

EVALUATION OF ANTIFUNGAL ACTIVITY IN ESSENTIAL OIL OF THE SYZYGIUM AROMATICUM (L.) BY EXTRACTION, PURIFICATION AND ANALYSIS OF ITS MAIN COMPONENT EUGENOL

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ABSTRACT

Antifungal properties of some essential oils have been well documented. Clove oil is reported to have strong antifungal activity against many fungal species. In this study we have evaluated antifungal potential of essential oil of *Syzygium aromaticum* (L.) against some common fungal pathogens of plants and animals namely, *Fusarium moniliforme* NCIM 1100, *Fusarium oxysporum* MTCC 284, *Aspergillus* sp., *Mucor* sp., *Trichophyton rubrum* and *Microsporum gypseum*. All fungal species were found to be inhibited by the oil when tested through agar well diffusion method. Minimum inhibitory concentration (MIC) was determined for all the species. Column chromatography was performed to separate the eugenol rich fraction from clove oil. Out of seven fractions maximum activity was obtained in column fraction II. TLC and HPLC data confirmed presence of considerable Eugenol in fraction II and clove oil. Microscopic study on effect of clove oil and column fraction II on spores of *Mucor* sp. and *M. gypseum* showed distortion and shrinkage while it was absent in other column fractions. So it can be concluded that the antifungal action of clove oil is due to its high eugenol content.

Key words: Essential oil, *Syzygium aromaticum*, Antifungal, Chromatography, Eugenol

INTRODUCTION

In the past few decades, a worldwide increase in the incidence of fungal infections has been observed as well as a rise in the resistance of some species of fungus to different fungicides in medicine and agriculture. The last two decades have witnessed a dramatic rise in the incidence of life threatening systemic fungal infections. The majority of clinically used antifungals have various drawbacks in terms of toxicity, efficacy and cost, and their frequent use has led to the emergence of resistant strains. Additionally, in recent years public pressure to reduce the use of synthetic fungicides in

agriculture has increased. Concerns have been raised about both the environmental impact and the potential health risk related to the use of these compounds (1).

The use of plant derived products as diseases control agents have been studied, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (5).

Essential oils have a long history of use as natural microbial agents and have recently been used in a number of pharmaceutical, food, and cosmetic products since these oils effectively inhibit the growth of a wide range of microorganisms, with fewer side effects than synthetic

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antimicrobial agents in humans. Despite the widespread use of essential oils, details about the exact mechanism of their antimicrobial action are yet to be explored. Thus, many researchers have recently attempted to identify the antimicrobial properties of essential oils. Clove oil has been widely investigated due to its popularity, availability, and high essential oil content (16).

Clove oil (*Syzygium aromaticum*) is widely used as a perfume and food flavoring (22), as a medicine for the treatment of asthma and various allergic disorders in Korea (12), and as a general antiseptic in medical dental practices (6). Ahmad *et al.* (2) evaluated antimicrobial activity of clove oil against a range of fungal pathogens including the ones for urogenital infection. Clove oil was found to possess strong antifungal activity against opportunistic fungal pathogens such as *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. The essential ingredient responsible for its antifungal activity is eugenol from the clove (15).

Eugenol is the main volatile compound of extracted oil from clove bud (*S. aromaticum* L.) that is used in traditional medicine, as a bactericide, fungicide (3, 4), anesthetic, and others (20). However, its antimicrobial activity was found to be higher against fungi than against bacteria (9). Besides eugenol (49–87 %), clove oil also contains β -caryophyllene (4–21 %), eugenyl acetate (0.5–21 %), smaller amounts of α -humulene along with trace amounts (<1 %) of 25–35 other constituents (20).

The purpose of this study was to examine the antifungal activity of clove essential oil extracted from floral buds against dermatophytes, such as *Trichophyton rubrum*, *Microsporum gypseum*, and plant pathogenic fungi such as *Fusarium moniliforme*, *Fusarium oxysporum*, *Aspergillus* sp. and *Mucor* sp. The major constituent of the clove oil (eugenol) was also purified and analyzed in this study through chromatographic techniques. Also the effect of clove oil on fungal spores was studied.

MATERIALS AND METHODS

Collection and identification of plant materials

The dried flower buds of clove plant used in this study were

purchased from local market of Bhopal, India. The buds were identified and authenticated by Dr. R.C. Rajak, Scientific Advisor, R&D, Kilpest India Ltd., Bhopal, India.

Extraction of volatile oil by steam distillation

Clove buds (200 g) were powdered in a grinder and it was subjected to steam distillation, using the Clavenger apparatus (Pyrex), as described by Harbone (10). Distillation was done for 4 hours and the oil was drained off and dried over anhydrous sodium sulphate (4).

Test organisms and preparation of inoculum

Mucor sp. and *Aspergillus* sp. were isolated from soil samples; *Microsporum gypseum* and *Trichophyton rubrum* were obtained from Fungal Germplasm Culture Collection Center, Department of Biological Sciences, R.D. University, Jabalpur, India. *Fusarium moniliforme* NCIM 1100 was procured from NCIM, NCL, Pune, India and *F. oxysporum* MTCC 284 was procured from MTCC, IMTECH, Chandigarh, India. All the fungal cultures were maintained on Potato Dextrose Agar (PDA), Himedia, India, slant medium supplemented with 0.1 % yeast extract and stored as active cultures at 4 °C for six months and revived thereafter. For the preparation of inoculum, all the test fungi were raised on Sabouraud Dextrose Agar (SDA), Himedia, by incubating the petriplates at 28±1 °C for 7 days in a BOD incubator. Fungal spore suspensions (100 ml) were prepared in sterile distilled water containing 0.05 % Tween-20.

Screening of antifungal activity and determination of MIC

Preliminary screening of antifungal activity of clove essential oil (EO) was done through agar well diffusion assay (17). Spore suspension of each isolate was prepared by taking a single fungal disc aseptically with a 5 mm cork borer from sporulating culture and mixed in 20 ml sterile distilled water to which 50 μ l of Tween-80 was added. The total number of spores was determined by counting under microscope using haemocytometer (Neubauer counting chamber). Fungal spore count was adjusted to 1×10^6 spores/ml by making appropriate dilutions (21). Petriplates containing SDA (Himedia) were inoculated aseptically with 500 μ l spore suspension through spread plate technique and kept in the

laminar flow for 30 min. Wells of 5 mm diameter were punched in the center of the petriplates by using sterile cork borer. Into each well, 10 μ l of the clove EO was dropped followed by 40 μ l of dimethyl sulphoxide (DMSO) so as to make the 50 μ l total volume in the well. Plates were kept in refrigerator at 4 °C for 3 h, to allow EO and DMSO to diffuse into the agar medium. Plates were finally incubated at 28 \pm 1 °C for 5 days. Ketoconazol at the concentration of 1 mg in 1 ml of DMSO was used as positive control. DMSO without clove oil was set as negative control. The diameter of the zone of inhibition around each well was measured in mm.

Minimum inhibitory concentration (MIC) was determined for all the fungal strains through agar dilution method. SDA plates were prepared with increasing concentration of clove EO from 1 μ l/ml to 12 μ l/ml (v/v). Tween-20 (0.05 %) was used to dissolve EO in the medium. Plates were inoculated in the center with mycelial disc (4 mm) and incubated for 5 days followed by measurement of mean radial colony diameters. Three replicates were made for each treatment and medium with Tween-20 alone was set as control. The plate with lowest concentration of clove EO, showing no visible growth was regarded as the MIC (13).

Separation of clove EO fractions through column chromatography

Clove EO was subjected to column chromatography for fractionation based on increasing solvent polarity. Silica gel (60-120 mesh) slurry was made in petroleum ether 40-60 °C and poured into a glass column (60 x 3 cm, ASGI, India) with sintered disc, to make an effective column of 45 x 3 cm size. Analytical grade solvents of Qualigens, India were used. Ten milliliter of clove oil was loaded onto column bed and eluted first with 300 ml petroleum ether followed by 300 ml each of ether:chloroform (5:5), chloroform:ethyl acetate (3:2), chloroform:ethyl acetate (5:5), chloroform:ethyl acetate (2:8) and lastly with methanol. Column fractions were collected in pre weighed conical flasks (500 ml) and were later concentrated by using rotary vacuum evaporator, buchi type

(Scientech, India) at 50 °C and finally weighed.

Antifungal assay of column fractions

Antifungal potential of all the six column fractions was determined through agar well diffusion assay. Fractions were equally diluted with 200 μ l ethanol in order to dissolve the crystallized fractions. Quantity used for bioassay was 5 μ l along with 45 μ l of DMSO, to make final volume of 50 μ l. Eugenol (95%) 5 μ l in 45 μ l DMSO (Oxford Chemicals, India) was used as positive control and DMSO (50 μ l) alone served as negative control.

Analysis of column fractions through TLC

Silica gel TLC was performed for analysis of column fractions along with eugenol standard. Silica plates of 1 mm thickness were prepared with silica gel H (Qualigens, India) by using TLC apparatus (Perfit India Ltd., India). Plates were activated in an oven (Tempo, India) for 2 hours at 65 °C. Six column fractions 5 μ l each were spotted with the help of Hamilton syringe (Germany) along with eugenol standard. Petroleum ether, toluene and ethyl acetate in a combination of 7:2:1 was used as developing solvent. When the solvent phase reached the top, the plate was air dried and exposed to iodine vapours in a glass chamber for 30 min. The plate was then taken out and photographed (11).

Analysis of clove EO through HPLC

HPLC analysis of clove oil and laboratory reagent (LR) grade eugenol (Oxford Chemicals, India) was done in isocratic mode by using Shimadu's prominent HPLC system using a reverse phase C18 column [Phenomenex Luna, 5 μ C18 (2) 100A (250 \times 4.60 mm)], eluent: methanol/acetonitrile (70:30), UV detector: 254 nm, flow rate 0.5 ml/min, column temperature 40°C. One hundred milligrams of EO was dissolved in 5 ml of mobile phase solvent and 20 μ l was injected. The eugenol content was quantified based on the peak areas obtained in HPLC analysis taking all the peak areas together as 100%.

Microscopic study of effect of clove EO and fraction II on fungal mycelium and spores

Fifty microliters of clove EO and fraction II were put on the center of the glass slides. Fresh fungal mycelium piece bearing spores was taken out of petriplate with inoculating needle and immersed in the drop of clove oil on slides. After 1 h of incubation at ambient temperature, the slides were examined under light microscope and photographed.

RESULTS AND DISCUSSION

The results of the preliminary screening shown in Fig. 1 clearly indicate that clove essential oil (EO) is highly antifungal towards all tested fungal species. Considerable variation in inhibition zone sizes ranging from 12-22 mm was observed among fungal isolates depending on their sensitivity towards clove EO. The order of sensitivity in the descending

array was *Mucor* sp. > *M. gypseum* > *F. moniliforme* > *T. rubrum* > *Aspergillus* sp. > *F. oxysporum*. The positive control ketoconazole was very effective even at low concentration as it showed higher inhibition zones against all the fungal strains. No inhibition zones were observed with in negative control DMSO.

Lee *et al.* (13) elucidated the antifungal activities of eugenol and nerolidol isolated from Japanese cypress oil in a guinea pig model infected by *M. gypseum*. Their results suggest that eugenol and nerolidol could apply supplementary antifungal agents. Nunez *et al.* (14) demonstrated that the association of clove oleoresin with concentrated sugar has a strong fungicidal effect against *C. albicans*, *P. citrinum*, *A. niger* and *T. mentagrophytes*. Ahmad *et al.* (2) reported clove oil to possess strong antifungal activity against *C. albicans*, *C. neoformans* and *A. fumigatus*.

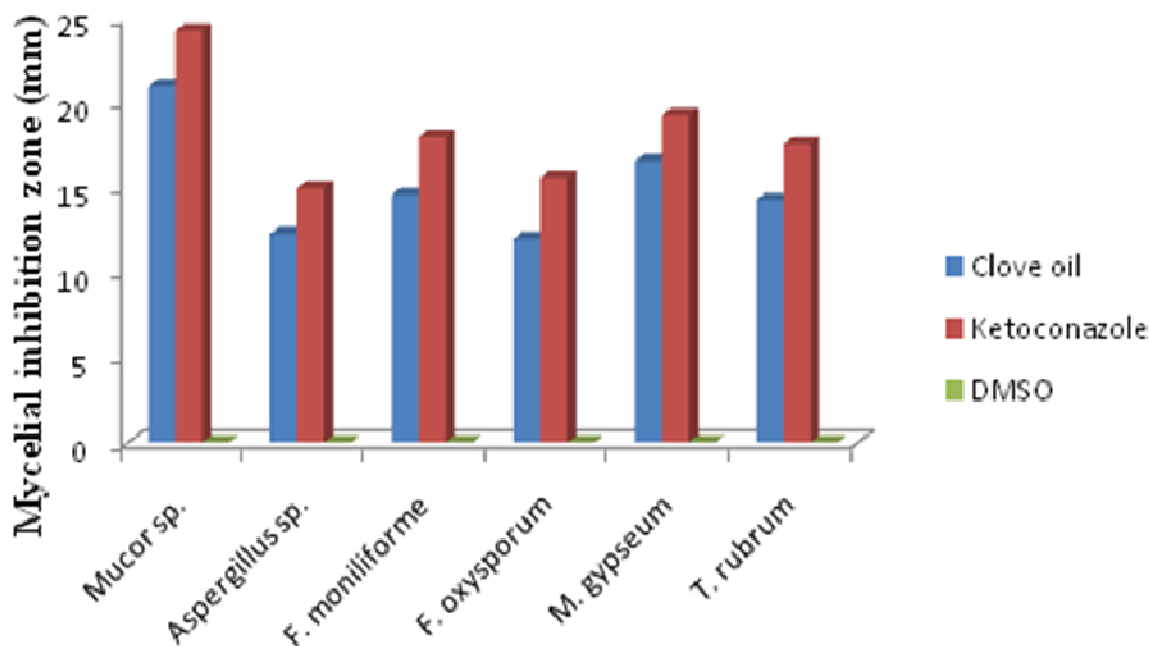


Figure 1. Mycelial inhibition (mm) of various fungal strains with clove EO (10 μ l), Ketoconazole (1mg/ml) and DMSO (10 μ l)

MIC of clove EO for the tested fungal strains was found to be from 9-12 $\mu\text{l/ml}$ (Table 1). *Mucor* sp., *M. gypseum* and *T. rubrum* were inhibited at 9 $\mu\text{l/ml}$ of clove oil followed by *Aspergillus* sp. and *F. oxysporum* at 10 $\mu\text{l/ml}$ and *F. moniliforme* at 12 $\mu\text{l/ml}$. Mycelial inhibition was concentration dependent as the colonies diameters were decreased with increasing quantity of EO. Except for *Mucor* and *Aspergillus* all other fungal strains exhibited considerable inhibition even at

1 $\mu\text{l/ml}$ concentration.

Pinto *et al.* (18) studied the antifungal activity of clove essential oil, obtained from *S. aromaticum*. They determined MIC to evaluate the antifungal activity of the clove oil and its main component, eugenol, against *Candida*, *Aspergillus*, and dermatophyte clinical and ATCC (American Type Culture Collection) strains. The essential oil and eugenol showed inhibitory activity against all the tested strains.

Table 1. MIC of clove oil against fungal strains after 5 days of incubation

Fungal strain	Mycelial diameter (mm)								
	Negative Control	Different conc. of clove oil ($\mu\text{l/ml}$) in medium							
		1	2	4	8	9	10	11	12
<i>Mucor</i> sp.	100	100	100	32 \pm 0.8	21 \pm 0.8	-	-	-	-
<i>Aspergillus</i> sp.	100	100	100	31.3 \pm 0.9	20.6 \pm 0.4	3.3 \pm 0.4	-	-	-
<i>F. moniliforme</i>	100	31.3 \pm 0.4	10 \pm 0.8	8.6 \pm 0.4	7.6 \pm 0.9	6.3 \pm 0.4	5.6 \pm 0.4	2.6 \pm 0.4	-
<i>F. oxysporum</i>	67.3 \pm 1.7	27.6 \pm 0.4	9.6 \pm 0.4	8.3 \pm 0.4	7.3 \pm 0.4	3.3 \pm 0.4	-	-	-
<i>Microsporium gypseum</i> .	19.3 \pm 0.9	4.6 \pm 0.4	4.3 \pm 0.4	3.6 \pm 0.4	3.3 \pm 0.9	-	-	-	-
<i>Trichophyton rubrum</i>	27.6 \pm 0.4	11 \pm 0.8	8 \pm 0.8	5.6 \pm 0.4	3.6 \pm 0.9	-	-	-	-

Figures are Mean \pm SD

'-' indicates absence of any visible growth

Table 2 shows that among the column eluents, highest quantity of 5.49 g was obtained in ethereal fraction followed by ether:chloroform (5:5) and chloroform:ethyl acetate (3:2). All other eluted components were in meager quantity. Non polar fractions were oily and light colored while polar fractions were solids and dark colored. The result of antifungal assay shown in Table 3 indicates that column fraction II & III possess

antifungal components, especially fraction II exhibited higher degree of inhibition, comparable to that of eugenol standard. This gave an indication that column fraction II may contain substantial amount of eugenol. It is also possible that some minor compounds present in fraction II may have promoted the inhibitory potential to some extent. In rest of the fractions no inhibition was observed.

Table 2. Quantity, consistency and color of six column fractions and clove

Column Fractions eluted with following solvent	Weight of samples (g)	Consistency/color
Ether – I	5.49	Light colorless Oil
Ether: Chloroform (5:5) – II	2.22	Viscous yellow oil
Chloroform: Ethyl acetate (3:2) – III	0.58	Semisolid yellow
Chloroform: Ethyl acetate (5:5) – IV	0.03	Brown solid
Chloroform: Ethyl acetate (2:8) – V	0.08	Brown solid
Methanol – VI	0.05	Dark brown solid

Table 3. Antifungal assay of six column fractions

Fungal species	Mycelial zone inhibition diameter (mm) in following column fractions (5µl)						Inhibition zone in eugenol 95% (5µl)
	I	II	III	IV	V	VI	
<i>Mucor sp.</i>	-	20.3	3	-	-	-	22.6
<i>Aspergillus sp.</i>	-	13.6	4.6	-	-	-	14
<i>F. moniliforme</i>	-	15	4	-	-	-	15
<i>F. oxysporum</i>	-	13.3	3	-	-	-	14.6
<i>Microsporium gypseum</i>	-	18	4.3	-	-	-	21.3
<i>Trichophyton rubrum</i>	-	16	3.6	-	-	-	17

'-' indicates absence of any visible zone

TLC results of column fractions (Lanes 1-6) along with eugenol standard (Lane 7) are shown in Fig. 2. Upon exposure of TLC plate to iodine vapors the result clearly shows presence of high quantity of eugenol in column fraction II. Small quantity of eugenol was also present in fraction III as a result of which little inhibition zones were also observed in this fraction. The non polar oily compounds in fraction I moved faster with developing solvent close to the solvent front. The highly polar compounds in fraction V travelled only few centimeters, and that of fraction VI didn't moved at all on TLC plate. The R_f value calculated for column

fraction II at lane 2 and for eugenol standard was 0.85 cm. The result of antifungal assay of column fractions and that of TLC proves that the antifungal activity of the clove oil is due to its high eugenol content.

Park *et al.* (16) found similar results in relation to clove EO. They performed TLC and column chromatography assay to identify the active antifungal components and their fractionation. They concluded that eugenol was the most effective antifungal constituent of clove oil against the dermatophytes *T. mentagrophytes* and *M. canis*.

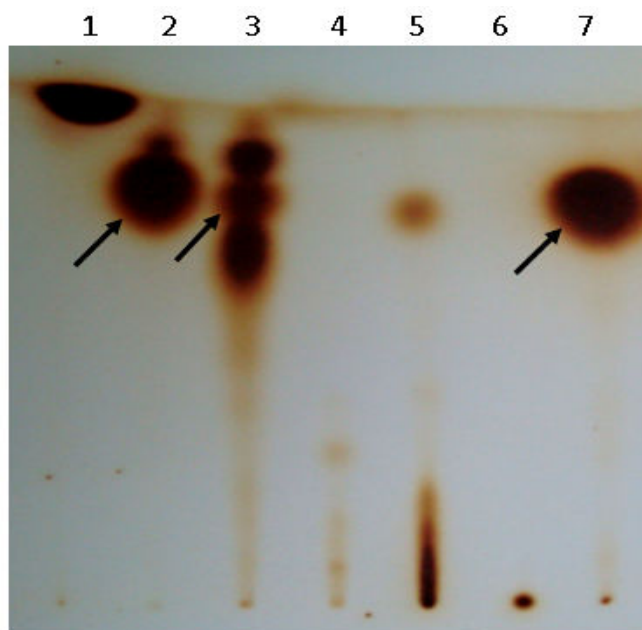


Figure 2. TLC plate showing column fractions (I, II, III, IV, V, VI) respectively in lane 1, 2, 3, 4, 5, 6. Lane 2 (column fraction II) showed presence of substantial amount of eugenol when compared with standard eugenol spot (5µl) at lane 7.

HPLC analyses of eugenol (LR grade) and clove oil are shown in Fig. 3A and 3B respectively. Peak areas of graphs depicted percentage of eugenol to be 90.55% represented by retention time 3.056 min. Clove oil, when compared with

eugenol, similar peak at a retention time of 3.045 min was observed which is 68.73%. Since, the principal component in clove oil is eugenol therefore; its strong antifungal activity may be attributed to eugenol.

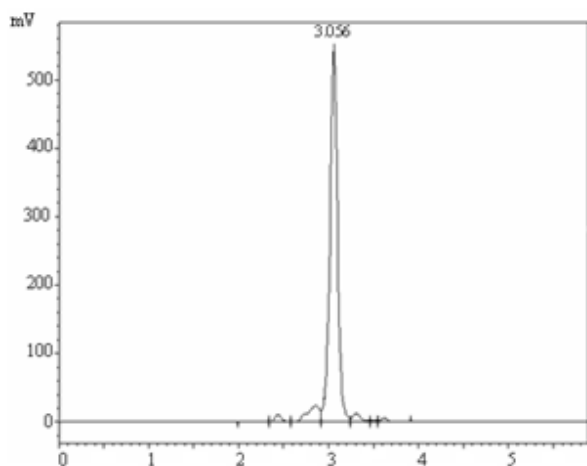


Fig. 3A

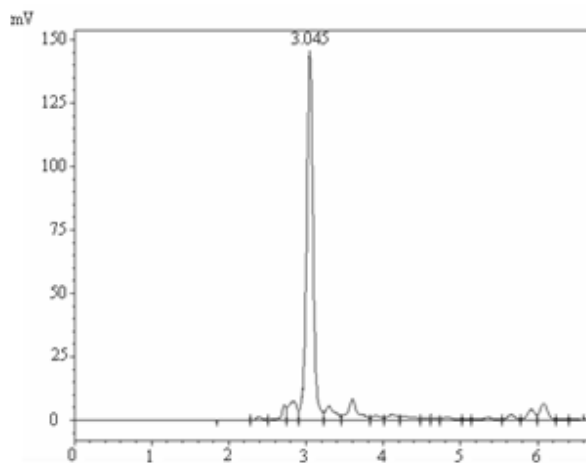


Fig. 3B

Figure 3. A: HPLC elution profile of eugenol (95% LR grade) with retention time of 3.056 min confirmed 90.55% eugenol. B: HPLC elution profile of clove oil confirmed presence of 68.73% eugenol with retention time of 3.045 min

Some authors have done HPLC for purification and analysis of essential oils. Benincasa (5) analyzed essential oils such as citrus by HPLC with standard columns in reversed and normal phase. Volatile and non-volatile fractions were investigated, and in the non-volatile fraction some coumarins were identified. Rauber *et al.* (19) developed a HPLC method for determination of citral in volatile oil of *Cymbopogon citratus*.

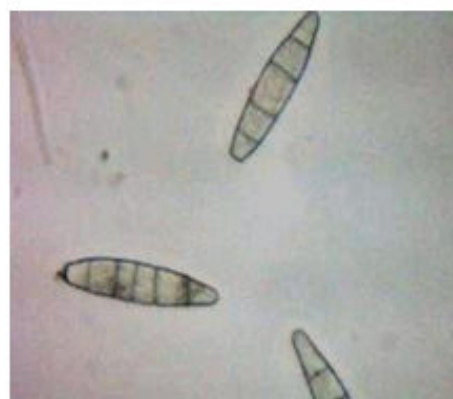
Pinto *et al.* (18) analyzed clove oil obtained commercially by gas chromatography and by gas-chromatography-mass spectroscopy. The essential oil showed high contents of eugenol (85.3 %). Ayoola *et al.* (4) has explained gas chromatography-mass spectrometry (GC-MS) analysis of the clove oil revealing that the components were eugenol, caryophyllene, eugenol acetate and alpha-humelene, with eugenol being the main component. Cheng *et al.* (8) revealed in antifungal tests that cinnamaldehyde, α -methyl

cinnamaldehyde, (*E*)-2-methylcinnamic acid, eugenol and isoeugenol exhibited strong antifungal activity against all fungi tested. Chami *et al.* (7) evaluated carvacrol and eugenol, the main (phenolic) components of essential oils of some aromatic plants for their therapeutic efficacy in the treatment of experimental oral candidiasis induced by *Candida albicans* in immunosuppressed rats. They claimed that, carvacrol and eugenol could be considered as strong antifungal agents and could be proposed as therapeutic agents for oral candidiasis.

After 1 hr of treatment of fungal mycelia bearing spores and conidia with clove oil, lysis and distortion of spores was observed in all the fungal isolates. The effect was profound and clearly visible in form of deformities/distortion of spore structure. In case of *M. gypseum* spores, leakage at multiple sites with balloon like swellings was observed (Fig. 4B and 5B). Similar effects were noted when spores were treated with standard eugenol.



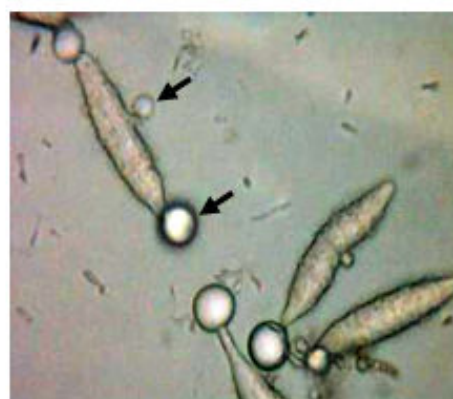
(4A)



(5A)



(4B)



(5B)

Figure 4. A: Showing *Mucor* sp. spores of control. 4B Showing deformities/ distortion of spores of *Mucor* sp. upon treatment with column fraction II. Fig. 5A: Showing *M. gypseum* spores of control. 5B Showing leakage of cellular components of spores of *M. gypseum* at tips and near center upon treatment with column fraction II

Park *et al.* (16) studied effect of clove oil on *Trichophyton mentagrophytes* with the help of transmission electron microscope. They observed the expansion of its endoplasmic reticulum near the cell membranes of a hyphal specimen treated with eugenol. In addition, the inner mitochondrial membranes were partially destroyed, with complete destruction of the cell wall. According to their results, they concluded that the antifungal activity of eugenol toward *T. mentagrophytes* is due to changes in fungal cell structure at the membrane level.

It is concluded, that the essential oil of *S. aromaticum* was active against all the tested fungal strains with low MIC values. Purification of active ingredient eugenol can be accomplished

through column chromatography. Analysis of column purified fractions through TLC and GC showed presence of major quantity of eugenol in fraction II. Microscopic study clearly indicated lytic effect of clove oil and column fraction II on fungal mycelium and spores. Hence the anti-fungal property demonstrated by the clove EO is attributed to the eugenol.

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REFERENCES

1. Abad, M.J.; Ansuategui, M.; Bermejo, P. (2007). Active antifungal substances from natural sources. *Arkivoc.*, 7, 116-145.
2. Ahmad, N.; Alam, M.K.; Shehbaz, A.; Khan, A.; Mannan, A.; Rashid, H.S.; Bisht, B.; Owais, M. (2005). Antimicrobial activity of clove oil and its potential in the treatment of vaginal candidiasis. *J. Drug Targeting*. 13, 555-561.
3. Arras, G.; Usai, M. (2001). Fungitoxic activity of essential oils against four post-harvest citrus pathogens: Chemical analysis of *Thymus capitatus* oil and its effect in sub-atmospheric pressure conditions. *J. Food Prot.* 64, 1025-1029.
4. Ayoola, G.A.; Lawore, F.M.; Adelowotan, T.; Aibinu, I.E.; Adenipekun, E.; Coker, H.A.B.; Odugbemi, T.O. (2008). Chemical analysis and antimicrobial activity of the essential oil of *Syzygium aromaticum* (clove). *African J. Microbiol. Res.* 2, 162-166.
5. Benincasa, M.; Buiarelli F.; Cartoni, G.P.; Coccioli, F. (1990). Analysis of lemon and bergamot essential oils by HPLC with microbore columns. *Chromatographia*. 30, 271-276.
6. Cai, L.; Wu, C.D. (1996). Compounds from *Syzygium aromaticum* processing growth inhibitory activity against oral pathogens. *J. Nat. Prod.* 59, 987-990.
7. Chami, N.; Chami, F.; Bennis, S.; Trouillas, J.; Remmal, A. (2004). Antifungal treatment with carvacrol and eugenol of oral candidiasis in immunosuppressed rats. *Braz. J. Infect. Dis.* 8, 217-226.
8. Cheng, S.S.; Liu, J.Y.; Chang, E.H.; Chang, S.T. (2008). Antifungal activity of cinnamaldehyde and eugenol congeners against wood-rot fungi. *Bioresource Technol.* 99, 5145-5149.
9. Hammer, K.A.; Carson, C.F.; Riley, T.V. (1999). Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol.* 86, 985-990.
10. Harbone, J.B. (1998). Essential oils. In: *Phytochemical Methods: A guide to modern techniques in plant analysis*, 3rd ed. Chapman and Hall, PA, USA. pp. 110-124.
11. Hostettmann, K. (1999). Strategy for the biological evaluation of plant extracts. *Pure App. Chem.* 70, 1109-1113.
12. Kim, H.M.; Lee, E.H.; Hong, S.H.; Song, H.J.; Shin, M.K.; Kim, S.H.; Shin, T.Y. (1998). Effect of *Syzygium aromaticum* extract on immediate hypersensitivity in rat. *J. Ethnopharmacol.* 60, 125-131.
13. Lee, S.J.; Han, J.I.; Lee, G.S.; Park, M.J.; Choi, I.G.; Na, K.J.; Jeung, E.B. (2007). Antifungal effect of eugenol and nerolidol against *Microsporum gypseum* in a guinea pig model. *Biol. Pharm. Bull.* 30, 184-188.
14. Nunez, L.; D'Aquino, M.; Chirife, J. (2001). Antifungal properties of clove oil (*Eugenia caryophyllata*) in sugar solution. *Brazilian J. Microbiol.* 32, 123-126.
15. Panizzi, L.; Falmini, G.; Cioni, P.L.; Morelli, I. (1993). Composition and antimicrobial properties of essential oil of four Mediterranean *Lamiaceae*. *J. Ethnopharm.* 39, 167-170.
16. Park, M.J.; Gwak, K.S.; Yang, I.; Choi, W.S.; Jo, H.J.; Chang, J.W.; Jeung, E.B.; Choi, I.G. (2007). Antifungal activities of the essential oils in *Syzygium aromaticum* (L.) Merr. Et Perry and *Leptospermum petersonii* Bailey and their constituents against various dermatophytes. *The J. Microbiol.*, 45, 460-465.
17. Perez, C.; Paul, M.; Bazerque, P. (1990). Antibiotic assay by agar - well diffusion method. *Acta. Bio. Med. Exp.* 15, 113 - 115.
18. Pinto, E.; Vale-Silva, L.; Cavaleiro, C.; Salgueiro, L. (2009). Antifungal activity of the clove essential oil from *Syzygium aromaticum* (*Eugenia caryophyllus*) on *Candida*, *Aspergillus* and dermatophyte species. *J. Med. Microbiol.* 58, 1454-62.
19. Rauber, C.S.; Guterres, S.S.; Schapoval, E.E.S. (2005). LC determination of citral in *Cymbopogon citratus* volatile oil. *J. Pharma. Biomed. Anal.* 37, 597-601.
20. Santos, A.L.; Chierice, G.O.; Alexander, K.S.; Riga, A.; Matthews, E. (2009). Characterization of the raw essential oil eugenol extracted from *Syzygium aromaticum* L. *J. Therm. Anal. Calorim.* 96, 821-825.
21. Yalentesfa, B.; Alemu, T.; Santhanam, A. (2010). Solid substrate fermentation and conversion of orange waste in to fungal biomass using *Aspergillus niger* KA-06 and *Chaetomium* Spp KC-06. *African J. Microbiol. Res.* 4, 1275-1281.
22. Zheng, G.Q.; Kenney, P.M.; Lam, L.K. (1992). Sesquiterpenes from clove *Eugenia caryophyllata* as potential anticarcinogenic agents. *J. Nat. Prod.* 55, 999-1003.



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