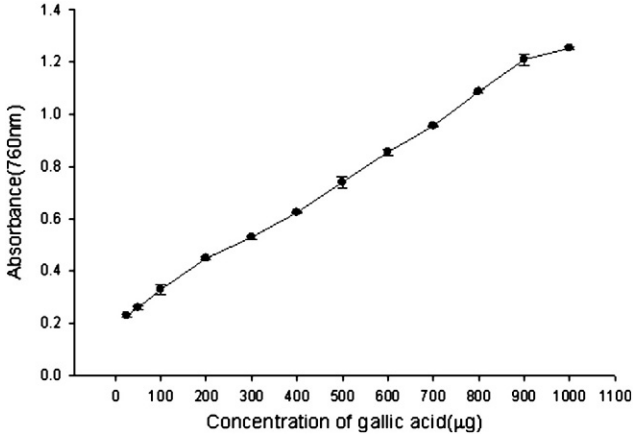

Fig. 2. Standard curve of gallic acid for the measurement of total phenolic content (TPC) of the oil.

4. Discussion

The findings of the present investigation demonstrate a comparative efficacy of *Zanthoxylum* essential oil, its two major components (linalool and methyl cinnamate) and five common organic acid preservatives as inhibitors of the growth of toxigenic *A. flavus* LHP-10 and aflatoxin B_1 secretion. In addition, the efficacy of the oil as an antioxidant by scavenging free radicals, and its safety profile on test animals, were assessed to establish its efficacy as a safe plant based food additive for enhancement of the post harvest shelf life of foods. Prior to testing of biological activity, the *Zanthoxylum* EO was characterized by GC–MS which showed the presence of 20 different compounds, linalool (56.10%) and methyl cinnamate (19.73%) being the

Table 2
Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Zanthoxylum* EO, major components and organic preservatives against *A. flavus* (LHP-10).

Name	MIC (μl/ml)	MFC (μl/ml)
Zanthoxylum alatum (EO)	1.25	2.50
Linalool	2.0	5.0
Methyl cinnamate	0.6	0.6
Benzoic acid	1.25	2.50
Propionic acid	1.50	>5.00
Formic acid	2.00	3.00
Acetic acid	2.00	4.00
Sorbic acid	1.00	1.50
DMSO	>5.00	nd

Nd, not determined.

Table 3
Effect of different concentrations of *Zanthoxylum alatum* EO and organic preservatives on mycelial biomass and aflatoxin B_1 production in SMKY medium.

Conc.	Essential oil		Benzoic acid*		Formic acid		Sorbic acid*		Acetic acid		Propionic acid	
	MDW	AFB_1	MDW	AFB_1	MDW	AFB_1	MDW	AFB_1	MDW	AFB_1	MDW	AFB_1
CNT	570.0 ± 34.03^{a}	1518.97 ± 128.40^{a}	570.0 ± 34.03^{a}	1518.97 ± 128.40^{a}	570.0 ± 34.03^{a}	1518.97 ± 128.40^{a}	570.0 ± 34.03^{a}	1518.97 ± 128.40^{a}	570.0 ± 34.03^{a}	1518.97 ± 128.40^{a}	570.0 ± 34.03^{b}	1518.97 ± 128.40^{b}
0.25	486.67 ± 26.84^{b}	1141.14 ± 105.42^{b}	506.33 ± 4.84^{b}	1213.65 ± 69.96^{b}	563.33 ± 34.92^{a}	1385.39 ± 63.74^{ab}	475.33 ± 12.20^{b}	1053.36 ± 34.98^{b}	493.00 ± 4.35^{b}	1064.80 ± 80.15^{b}	501.67 ± 1.76^{c}	1194.57 ± 66.87^{c}
0.50	412.00 ± 10.07^{c}	893.06 ± 40.21^{c}	440.00 ± 9.53^{c}	946.49 ± 31.24^{c}	488.33 ± 4.05^{b}	1206.02 ± 27.52^{bc}	400.67 ± 0.88^{c}	809.10 ± 10.09^{c}	439.00 ± 8.02bc	858.72 ± 34.35^{bc}	453.00 ± 2.52^{cd}	965.58 ± 31.24^{cd}
0.75	213.67 ± 5.90^{d}	374.02 ± 33.27^{d}	388.67 ± 4.97^{d}	725.13 ± 27.51^{d}	444.00 ± 6.02^{bc}	1068.62 ± 33.92^{cd}	374.00 ± 9.54^{c}	442.72 ± 38.73^{d}	404.33 ± 2.85^{c}	706.05 ± 42.50^{cd}	407.33 ± 2.73^{d}	770.94 ± 37.59^{d}
1.00	95.67 ± 3.93^{e}	80.14 ± 28.81^{e}	241.33 ± 20.90^{e}	446.52 ± 63.05^{e}	398.66 ± 1.45^{c}	912.14 ± 13.76^{de}	00.00^{d}	00.00^{e}	383.67 ± 8.69^{c}	541.94 ± 31.24^{de}	617.67 ± 9.78^{ab}	1644.92 ± 140.28^{b}
1.25	00.00^{f}	00.00^{f}	00.00^{f}	00.00^{f}	379.67 ± 4.09^{c}	805.28 ± 10.10^{e}	00.00^{d}	00.00^{e}	171.00 ± 15.01^{d}	320.59 ± 11.45^{e}	637.67 ± 2.85^{a}	1954.05 ± 10.10^{a}
1.50	00.00^{f}	00.00^{f}	00.00^{f}	00.00^{f}	303.33 ± 4.67^{d}	465.61 ± 15.27^{f}	00.00^{d}	00.00^{e}	17.00 ± 2.65^{e}	00.00^{f}	00.00^{e}	00.00^{e}
1.75	00.00^{f}	00.00^{f}	00.00^{f}	00.00^{f}	36.67 ± 11.05^{e}	00.00^{g}	00.00^{d}	00.00^{e}	00.00^{f}	00.00^{f}	00.00^{e}	00.00^{e}
2.00	00.00^{f}	00.00^{f}	00.00^{f}	00.00^{f}	00.00^{f}	00.00^{g}	00.00^{d}	00.00^{e}	00.00^{f}	00.00^{f}	00.00^{e}	00.00^{e}

Conc., concentration (μl/ml;* mg/ml); CNT., control; MDW, mycelial dry weight (mg); AFB1, aflatoxin B1 content (μg/L).value are mean (n = 3) ± SE. the means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

Table 4
Effect of different concentrations of linalool and methyl cinnamate on mycelial biomass and aflatoxin B_1 production in SMKY medium.

Conc.	Linalool		Methyl cinnamate	
	MDW	AFB_1	MDW	AFB_1
CNT	570.0 ± 34.03^{a}	1518.97 ± 128.40^{a}	570.00 ± 34.03^{a}	1518.97 ± 128.40^{a}
0.2	557.0 ± 09.0^{a}	1331.00 ± 71.55^{b}	414.00 ± 13.00^{b}	1001.92 ± 28.52^{b}
0.4	478.0 ± 10.0^{b}	858.71 ± 28.62^{c}	49.50 ± 1.5^{c}	21.46 ± 7.15^{c}
0.6	423.0 ± 05.3^{c}	636.87 ± 50.09^{d}	00.00^{d}	00.00^{c}
0.8	161.5 ± 06.5^{d}	78.71 ± 21.46^{e}	00.00^{d}	00.00^{c}
1.0	085.5 ± 0.50^{e}	00.00^{e}	00.00^{d}	00.00^{c}
1.25	031.5 ± 03.5^{f}	00.00^{e}	00.00^{d}	00.00^{c}
1.50	022.0 ± 01.0^{f}	00.00^{e}	00.00^{d}	00.00^{c}
1.75	017.7 ± 02.1^{f}	00.00^{e}	00.00^{d}	00.00^{c}
2.00	00.00^{g}	00.00^{e}	00.00^{d}	00.00^{c}

Conc., concentration (μl/ml); CNT., control; MDW, mycelial dry weight (mg); AFB1, aflatoxin B1 content (μg/L).value are mean (n = 3) ± SE. the means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

major components of the oil. The chemical profile of essential oil of a particular plant species varies with seasons, geographical conditions, plant parts used, time of harvesting and method of isolation (Boehme et al., 2008; Burt, 2004; Ngassoum et al., 2003; Shukla et al., 2009) and these chemotypic variations significantly affect the biological efficacy of the oil. Hence, *Zanthoxylum* EO with a known chemical profile was tested in the present investigation. During antimicrobial testing, the *Zanthoxylum* EO completely checked the growth of a toxigenic strain of *A. flavus* at 1.25 μl/ml and was recorded to be more efficacious than the organic acid preservatives (benzoic acid, propionic acid, formic acid and acetic acid) as their MIC for fungal growth inhibition was higher than that of the oil. Only sorbic acid exhibited a lower MIC (1.00 μl/ml) for inhibition of growth of the toxigenic strain of *A. flavus*. Methyl cinnamate, the major component of the *Zanthoxylum* oil, with an MIC at 0.6 μl/ml was found to be more efficacious than the oil alone or the five organic acid preservatives. However, linalool, the another major component of the *Zanthoxylum* oil had an MIC of 2.0 μl/ml which was higher than that of the oil alone against *A. flavus* LHP-10. This clearly shows that the main component responsible for antimicrobial efficacy of *Zanthoxylum* oil was methyl cinnamate. Hence, it may be concluded that the higher MIC of the *Zanthoxylum* oil over methyl cinnamate for inhibition of fungal growth could be because of negative effects of remaining components masking the activity of methyl cinnamate in the oil.

The MIC and MFC of methyl cinnamate against the test fungus were same (0.6 μl/ml) which showed it was fungicidal even at its MIC. On contrary, the MFC of *Zanthoxylum* oil, linalool, and the organic acid preservatives was higher than their MIC values. Hence, their

Table 5
Fungitoxic spectrum of *Zanthoxylum alatum* EO against twelve storage molds.

Test molds	(%) Percent inhibition	
	(1.25 μl/ml)	(2.5 μl/ml)
Aspergillus flavus	100.00^{a}	100.00^{a}
Aspergillus niger	89.61 ± 0.19^{cd}	100.00^{a}
Aspergillus terreus	100.00^{a}	100.00^{a}
Aspergillus candidus	100.00^{a}	100.00^{a}
Aspergillus sydowi	88.74 ± 0.54^{d}	100.00^{a}
Aspergillus fumigatus	100.00^{a}	100.00^{a}
Alternaria alternata	91.18 ± 0.75^{c}	100.00^{a}
Cladosporium cladosporioides	93.40 ± 0.28^{b}	100.00^{a}
Curvularia lunata	89.83 ± 0.39^{cd}	100.00^{a}
Fusarium nivale	100.00^{a}	100.00^{a}
Penicillum italicum	100.00^{a}	100.00^{a}
Trichoderma viride	100.00^{a}	100.00^{a}

Values are mean (n = 3) ± SE. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

Table 6
Antioxidant activity of *Zanthoxylum alatum* EO, linalool, methyl cinnamate and organic acid preservatives.

Samples	DPPH (IC_{50})	β-carotene/linoleic acid inhibition (%)
Essential oil	$5.6 \pm 00.06^{h\dagger}$	29.08 ± 0.31^{c}
BHT	$7.80 \pm 0.15^{h*}$	65.35 ± 0.34^{b}
BHA	$5.03 \pm 0.08^{h*}$	80.16 ± 0.57^{a}
Acetic acid	$358.67 \pm 00.66^{d\dagger}$	nd
Benzoic acid	$452.33 \pm 01.45^{c\ddagger}$	nd
Formic acid	$154.33 \pm 01.20^{g\dagger}$	nd
Propionic acid	$475.00 \pm 00.57^{b\dagger}$	nd
Sorbic acid	$306.66 \pm 00.88^{e\ddagger}$	nd
Linalool	$299.66 \pm 00.87^{f\dagger}$	nd
Methyl cinnamate	$636.66 \pm 03.30^{a\dagger}$	nd

‡ mg/ml; †μl/ml; * μg/ml; nd not determined; value are mean (n = 3) ± SE. the means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

toxicity was fungistatic in nature at their MIC. Therefore, during practical application as plant based antimicrobial, methyl cinnamate may be efficacious in permanent disinfestation of fungi present on some food items. However, the *Zanthoxylum* oil, linalool, and the organic acid preservatives, because of their fungistatic nature, could be useful in inhibition of growth of food borne fungi.

The MIC of *Zanthoxylum* EO recorded in the present investigation was significantly lower than some fungicides such as Nystatin (1.85 μl/ml) and Wettasul-80 (2.78 mg/ml) (Prakash et al., 2010) and earlier reported antimicrobial oils such as *Daucus carota* (Tavares et al., 2008), *Distichoselinum tenuifolium* (Tavaresa et al., 2010) and *Sesuvium portulacastrum* (Magwa et al., 2006). Because of the lower MIC, a smaller amount of the *Zanthoxylum* essential oil would be required for controlling food borne microorganisms.

During testing of antiaflatoxigenic efficacy, methyl cinnamate, *Zanthoxylum* oil, benzoic acid, sorbic acid, and propionic acid checked aflatoxin secretion by *A. flavus* LHP-10 at their MIC preventing fungal growth. This may be due to similar mode of action on fungal growth inhibition and aflatoxin suppression as has been reported in the case of sweet basil oil (Atanda et al., 2007). However, linalool, formic acid and acetic acid showed inhibition of aflatoxin B_1 production at a concentration lower than their MIC for fungal growth. This may be due to their different mode of action on fungal growth inhibition and aflatoxin suppression as has been reported in the oils of *Piper betle* and *Lippia alba* (Prakash et al., 2010; Shukla et al., 2009). Thus, the inhibition of AFB_1 production cannot be completely attributed to reduced fungal growth, but may be due to the inhibition of carbohydrate catabolism in *A. flavus* by acting on some key enzymes, reducing its ability to produce aflatoxins as has been reported by Tian et al. (2011). To the best of our knowledge the efficacy of *Zanthoxylum* oil and methyl cinnamate in inhibition of aflatoxin secretion has not been reported previously although the antifungal activity of *Zanthoxylum* oil has been reported in inhibition of growth of toxigenic strains of *A. flavus* and *A. parasiticus* (Dube et al., 1990).

Table 7
Percent protection on fruit sample of *Piper nigrum* fumigated with *Zanthoxylum alatum* essential oil.

Mold isolates	No. of fungal isolates		
	Control	1.25 μl/ml	2.5 μl/ml
Aspergillus flavus	234	72	29
Aspergillus niger	63	32	17
Fusarium oxysporum	7	–	–
Fusarium nivale	12	4	–
Penicillium italicum	10	2	–
Cladosporium cladosporioides	18	6	1
Total isolates	344	116	47
% protection		66.27	86.33

Both mycelial biomass and AFB_1 production exhibited a statistically significant declining trend with increasing concentration of the *Zanthoxylum* EO, methyl cinnamate, linalool, and the preservatives. An increase the aflatoxin content at certain higher concentrations of propionic acid indicates that this compound did not act in a typical dose response manner. A similar increase in mycotoxin production at a higher concentration has been also reported by Paterson (2007). This may be due to inter-strain variability in the quality and quantity in a particular inoculum within natural population. The finding is in accordance with earlier report (Locke et al., 2001) suggesting that some fungal species or strains may be reacting differently to specific fungicides/preservatives leading to stimulation of mycotoxin synthesis at certain higher concentrations. The *Zanthoxylum* EO also showed a broad fungitoxic spectrum. The findings thus strengthen the possibility of its economic application at a low concentration for protection of food commodities from spoilage by various storage molds.

Apart from microbial infestation and mycotoxin contamination, the shelf life of food items is also reduced by toxic reactive oxygen species (ROS) molecules causing oxidative stresses and biodegradation. In addition to other factors, the metabolic products of aflatoxin B_1 (AFB_1-8,9-exoepoxides) are also reported to be responsible for the stimulation of the lipid peroxidation by enhancement of highly reactive molecules (ROS) (Choy, 1993). Therefore, to prolong the shelf life of foods, an ideal preservative should be antimicrobial, antimycotoxigenic as well as antioxidant. Most of the prevalent preservatives are only antimicrobial in action and some of them may also stimulate aflatoxin biosynthesis (Jayashree and Subramanyam, 2000). Oxidative stress has also been recognized as one of the major factors responsible for aflatoxigenesis (Kim et al., 2005). Hence, in the present investigation, the antioxidant activity of *Zanthoxylum* EO, methyl cinnamate, linalool and the five organic acid preservatives was also assessed and compared with BHT and BHA. DPPH was used in the present investigation as it has the advantage over laboratory generated free radicals such as the hydroxyl radicals and superoxide anions because it remains unaffected by certain side reactions such as metal ion chelation and enzyme inhibition brought about by different additives (Amarowicz et al., 2010). Only the *Zanthoxylum* EO and common antioxidants (BHT and BHA) showed antioxidant activity by DPPH radical assay. Their antioxidant activity was again tested through β-carotene/linoleic acid assay so as to confirm their ability to prevent lipid peroxidation by reacting with chain propagating peroxyl radical faster than the reaction of these radicals with protein or fatty acid side chain. In the β-carotene linoleic acid assay, free radicals produce by the oxidation of linoleic acid are responsible for the bleaching of characteristic yellow color of β-carotene (used as a marker). Antioxidant activity of essential oil prevents the bleaching of β-carotene by neutralizing the free radicals present in the system (Bamoniri et al., 2010; Ebrahimabadi et al., 2010). The *Zanthoxylum* EO exhibited better antioxidant activity than that reported earlier for essential oils of *Stachys inflate* (Ebrahimabadi et al., 2010), *Satureja montana*, and *Satureja subspicata* (Cavar et al., 2008), *Semenovia tragioides* (Bamoniri et al., 2010) and *Ocimum gratissimum* oil (Prakash et al., 2011). Contrary to the results of antimicrobial and antiaflatoxigenic efficacy where methyl cinnamate was more efficacious, the significant antioxidant activity in the *Zanthoxylum* EO may be due the synergism between its components, as neither of the two major components (methyl cinnamate and linalool) individually showed significant free radical scavenging activity. The high phenolic content of the oil may be responsible for the antioxidant activity acting as scavenger of the free radicals as reported by some earlier workers (Gholivand et al., 2010). Moreover, the plant based aromatic essential oils due to their high phenolic content disrupt cell membranes of the fungi leading to leakage of specific ions or causing inhibition of membrane bound enzymes (Lanciotti et al., 2004).

The LD_{50} of the *Z. alatum* oil, was recorded to be 6124 μl/kg body weight which is higher than some well known botanicals such as azadirachtin (>5000 mg/kg), pyrethrum (350–500 mg/kg) and carvone (1640 mg/kg) and some commercial fungicides including Bavistin (1500 mg/kg) and Wettable Sulfur (5000 mg/kg) (Prakash et al., 2011). The LD_{50} of weak organic acid preservatives are: sorbic acid (3200 mg/kg) (http://www.sciencelab.com/msds.php?msdsId=9925055), propionic acid (3500–4300 mg/kg.), formic acid (700 mg/kg), acetic acid (3530 mg/kg) and benzoic acid (2000–2500 mg/kg) (Patnaik, 2007). The LD_{50} of the *Z. alatum* oil is also higher than that its major components: the LD_{50} of linalool is 3120 mg/kg (http://www.inchem.org/documents/sids/sids/78706.pdf) and that of methyl cinnamate 2610.00 mg/kg (http://www.thegoodscentscompany.com/data/rw1417571.html). The high LD_{50} value of the oil indicates its favorable safety profile and negligible mammalian toxicity. The efficacy of *Zanthoxylum* EO as a post harvest antimicrobial is being reported for the first time. As the oil showed remarkable in vivo efficacy in protecting the fumigated black pepper fruits from fungi growth, further large scale trials are required before it can be recommended as a plant based preservative in post harvest processing of fruits. The long ethnomedicinal use of *Z. alatum* in the Indian system of medicine (Jain et al., 2001; Tiwary et al., 2007) is additional evidence of the low mammalian toxicity of the *Zanthoxylum* EO should it be recommended as food additive.

Hence, due to antimicrobial, antiaflatoxigenic, antioxidant activities, persistence of efficacy in food systems during in vivo trials and favorable safety profile, which are required for an ideal preservative, *Zanthoxylum* EO may attract the attention of scientific community and food industries in place of synthetic preservatives for possible application in the enhancement of shelf life of various food items by protecting them from molds, aflatoxin contamination as well as oxidative degradation during storage. After detailed in vivo trials using modern encapsulation technology, the EO can be easily released as a fumigant in storage containers at practical doses for application. In addition development of resistant microorganisms which can occur with organic acid preservatives (Brul and Coote, 1999) may be solved due to synergism between different components of the oil. Because of high yield of the oil (1.8% (v/w)) and availability of the raw material in bulk, the *Zanthoxylum* EO may constitute an economical source of plant based food additive during post harvest processing and storage of various foods.

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