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# Influence of *Thymus serpyllum* essential oil on *Aspergillus parasiticus* morphology and aflatoxins production

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## ABSTRACT

Antifungal activities of the *Thymus serpyllum* L. oil was studied with special reference to inhibition zones diameter, Minimal inhibitory (MIC) and Minimal fungicidal (MFC) concentrations of the oil. The antifungal efficacy of this essential oil was evaluated in vitro upon *Aspergillus parasiticus* growth and mycotoxin production. The essential oil from *T. serpyllum* obtained by hydro distillation was analyzed by GC/MS. The major components of *T. serpyllum* oil was thymol ( 64.2 % ) , β – phellandrene ( 13.5 % ) , cis-sabinene hydroxide ( 8.09 % ) 1,8 – cineole ( 1.9% ), and β – pinene ( 1.3% ) . Static growth effects of the above oil against *A.parasiticus* were at 250 ppm and lethal effects of *T. serpyllum* was 500 ppm of the oil. Aflatoxin production was inhibited at 250 ppm. Transmission electron microscopy (TEM) of *A. parasitcus* exposed to 250 ppm of the oil showed irreversible damage to cell wall, cell membrane , and in the cellular organelles. It is concluded that essential oil could be safely used as preservative materials on some kinds of foods at low concentrations to protect them from fungal infections.

Keywords: *Thymus serpyllum*, aflatoxins, *Aspergillus parasiticus*

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## INTRODUCTION

Since fungicides are very expensive and cause serious environmental pollution. Control strategies are today directed towards the replacing the use of hazardous chemical fungicides by environmentally friendly natural products [33].

Essential oils of several plants are frequently used in the preparation of cosmetics, perfumes, antiseptics and as an active ingredient in certain medicines. Essential oils are also used for purification of environment since they possess antimicrobial properties. Anti-microbial activity of essential oils has been reported by various workers and has been reviewed time to time; [10, 12, 14, 17, 18, 21, 22, 25, 30, 39, 49, 50 and 52].

*Thymus serpyllum* L. (F. Labiatae) is a grey dwarf shrub rarely exceeds 40 cm in height. Stem quadrangular erect, woody much branched. Leaves small oval, rolled margins and downy undersurface flowers small pink, arranged in a corymbs. The plant has a strong characteristic smell. It grows wildly in Garian, Tarhuna and Tripoli [28].

Various species of thyme have been reported to possess antifungal properties [4, 5, 40, 7, 8, 9, 32 and 47]. There is few reports on fungicidal activity of essential oil from *Thymus serpyllum* on A. parasiticus.

However, the essential oil of T. striatus and its major component, thymol, was analyzed for potential antifungal activity against plant, animal, and human pathogenic fungi from different genera including Aspergillus .The oil showed a strong inhibitory effect against all fungi investigated [13 and 47]. The presence and growth of fungi in food may cause spoilage and result in a reduction in quality and quantity. The use of natural antimicrobial compounds is important not only in the preservation of food but also in the control of human and plant diseases of microbial origin [2, 16, 23, 37, 42, 44 and 46].Some Aspergillus species are xerophilic fungi and are responsible for many cases of food and feed contamination [1, 26].

Aflatoxin – producing moulds are widely distributed in nature and frequently contaminate human food resources .Aflatoxins are secondary metabolites produced by toxigenic strains of A. flavus and A. parasiticus. These fungi grow rapidly on a variety of natural substrates and consumption of contaminated food cause serious health hazards to human and animals. They are able to produce aflatoxins in food feedstuffs [7, 12, and 31]. Aflatoxin B1 is produced by Aspergillus species on agricultural commodities [31]. Chemicals are currently used to limit the growth of hazardous fungi like *A.parasiticus* in stored foods. [36] demonstrated that 0.9-1% ammonia inhibited fungal growth together with aflatoxin production.

The use of spices and their non – toxic derivatives as alternative preservative agents is considered by consumers as safe. The limited knowledge concerning antimicrobial activity and the mechanism of plant extracts has led us to address such issues. Most of these studies have been conducted using essential oils in microbiological media; consequently, little is understood about mechanism of their effectiveness. *A.parasiticus*, as food contaminant, has still received

little attention as far as essential oils are concerned. [3] Reported more than 50% inhibition of aflatoxin production at 50% (v/v) need extract concentration.

In this investigation, we decided to study the mechanism of fungicidal activity of essential oil from Libyan *T.serpyllum* on *A.parasiticus* both in vitro and its ultra structural level effect.

## MATERIALS AND METHODS

### Micro-organism and cultivation conditions

*Aspergillus parasiticus* NRRL 2999 was employed in this study. Potato Dextrose Agar slants were used for its growth and maintenance. Spore suspensions were prepared in sterile yeast extract sucrose (YES) broth to a concentration of approximately $10^8$ spores/ml. Subsequent dilutions were made from this suspension, which were then used in the tests. YES broth also served as aflatoxin production medium.

### Plant material

*Thymus serpyllum* was collected from open areas of Garian and Tarhuna of Libya during May-June (2010).

### *T. serpyllum* oil extraction.

350 g fresh leaves were hydro distilled for 90 min in full glass apparatus. The oil was isolated using a Clevenger-type apparatus. The extraction was carried out for two hours after four h maceration in 500 mL of water. The oil was stored in dark glass bottles in a freezer until they were used. Methanol was selected as a diluting agent for the oils as it did not exhibit antifungal activity when tested *A.parasiticus*.1/2, 1/4, 1/8, and 1/16 dilutions of oil was made with methanol. These dilutions were used in antifungal analysis. Undiluted oil was taken as $1^{st}$ dilution. This solvent also served as control.

### *T. serpyllum* oil GC/MS:

The essential oil was analyzed by GC (9-A-Shimadzu) and GC/MS (Varian-3400) column:{DB-1(dimethy1 poly- siloxane), 60m x 0.25mm fused silica capillary column, film thickness 0.25 µm film thick)} using a temperature program of 50-250$^{o}$C at a rate of 4$^{o}$C/min, injector temperature 250 $^{o}$C, carrier gas: helium ( 99.99%), inlet pressure 3 kg /cm$^2$ .The constituents were identified by comparison of their mass spectra with those in the computer library and with authentic compounds. The identifications were confirmed by comparison of their retention indices with those of authentic compounds.

### Antifungal analysis

Diameters of inhibition zones using disc diffusion method , the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the oil dilutions were performed for 50 µ from each of various dilutions of the oil when added to 5 ml of YES broth tubes containing $10^7$ spores/ml. The tubes were then incubated on a rotary shaker at 150 rpm and 28ºC for 6h to evenly disperse the oil throughout the broth in tubes. After at 48h incubation at 28ºC, the tubes were examined visually for fungal growth precipitation compared to the reference antibiotic .The highest dilution (lowest concentration), showing no visible precipitation, was regarded as MIC. Cells from tubes showing no growth were subculture on potato dextrose agar plates to determine if the inhibition was reversible or permanent. MFC was determined as the highest dilution (lowest concentration) at which no growth occurred on the plates. [11]

**Determination of mycelia weight**

Flasks containing mycelia were filtered through Whatman filter No.1 and then were washed with distilled water. The mycelia were placed on pre weighed Petri plates and were allowed to dry at 60 $^oC$ for 6 h and then at 40 $^oC$ overnight. The flasks containing dry mycelia were weighed.

Percent growth inhibition on the basis of dry weight = Control weight – Sample weight ÷ Control weight × 100.

**Measurement of aflatoxins: [35]**

Aflatoxin extraction was performed routinely with solvent extraction [3, 43]. Silica gel-GF pre-coated sheets were used for analysis of aflatoxins produced by the fungal strain. The toxin was measured spectrophotometrically in aflatoxin fraction eluted from silica gel . Shimadzu UV-2501PC Spectrophotometer.

**Transmission electron microscopy**

500 µ l of *T. serpyllum* oil at 125 and 250 ppm was added to 50 ml of each spore suspension containing $10^7$ spores/ml and were then incubated on a shaker at 30 $^oC$ for 72 h. Samples were then taken and processed for transmission electron microscopy (TEM). Mycelial samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate-buffer (pH 7.2) for 2 h at room temperature. They were washed three times, each time for 15 min, in cacodylate- buffer. Specimens were then post- fixed for 2 h in 1% Osmium tetroxide (Os $O_4$) dissolved in cacodylate- buffer at room temperature and washed cacodylate-buffer ( three times, 15 min each). Samples were dehydrated in a graded series of ethanol (40%, 60%, 75%,80%, and 95%, two times for 15 min each and two times for 30 min each in 100% ethanol). Fixed mycelia were processed in graded propylene oxide: araldite and finally were embedded in araldite. The polymerization of araldite to form specimen blocks was accomplished in an oven at 45$^oC$ for 24 h and then at 70 $^oC$ for 48 h. The specimen blocks were hand trimmed with a razor blade and sectioned with an ultra microtome with 1 µ m thickness (sections appearing blue color under

ultratome) for light microscopic observation and 0.1 µm (sections appearing blue color under ultra tome) for transmission electron microscopic observation. The ultrathin sections were placed on 200 mesh copper grids. The sections were stained with 12.5% alcoholic uranyl acetate [ $UO_2(CH_3COO)_2 .2H_2O$] in methanol for 20 min and then with lead citrate [25mg lead citrate [$Pb_3(C_6H_5 O_7)_{2.} 3 H_2O$] dissolved 1 ml 1 N sodium hydroxide and the final volume was made to 10 ml by adding 9 ml double distilled water], and then were washed with double distilled water for 1 min, dried under reading lamb for 30 min and viewed with a JEOL 100 (Japan) Transmission Electron Microscope ( TEM) operating at 80 kV.

All chemicals from Merck were of analytical grade.

## RESULTS AND DISCUSSION

### T.Sepyllum chemical composition:

Essential oil extracted from *T. serpyllum* leaves yielded 1.23% (w/w) oil. The oil contents were in expected range. Chemical analysis of the components of the oil led to identification of 18 components in *T. serpyllum* oil (Table 1). The major components of *T. serpyllum* oil was thymol (62.02%), β-phellandrene (13.50%), cis-sabinene hydroxide (8.09%), 1,8 –cineole (1.9%), and β-pinene ( 1.3%). The chemical constituents are similar to other varieties of thyme but at different concentrations.. This indicates that highly nutritious medium such as YES could not support fungal cells resistance against the oil. The antifungal effect of the thyme oil could be related to several components known to have biological activities, such as thymol as the most prevalent component [48]. Thymol (64.2%) and β-phellandrene (13.50%) accounted for the most abundant components of essential oil of T. serypllum oil.

**Table 1: Chemical composition of essential oil from *T. serpyllum* analyzed by (GC-MS).**

| No. | *T. serpyllum* compounds | Retention index | area% |
|---|---|---|---|
| 1 | Tricyclene | 909 | 0.74 |
| 2 | α-Thujene | 916 | 0.42 |
| 3 | α -pinene | 929 | 0.27 |
| 4 | Jerbenene | 949 | 0.28 |
| 5 | β-Pinene | 967 | 1.30 |
| 6 | Myrcene | 982 | 0.30 |
| 7 | α -Phellandrene | 995 | 1.22 |
| 8 | β -Phellandrene | 1005 | 13.5 |
| 9 | 1,8-Cineole | 1009 | 1.9 |
| 10 | Cis-Sabinene hydroxide | 1036 | 8.09 |
| 11 | n-Octanol | 1039 | 0.85 |
| 12 | Trans-Thujene | 1110 | 0.28 |
| 13 | (z)-Tagetone | 1134 | 0.75 |
| 14 | Cis-Sabinene hydrate acetate | 1214 | 0.70 |
| 15 | (E)-Cinnamaldehyde | 1256 | 0.83 |
| 16 | Cis-Sabinene hydrate | 1096 | 0.29 |

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| | | | |
|---|---|---|---|
| 17 | Thymol | 1267 | 64.2 |
| 18 | Germacrene D | 1472 | 1.41 |

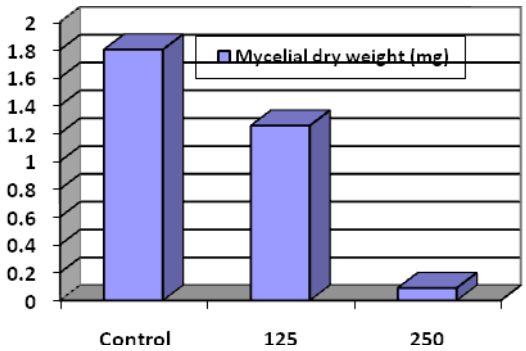
**_T. serpyllum_ MIC and MFC**

Unpublished experiments were carried out in vitro to investigate antifungal action of the *T. serpyllum* essential oil. It was tested on potato dextrose agar plates ( previous unpublished data.). A. parasiticus growth inhibition zones of 15, 18, 29, 48 mm were observed at 125, 250,500, 1000 ppm of *T. serpyllum* oil. MIC and MFC techniques were employed to assess fungi static and fungicidal properties of the oil. Static effects of the above oil against A. parasiticus were at 250 ppm and highest effects were observed at 500 and 1000 ppm of *T. serpyllum* oil. even with YES medium, more nutritious medium than Czapek-Dox Agar, indicate complete inhibition of A. parasiticus at 0.05% (500 ppm) and 1% (1000 ppm) of T. serypllum oil.

**Aflatoxin suppression by _T. serpyllum_ oil.**

Fig 1A showed that there was a gradual decrease in mycelial growth with increase in oil concentration due to the increased thymol present in the essential oil compared to the control. Aflatoxin production was inhibited at 250 ppm of oil (Fig. 1B). So, the extent of inhibition of fungal growth and mycotoxin production was dependent on the concentration of essential oil used . [48] reported complete inhibition of A. flavus, A. parasiticus, and A. ochraceus by the oils of thyme and cinnamon (<500 ppm), marigold ( < 200 ppm), spearmint, basil, ( 3000ppm). However, they did not specify chemical composition of their thyme oil. Growth of A. parasiticus NRRL 2999 was reported to be completely inhibited by thyme extracts at the 2% level in Czapek-Dox Agar [38].

It may be deduced that fungal growth inhibition and subsequent mycotoxin production were related mostly to thymol content of the oil (Fig.1A,B).




(A)

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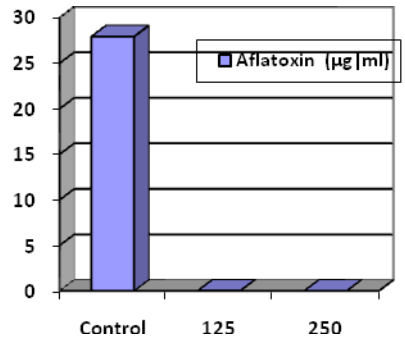
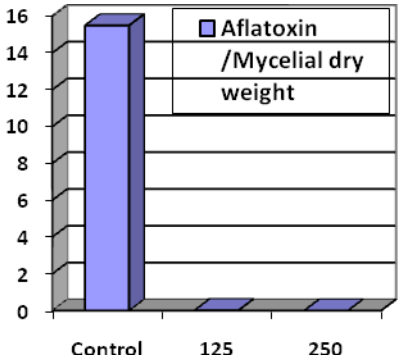



(B)




(C)

**Fig 1(A, B, C): Effect of thyme essential oil on A. parasiticus dry weight and aflatoxin production.**

## A. parasiticus Transmission electron microscopy (TEM)

TEM of untreated A. parasiticus revealed intact mycelia with healthy structure, smooth cell wall, and cell membrane ( Fig.2),in the contrary to the mycelium treated with thyme oil showed alterations in the morphology of the hyphae, which appeared severely collapsed, and a reduction in conidiation (fig 3). TEM of A. parasiticus exposed to 250 ppm of the oil showed severe damage to cell wall, cell membrane, and cellular organelles such as mitochondria which seem to be destroyed (Fig.3 and 4).

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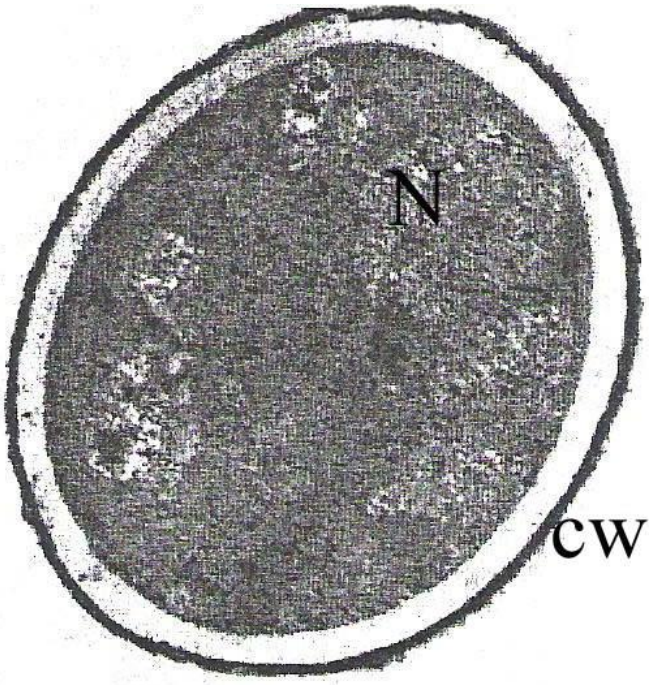


**Fig.(2). TEM.*A.parasiticus* cell (control) , CW, cell wall and N, nucleus 800X , Nucleus.**

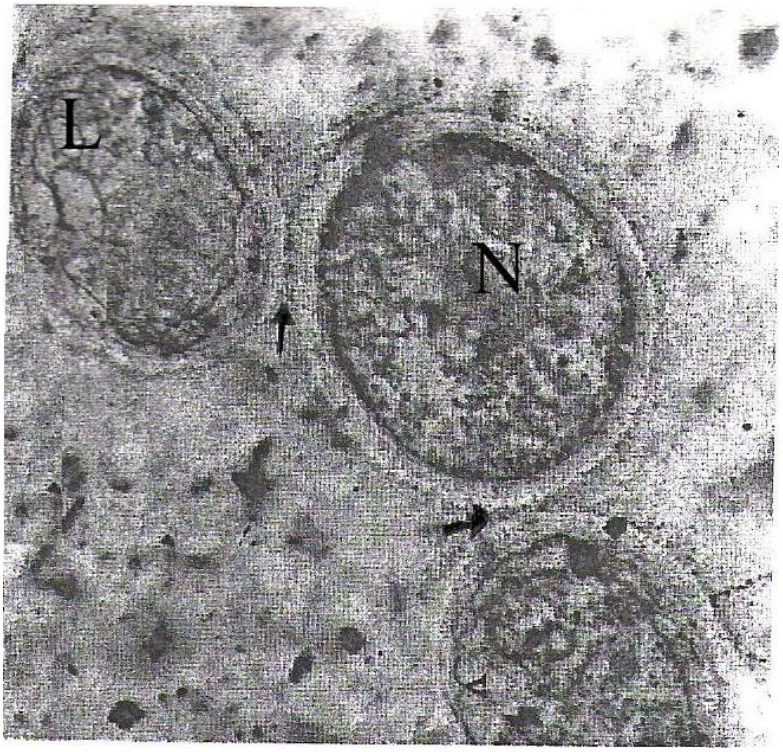


**Fig.(3).TEM graph 10,000x ,*A.parasiticus* exposed to 125 ppm of essential oil from T . serpyllum showing damage to the organelles , destruction of cytoplasm , folding of cell membranes ( arrow head).The cells seem to be approaching each other by sharing their membranes ( arrows).N, nucleus**

[24] came to the conclusion that the loss of aflatoxigenic capabilities in the non-aflatoxigenic variants of A. parasiticus is due to the alterations in the conidial morphology of the fungus, suggesting that the regulation of aflatoxin synthesis and conidiogenesis may be interlinked.

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Fungal growth inhibition was reported to be associated with the degeneration of fungal hyphae after treatment with T. vulgaris L., Lavandula R.C., and Mentha piperita L. essential oils with the oil of thyme being more effective than that of lavender or mint [52]. Our observations show that main target of the oil were the cell wall and cell membrane (Figs. 3 and 4). The plasma membrane of A. parasiticus, in the presence of thyme essential oil at 250 ppm, was seen to be irregular, dissociated from the cell wall, invaginated and associated with the formation of lomasomes ( Fig. 3). These lomasomes(membranosome: all boundary structure containing a membrane component ) are usually found in fungi treated with imidazole components [34, 45]. The marked action of oil components might have conferred lipophilic properties and the ability to penetrate the plasma membrane [27]. A synthetic antifungal alcohol, Econazole, has been reported to find its target in the plasma membrane of Microsporum canis cells which dissociated from the wall after a 4 h treatment at 100 mg/Las recorded by [34]. The thyme oil under this investigation showed shorter effective time and effective lower concentrations. Similar to the findings of Watanabe [51] who observed, the effect of benacomicin A, a synthetic antifungal agent with mannan affinity, that affects wall synthesis, revealed disruption of S. cerevisiae plasma membrane in TEM.

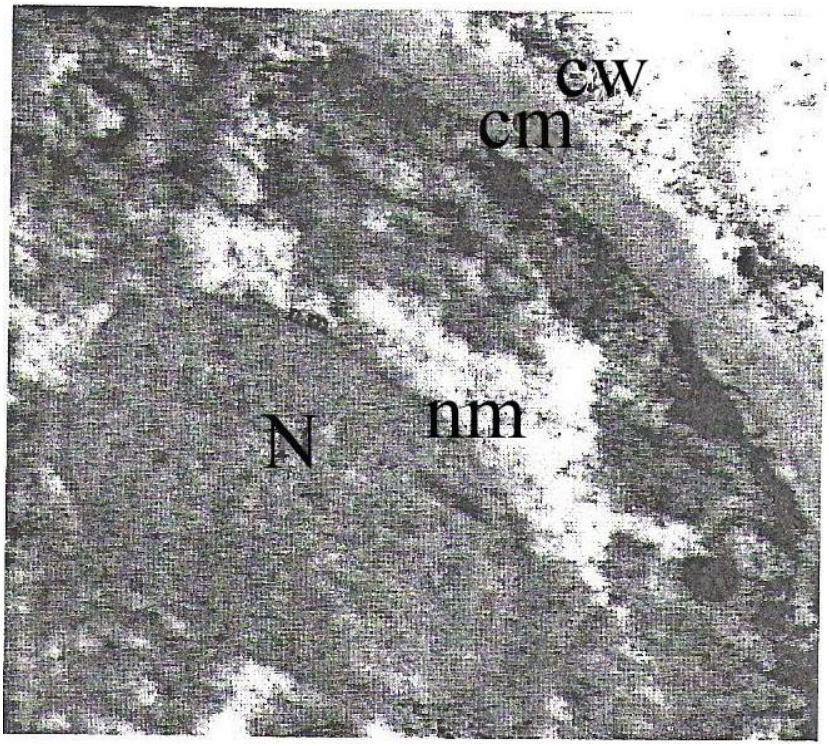


**Fig 4: EM graph 32,000x, *A.parasiticus* exposed to 250 ppm essential oil from T .serpyllum showing damage to the cell wall ( CW), nuclear membrane ( nm) and cell membrane(cm).**

These observations bring further evidence that essential oil should find a practical application in the inhibition of mycotoxin production in food products. Essential oils could be safely used as preservative materials on some kinds of foods, such as thyme oil which inhibited growth and aflatoxin production of A. parasiticus at low concentrations, leading to deleterious

cellular morphological alterations, which become irreversible at 250 ppm, and could be added to food stuffs in storage to protect them from fungal infections.

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