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Full Length Research Paper

## Survey of *Cryptococcus neoformans* in pigeon (*Columba livia*) excreta in Public Square in Umuarama, Paraná, Brazil

Gilneia Rosa, Luiz sérgio Merlini\*, Wellington Henrique Bessi, Arianne Peruzo Pires Gonçalves, Lorena Zacharias Silva, Pamela Roberta Perussi, Paulo Henrique Sposito and Pinto Neto Adalgiza

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**Cryptococcosis is a common opportunistic fungal infection caused by encapsulated yeast of the *Cryptococcus* genus, mainly by the *Cryptococcus neoformans* and *Cryptococcus gattii* species. The fungus is acquired through the inhalation of environmental propagules or, more rarely, through organ transplants in immune-compromised individuals. Domestic pigeons (*Columba livia*) are related to the infection, mainly as natural reservoirs for the fungus, a fact that is relevant due to the large concentration of these animals in public areas, mainly in places of large circulation of people. In order to isolate and diagnose *Cryptococcus* spp., a total of 50 excretion samples from pigeons from five public squares located in the central region of Umuarama, PR, were analyzed, being 10 samples from each square, totaling 50. From the analyzed samples, 100% (50) were negative for the capsulated yeast. Even though all samples were negative, the creation of educative campaigns aiming to raise awareness of the population on the risk of acquiring cryptococcosis and the importance of not feeding the pigeons in leisure areas, the main strategy in the population control of pigeons, were suggested.**

**Key words:** *Cryptococcus neoformans*, yeast, pigeons, zoonosis, public square.

### INTRODUCTION

Cryptococcosis is a systemic nature of mycosis caused by *Cryptococcus* fungus genus, whose infection occurs through inhalation of fungus present in the feces of pigeons (Sidrim and Rocha, 2010). Fungi from the *Cryptococcus* genus are composed of 100 species, with two clinically relevant, the *Cryptococcus neoformans* and *Cryptococcus gattii*. This is an sexual encapsulated yeast fungus of the *Filobasidiella neoformans* basidiomycete

(Del Poeta and Casadevall, 2010).

The fungus presents tropism via the central nervous system (CNS), respiratory and integumentary system. The commitment of the immune response is the main predisposing factor for the occurrence of the disease, both in healthy people and immunodepressed patients, bearers of the acquired immunodeficiency syndrome (AIDS) (Pappalardo and Melhem, 2003).

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Mortality by cryptococcosis is estimated at 10% in developed countries, and it can reach 43% in developing countries (Kon, 2008). Within the characteristics observed in *C. neoformans* studies, the presence of capsules is observed. This structure is used in the identification and studies based on antigens that are specific to the mucopolysaccharide capsule. *C. neoformans* has been divided into three varieties and five serotypes, with serotype A belonging to the *grubii* variety (Franzot et al., 1999), serotype D to the *neoformans* variety, and B and C to the *gattii* variety (Sorrell, 2001).

Cryptococcosis is among the emergent fungal infections with significant lethality and morbidity, mainly in the form of meningoencephalitis, which is usually secondary to skin or lung processes, in either immunodepressed or immunocompetent individuals (Lacaz, 2002).

Environments with large concentration of excreta favor the dissemination of the yeast, since the infection by *C. neoformans* is acquired through the inhalation of environmental propagules by dehydrated yeast that can be easily dispersed in the air (Filiú et al., 2002). Due to the high concentration of *C. neoformans* in pigeon excreta, together with the high density of these birds in urban areas and the severity of human cryptococcosis, there is a growing interest in the study of the relationship among the birds, their excreta and the disease in humans (Huffnagle et al., 1999).

From a clinical and epidemiological point of view, cryptococcosis can be opportunistic, cosmopolitan, associated with cellular immune-depression conditions caused by *C. neoformans*. According to Lacaz (2002), cryptococcosis is fundamentally opportunistic, and immunodepression is the relevant factor for the high frequency of this infection.

The objective of this paper was to identify the presence of *C. neoformans* in pigeon excreta collected from squares in the central region in the city of Umuarama, Paraná.

## MATERIALS AND METHODS

The city of Umuarama is located in the northwestern region in the state of Paraná, Brazil (latitude 23° 47' 55 south and longitude 53° 18' 48 west) and has 106,387 inhabitants (IBGE, 2012), with population miscegenation of several ethnicities, with different behavioral and cultural habits.

The city has a total of 10 squares, between central and peripheral ones. For the sampling, five squares were selected (Unipar, Artur Thomas, Santos Dumont, Miguel Rossafa and Rodoviária), as shown in Figure 1, where 10 samples were collected from each square, totaling 50 samples. For the inclusion of the square, the criteria of people flow and large concentration of pigeons were used, these places are located in the central area of the city, with a concentration of shopping streets, which increases the flow of personnel and there is visual observation of the presence of pigeons in these squares.

The collections took place from March to September 2014, and random samples of pigeon feces were chosen in these squares.

With the aid of spatulas and gloves, approximately five grams of old and dry excreta were collected, since they offer substrate that is favorable to the growth of yeast and present lower amount of bacteria, since there is no nutritional competition among these microorganisms (Reolon et al., 2004). The samples were collected on alternate days, between 8 and 9 a.m., in different temperature and humidity conditions, with a few samples being exposed to sun radiation, while others were in the shadows, the minimum temperature was 24.7°C and maximum was 38.7°C.

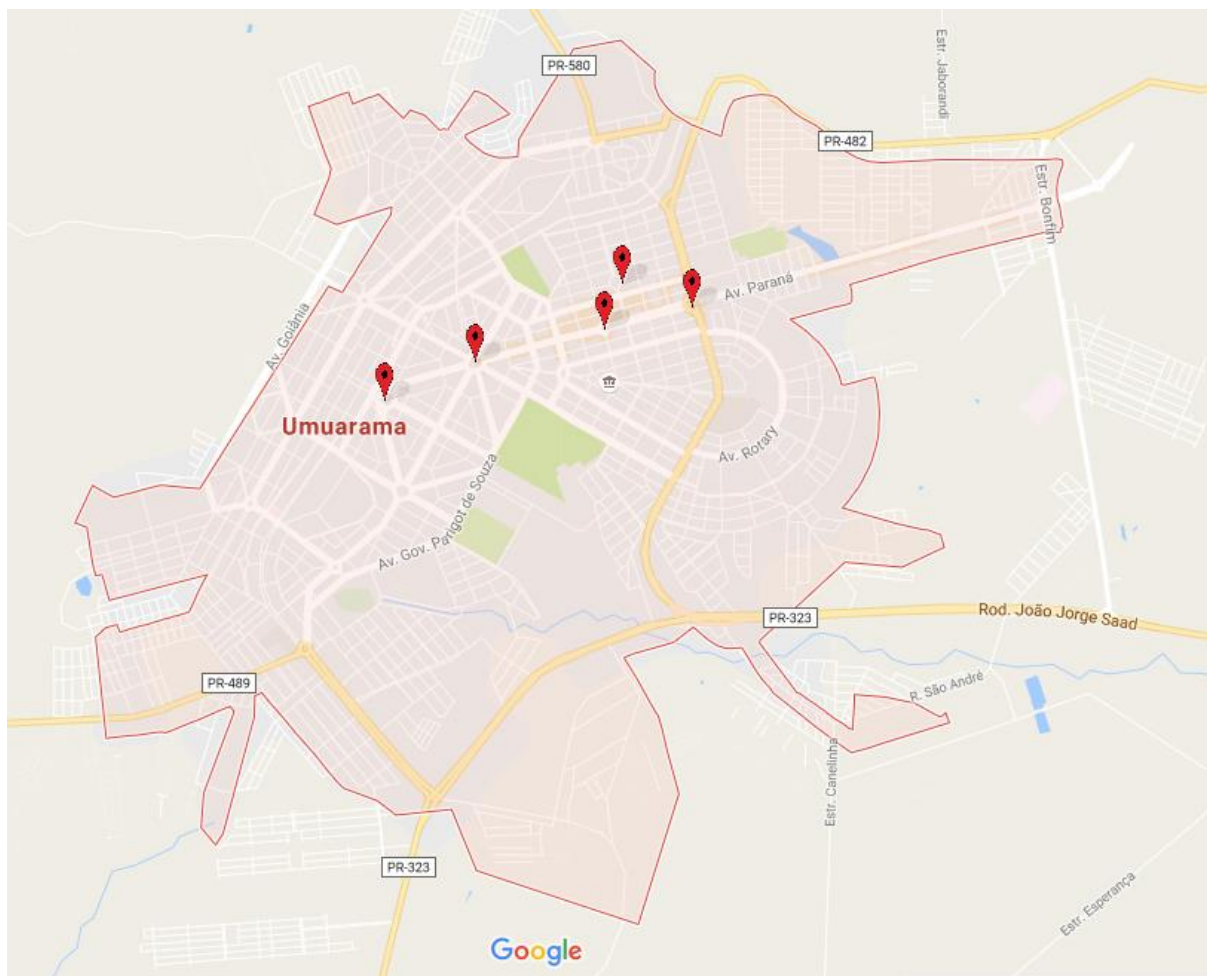
After collection, the excreta were stored in sterile containers, duly identified and forwarded to the Laboratory of Culture and Microbiology at Universidade Paranaense - UNIPAR, where they were stored at room temperature for 48 h. The samples were homogenized with 2 mL sterile saline solution, the supernatant was aspirated and sown onto Petri dishes; each sample was sown on a plate containing Agar Niger culture medium, as indicated in Santos et al. (2009). The plates were incubated for seven days, at 29 to 30°C (Machado et al., 1993). In order to confirm colonial growth in culture medium with *Cryptococcus* spp., coloration was observed, which ranges from light beige to brown. These colonies were applied to blades microscopic analysis of the fungus and stained with China dye, which is the standard dye for visualization of the fungal capsule, and later analyzed under an optical microscope with 1000x magnification. Colonies with yeast characteristics were sent to urease test, an enzyme that aids the diagnosis (Fisher and Cook, 2001).

## RESULTS AND DISCUSSION

Fifty stool samples from pigeons were analyzed, the samples showed no positive results for the isolation and diagnosis of *Cryptococcus* spp. and *Cryptococcus neoformans* is absent in all samples. Environmental sources of *C. neoformans* are very diversified and related to a few organic substrates, among them bird excreta and vegetables. This species is the main etiological agent of cryptococcosis, a disease considered by a few authors as a fatal infection, which can be transmittable to humans in locations with high number of pigeons or by the contact with the corresponding niche through inhalation of a large amount of propagules (Lazera et al., 1993).

The use of the urease test for diagnosing *Cryptococcus* is due to the capacity of the fungus in hydrolyzing urea through a metalloenzyme, resulting in ammonia and carbamate, with a change in the pH of the medium, changing its color from yellow to pink. This ability for hydrolyzing urea is important to differentiate other yeast species, such as *Candida* spp. (Sidrim and Rocha, 2010). The use of the Niger medium provides better visualization and characterization of the colony than the traditional methods for fungal culture. The capacity for producing melanin with the formation of brown colonies in Niger seed extract media is a characteristic of *C. neoformans* and *C. gattii*, not happening in other species from the *Cryptococcus* genus, nor other yeasts of medical interest (Santos et al., 2009).

There are several studies identifying the presence of the *C. neoformans* fungus in bird excreta. However, there are few reports of cryptococcosis in pigeons (Mitchell and



**Figure 1.** City map of Umuarama, with the location of the five cities (Unipar, Artur Thomas, Santos Dumont, Miguel Rossafa and Rodoviária) where the samples were collected in 2014.

Perfect, 1995), that are resistant to the invasion of the fungus due to their elevated body temperature (Reolon et al., 2004).

According to Bulmer (1990), the problem is the long viability of *C. neoformans* in dry excreta, of approximately two years. Based on this information, old buildings and towers of old churches can be considered potential sources of *C. neoformans* and must be periodically inspected by public health authorities.

*C. neoformans* is cosmopolitan and opportunistic, especially affecting immunodepressed individuals (Sorrell, 2001). *C. gattii* has a stricter distribution, being usually associated to tropical and subtropical areas (Boekhout et al., 2001). Studies show a variation in the isolation of *C. neoformans* from pigeon excreta ranging from 24.7 to 4.6% (Faria et al., 2010).

Several researchers have found environmental sources of *Cryptococcus* in different regions in Brazil. Silva and Capuano (2008) reported the occurrence of *Cryptococcus*

spp. and parasites of public health interest in pigeon excreta in the city of Ribeirão Preto, São Paulo. In Brazil, clinical and epidemiological studies have shown the importance of cryptococcosis by *C. gattii* for the CNS in young adults of both genders and in children in the North and Northeast regions, with a lethality of 35 to 40% (Correa et al., 1999).

Regarding the isolation of *Cryptococcus* spp. in pigeon excreta, the positivity in our study was low in relation to similar studies performed in Brazil. In the southern region, Reolon et al. (2004) detected the fungus in 100% of the samples in Porto Alegre and Faria et al. (2010) recorded positivity of 26.9% in environmental samples in Pelotas. Abegg et al. (2006) analyzed excreta from 59 captivity bird species kept in cages in a zoo in Rio Grande do Sul, where the birds belonged to 12 different orders, 38 samples found positive for *C. neoformans* in environments with Psittaciformes.

Yamamura et al. (2013) isolated pathogenic fungi from

the *C. neoformans* and *C. gattii* species in the environment represented by the soil where there is a large movement of passers-by in the central region of Londrina, such as parks, squares and streets. It was isolated in eight (2.2%) of 360 samples. These fungi were isolated from the soil with pigeon excreta and vegetable matter near trees in unsheltered areas, and exposed to constant rain and sunlight.

In Lages/SC, Menezes et al. (2014) analyzed 195 samples of pigeon excreta from six squares located in the central region in the city of Lages, SC. From the analyzed samples, 7.69% (15) were positive for the capsulated yeast. In Cuiabá- MT, Takahara (2013) reported the occurrence of 6.6% *C. neoformans* in 122 samples of dry pigeon excreta collected in 49 locations in the city. Correa et al. (2011) found 100% positive samples for *C. neoformans* in excreta from *Columba* sp. in the city of Cacoal, Rondônia, Brazil.

It is of pivotal importance to guide the population not to feed the pigeons so that there is no proliferation of these animals near homes, schools, squares, churches, sheds, silos and others; as well as closing locations where they are likely to shelter and build their nests (Lima and Lima, 2013).

Cryptococcosis occurs as the first opportunistic manifestation in approximately 4.4% of AIDS cases in Brazil. The prevalence of cryptococcosis associated with AIDS is estimated to be between 8 and 12% in reference centers in the southeastern region (Brasil, 2012). Delgado et al. (2005) and Lindenberg et al. (2008) reported that infection by *C. gattii* in humans and animals has a broader geographical occurrence than what has been habitually described, and its clinical and epidemiological aspect is not well known, therefore, it is necessary to differentiate it from opportunistic cryptococcosis by *C. neoformans*. In regions with large endemism, it is significantly associated with AIDS. However, the authors stated that, on the other hand, *C. neoformans* is capable of causing fatal infection in apparently normal individuals.

Environmental factors might influence in the isolation and diagnosis of the yeast, since the weather in a certain region, allied to temperature, humidity and exposure to sun radiation can influence the recovery and isolation of fungal strains from environmental origin (Quintero et al., 2005).

One of the main strategies is the control of pigeons, and a preventive measure is to damp the locations where there are large accumulation of pigeon feces to avoid the fungus to be dispersed by aerosol. There is no need for notification or isolation of the sick individuals (Hill et al., 1995).

The Ministry of Health does not consider Cryptococcosis as a disease of mandatory notification; therefore, there are no statistical data on its incidence in the city of Umuarama, Paraná. The multiplication of the fungus in

feces and environments with lack of cleaning, together with an increase in temperature, provides the fungus basidiospores, by aerogenous route from the excreta, the contamination of *Passeriformes* and *Psittaciformes* (Lugarini et al., 2008), yeast reservoirs, as well as contamination of other environments near pigeon niche (Rosario et al., 2008).

## Conclusion

The low occurrence in the isolation of *C. neoformans* in this study does not exclude the risk for human health, since pigeons are part of the landscape in urban centers, such as in the city of Umuarama, Paraná; there is no need to carry out survey in all the squares of the municipalities in the future.

## Conflict of interests

The authors have not declared any conflict of interest.

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## Full Length Research Paper

# Induction of defense mechanisms from filtrates of saprophytic fungi against early blight disease in tomato

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The induction of defense enzymes presents efficient plant disease management when triggered by metabolic products of microorganisms. The aim of the present study is to select filtered saprobes fungi used for the management of early blight of tomato by inducing pathogenesis related to tomato plant. Filtrates of the fungi *Curvularia eragrostidis*, *Curvularia inaequalis*, *Memnoniella echinata* and *Pseudobotrytis terrestris* were cultured in potato and dextrose (PD) media and maintained in a growth chamber at  $25 \pm 2^\circ\text{C}$ , with a photoperiod of 12 h light. After 20 days, the mycelial mass was removed through filtration. Concentrations (0, 5, 10, 15 and 20%) of filtrates and Acibenzolar-S-Methyl were applied in the 3rd tomato leaf 3 days before inoculation with *Alternaria solani*. Then the disease severity was analyzed calculating the area under the disease progress curve. The activity of the enzymes, catalase, guaiacol peroxidase and phenylalanine ammonia-lyase (locally and systemically) at plant control (72 h before inoculation) and 0, 24, 48, 72 and 96 h after inoculation with *A. solani* was analyzed. *C. eragrostidis*, *C. inaequalis*, *M. echinata* and *P. terrestris* filtrates reduced the AUDPC, both locally and systemically. Greater activity of phenylalanine ammonia-lyase was observed in plants treated with *C. eragrostidis* (local and systemic) and *C. inaequalis* filtrates (local). The filtrate of *P. terrestris* promoted greater catalase activity, either locally or systemically. The filtrate of *M. echinata* increased peroxidase activity locally and systemically. The filtrates tested are resistance inducers used for the management of tomato early blight, although further testing is necessary to identify elicitors present in filtrates.

**Key words:** *Alternaria solani*, alternative control, proteins related to pathogenesis, *Lycopersicon esculentum*.

## INTRODUCTION

*Lycopersicon esculentum* is one of the most important crops in the world agricultural scenario. It constitutes an important product for "in natura" trade and extract industry. It generates direct and indirect employment. However, tomato production is considered a risky activity

because the culture is affected by many diseases. Among these, tomato early blight, caused by the fungi *Alternaria solani*, is a major disease which causes great losses for producers, as it is a highly destructive disease and of rapid proliferation. It focuses on leaves, stems,

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petioles and fruits of tomato. Studies conducted by Tofoli et al. (2003) on conditions of Brazil observed that early blight, without control, can cause 57% loss in yield of commercial fruits and cause qualitative losses (55%) through burning of fruits caused by defoliation.

Crops that are highly susceptible to pathogens require the adoption of constant pesticide applications during cultivation. The awareness of the problems generated by the adoption of isolated methods for disease management by chemical methods has increased research technologies such as inducing plant resistance to pathogens.

Induction of resistance is a method that promotes the reduction of disease severity. This method consists of sensitizing the plant to activate its defense mechanisms by an elicitor agent, and preparing the plant for the pathogen arrival, its infection and colonization (Conrath et al., 2002). The activation of plant defense system involves an increase in the enzymes activity related to pathogenesis, such as peroxidase, phenylalanine ammonia-lyase and catalase (Garcia-Cristobal et al., 2015; Maschietto et al., 2016).

Among the reported elicitors, there are biotic agents such as microorganisms, which can activate plant defense mechanisms (Chowdappa et al., 2013). Resende et al. (2015), exploring the biodiversity of saprobe fungi present in semi-arid North-east of Brazil, observed that conidial suspension of *Curvularia inaequalis* was able to potentiate sorghum resistance against *Colletotrichum sublineolum* infection. Filtrates of pathogenic fungi, non-pathogenic and saprobes are able to activate the defense system because they contain molecules: proteins, oligosaccharides, oligopeptides, toxins and others. They function as microorganism recognition signature, which is perceived by protein receptors present in the cell membrane of the plant cell (Dubery et al., 2012).

In proposing research to identify inducers of plant resistance to pathogens, it is important to note the potential for inhibiting or reducing the symptoms of pathogen attack and to study the biochemical changes occurring in the host. Further, persistence of defense response(s) involved, as well as systemic signaling should also be considered (Choudhary and Johri, 2007).

This current work aimed to study the effect of different concentrations of saprophytic fungal filtrates in induction of defense related enzymes against early blight disease of tomato.

## MATERIALS AND METHODS

The experiments were conducted in the Laboratory of Alternative Control and Induction of Resistance at the State University of Maringá, from January 2012 to December 2013, in the City of Maringá, State of Paraná.

### Preparation of *A. solani* inoculum

The pathogen *A. solani* was obtained from the State University of

Western Paraná, and plated on Potato, Dextrose, and Agar (PDA) at  $25 \pm 2^\circ\text{C}$  for 2 to 3 days to obtain pure colonies. The pure phytopathogen was kept in a growth chamber at  $25 \pm 2^\circ\text{C}$  in PDA, with a photoperiod of 12 h light.

### Preparation of filtrates from fungal saprobes

Isolates of saprobes fungi from Brazil's semi-arid Northeast (*Memnoniella echinata*, *Curvularia eragrostidis*, *C. inaequalis* and *Pseudobotrytis terrestris*) ceded by the State University of Feira de Santana, and deposited in CCMB (Bahia Microorganisms Collection) were grown on PDA. They were kept in a growth chamber at  $25 \pm 2^\circ\text{C}$ , with a photoperiod of 12 h light.

The filtrates were obtained by subculturing fungal mycelia disc grown on PDA to 100ml of Potato dextrose broth sterilized at  $120^\circ\text{C}$  and 1 atm for 20 min. They were incubated in a growth chamber at a temperature of  $25 \pm 2^\circ\text{C}$  with a photoperiod of 12 h of light for 20 days. Later, the broth containing fungal liquid metabolites was filtered through Whatman paper No. 1.

### Effect of fungal filtrate on early blight incidence and defense related enzymes

To study the influence of filtrates' concentrations and induced biochemical aspects, tomato seeds from the cultivar Santa Cruz Kada were used in a 4x4 split-plot design with a negative control (sterile distilled water) and a positive control (ASM) at  $5 \text{ g } 100 \text{ L}^{-1}$ . The first factor consisted of filtrates of the fungi *P. terrestris*, *C. inaequalis*, *C. eragrostidis* and *M. echinata*, and the second factor consisted of 0, 5, 10, 15 and 20% concentrations; there was a total of 18 treatments with five replicates, and plots constituted of a plant. Treatments were applied in the 2nd and 3rd true leaves, three days before inoculation with *A. solani* pathogen, when the plants had five leaves. The inoculation was performed in  $1 \times 10^4$  conidia  $\text{ml}^{-1}$  (Balbi-Penã et al., 2006), and then the plants were kept in a humid chamber in a greenhouse for 12 h.

The severity evaluation of tomato early blight started from the observation of the first disease symptoms in plants. It was severe in leaflets that were treated and inoculated (3rd leaf) and also in those untreated and inoculated (4th leaf), using the diagrammatic scale of Boff (1988). The evaluations were conducted every 5 days for 25 days; there were a total of 5 ratings. For severity evaluation, the area under the disease progress curve (AUDPC) was calculated according to Campbell and Madden (1990). The experiment was conducted in a completely randomized design with four replications. The AUDPC data were submitted to regression analysis ( $p < 0.05$ ), with one control and additional treatment.

### Biochemical analysis

To evaluate the occurrence of biochemical changes on tomato plants treated with filtrates from saprobes fungi, the specific activity of guaiacol peroxidase, phenylalanine ammonia-lyase and catalase was analyzed.

For biochemical analysis, leaflets were collected from the 3rd leaf (treated and inoculated) to determine the local effect, and also from the 4th leaf (untreated) to observe the systemic effect. The leaves were harvested at 0, 24, 48, 72 and 96 h after inoculation (hpi) with *A. solani* for all treatments, including non-inoculated plants. The leaflets were weighed and stored at  $-20^\circ\text{C}$  to perform the subsequent biochemical analysis. The enzyme extract was obtained and stored at  $-20^\circ\text{C}$  to determine the total content of protein and activity of guaiacol peroxidase, phenylalanine ammonia-lyase and catalase.

Total proteins were quantified (Bradford, 1976) and the activity of



guaiacol peroxidase (GPOX) was determined by the method of Lusso and Pascholati (1999); the specific activity was expressed in absorbance units  $\text{min}^{-1} \text{mg}^{-1}$  protein. Catalase activity was determined by Góth method (1991) as adapted by Tomanková et al. (2006) and specific activity was expressed in absorbance unit  $\text{min}^{-1} \text{mg}^{-1}$  protein. The activity of phenylalanine ammonia-lyase enzyme was determined by colorimetric quantification of trans-cinnamic acid released from the substrate of phenylalanine, according to Umesha (2006) and the results were expressed as  $\mu\text{mg}$  trans-cinnamic acid  $\text{min}^{-1} \text{mg}^{-1}$  protein.

The results were submitted to statistical method of surface response ( $p < 0.05$ ) using SAS statistical software, to determine the mathematical model that best fits the data using the formula  $Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$ . This mathematical model of equation was used in the Statistica Six Sigma program for building graphics.

## RESULTS AND DISCUSSION

In analyzing the influence of different concentrations of saprobe fungi filtrates on the management of early blight in the 3rd leaf, there was a cubic regression model, for all treatments. There was reduction of 89, 83, 92 and 91% of disease at 8, 20, 8, and 7% of the filtrates of *C. eragrostidis*, *C. inaequalis*, *M. echinata* and *P. terrestris*, respectively. Comparing the estimated concentrations of filtrates which promote greater reduction of AUDPC on tomato early blight with ASM, there was a reduction of 79, 63, 81 and 83% in the 3rd leaf with the application of filtrates of *C. eragrostidis*, *C. inaequalis*, *M. echinata* and *P. terrestris*, respectively (Figure 1A).

In the 4th untreated leaf, AUDPC of tomato early blight was adjusted to the cubic regression model when filtrates of *C. eragrostidis* and *C. inaequalis*, *M. echinata* and *P. terrestris* were applied. The lowest values of AUDPC were observed at 8, 7, 7 and 7% with a reduction of 81, 70, 82 and 81%. When compared with the ASM inducer, there was a decrease of 44, 10, 13 and 10% in the 4th leaf with filtrates of *C. eragrostidis*, *C. inaequalis*, *M. echinata* and *P. terrestris*, respectively (Figure 1B).

The application of *C. inaequalis* filtrate on the 3rd leaf (treated) originated a quadratic response in a surface response to the guaiacol peroxidase activity (GPOX activity), catalase (CAT) and phenylalanine ammonia-lyase (PAL) (Figure 2 IA, IIA and IIIA). The influence of the concentrations of filtrates and/or collection time after inoculation with the pathogen is dependent on the enzyme studied.

In the analysis of the activity of GPOX, there was an interaction between the filtrate concentration and time after inoculation with the pathogen. There was a minimum critical point calculated at 48 h with the application of 10% of filtrate; there was reduction in enzymatic activity when compared with untreated plants. Enzyme activity increased at 72 h, with maximum observed at 96 hpi with *A. solani* at all concentrations; although greater one was also observed in untreated plants (Figure 2 IA).

For CAT activity, it was only the effect of time that was observed after inoculation with the minimum and maximum

critical points calculated at 28 and 96 hpi, respectively, independent of the filtrate concentration in the treated leaf (Figure 2 IIA). Interaction between time and concentration was observed for PAL activity. There was an increase at 0 h after inoculation and maximum increase was observed at 72 h at 20% concentration; there was 2-fold increase in the enzyme activity compared to untreated plants control (Figure 2 IIIA).

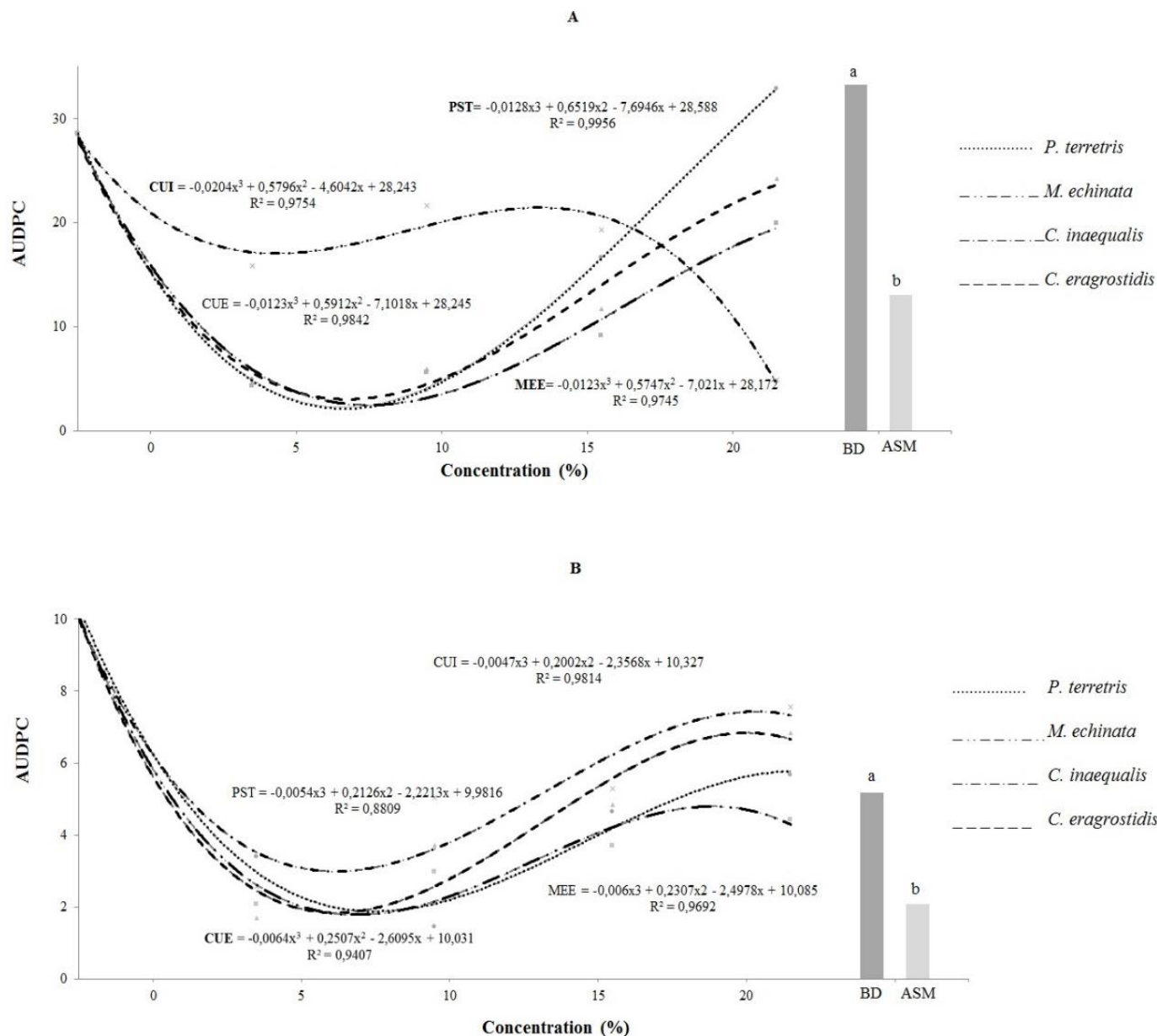
From the analysis of systemic effect on the 4th leaf treated with filtrate of *C. inaequalis*, it can be observed that the GPOX response was adjusted to a linear model and the CAT and PAL enzymes to a quadratic by the surface response method (Figure 2 IB, IIB and IIIB).

Considering the GPOX response, there was an increase in the activity of enzyme after inoculation; it was maximum at 96 h independent of the filtrate concentration from *C. inaequalis* (Figure 2 IB). For CAT, there was concentrations influence, with two maximal activities, one observed at 0 h of inoculation with the pathogen and another 96 hpi, independent of *C. inaequalis* filtrate concentration (Figure 2 IIB). In untreated leaves, the PAL activity increased at 0 h with 2-increase 80 hpi, at estimated concentration of 16% of *C. inaequalis* filtrate (Figure 2 IIIB).

Increase in the activity of both GPOX and CAT was observed (local and systemic) from 72 h in tomato plants treated with filtrate of *C. inaequalis*, after inoculation with *A. solani*. This may be related to the recognition of phytopathogens by the receptors present in the host. These activate a cascade of signaling and transduction of signals which culminate in increasing the activity of these defense enzymes. Such response occurs because the plants have a defense system able to recognize molecular patterns of the pathogen or the one the plant itself originates during the attack (Dubery et al., 2012).

The increase of enzymes such as CAT and GPOX has been reported in many studies about phytopathogen-host interaction, including *A. solani* and *S. lycopersicum*. Here, the defense mechanisms activation in plants can involve the increase of enzymes related to detoxification of reactive oxygen species generated during the plant attack by pathogens, such as GPOX and CAT (Apel and Hirt, 2004). Similar results were found in this study, allowing one to infer that the increase in guaiacol peroxidase does not have a direct relation in the reduction of AUDPC on tomato early blight observed both locally and systemically when the filtrate of *C. inaequalis* was applied. This is because there was an increased pattern of activity of GPOX and CAT in negative control (Figure 1A and B).

The increase in the activity of phenylalanine ammonia-lyase observed locally and systemically (Figure 2 IIIA and IIIB) explains the decrease in tomato early blight (Figure 1A). This increase may be related to the recognition of elicitors present in the filtrate of *C. inaequalis*. According to Henry et al. (2012), in the recognition of elicitors related to pathogen, the plants are also able to notice

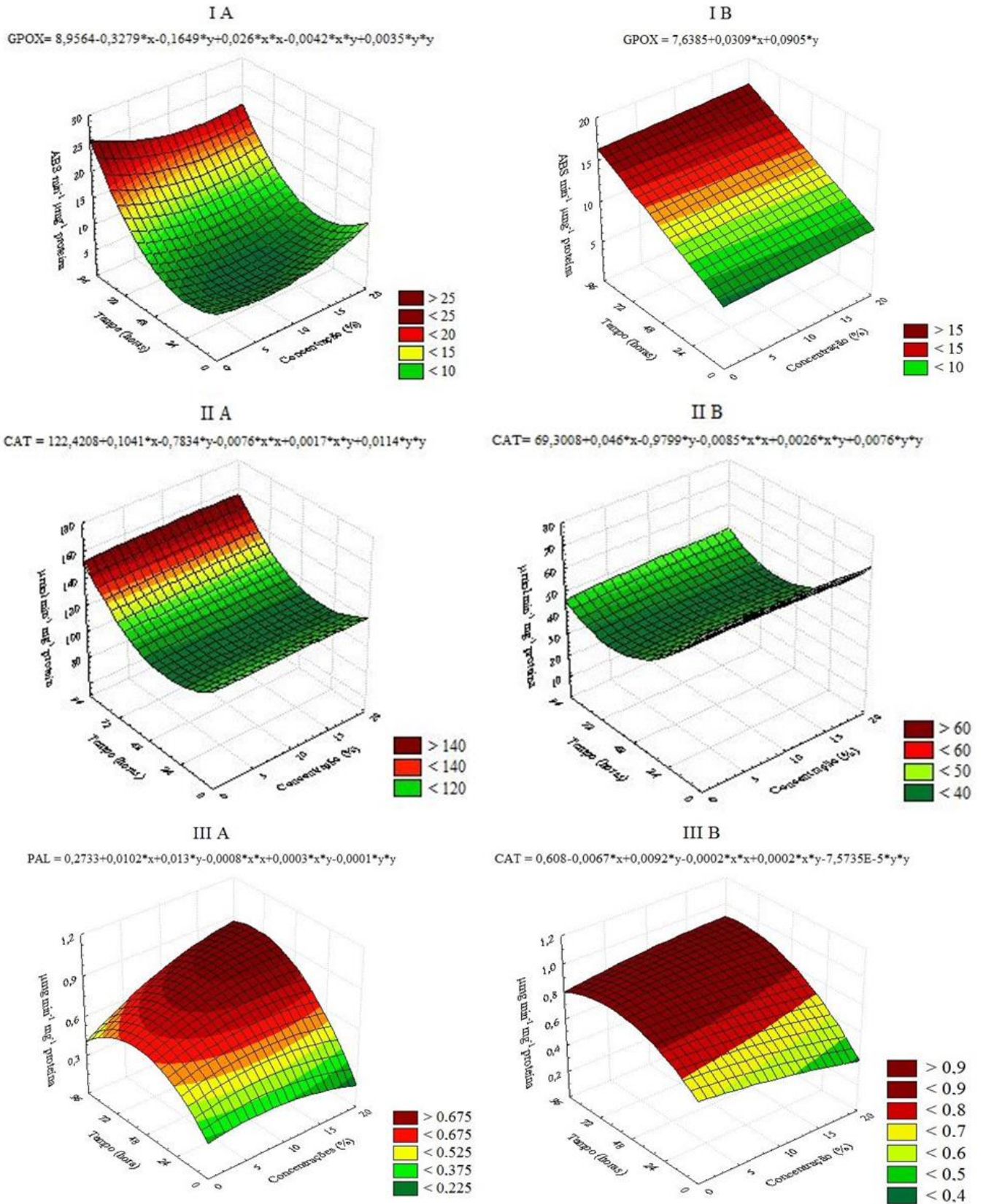


**Figure 1.** Area under the disease progress curve (AUDPC) of tomato early blight in 3rd leaf-treated (A) and 4th leaf-untreated (B) with concentrations of 5, 10, 15 and 20% of the filtrates from saprobes fungi, Maringa, 2013. \*Columns followed by the same letter do not differ by T test at 5% of probability.

those that originate from non-pathogenic microorganisms; they activate resistance mechanisms, such as PAL (Figure 2 IIIA and B).

Akram and Anjum (2011) also found that *Bacillus* strains activate defense responses, by increasing PAL activity and consequently the accumulation of phenolic compounds in tomato plants, which were resistant to the phytopathogen *Fusarium oxysporum*. According to Cavalcanti et al. (2007), the early activation of the defense mechanisms, such as the PAL enzyme, are critical to plant's resistance to pathogen, because it acts

by converting the phenylalanine amino acid to trans-cinnamic acid. This conversion starts with carbon channeling from the primary metabolism in phenylpropanoid to the secondary metabolism of plants. This includes salicylic acid formed by the benzoic acid which is also a precursor in the synthesis of several phenylpropanoids such as lignin, coumarins and flavonoids (Lister and Lancaster, 1996). In this study, there was a reduction of early blight of about 63 and 10%, indicating the efficiency of *C. inaequalis* filtrate when tested locally and systemically, respectively.



**Figure 2.** Activity of guaiacol peroxidase (I), catalase (II) and phenylalanine ammonia-lyase in leaves (III) treated - local (A) and untreated - systemic (B) with filtrate concentration of *Curvularia inaequalis*, 72 h before inoculation of *Alternaria solani* and harvested at 0, 24, 48, 72 and 96 hpi, Maringá, 2013.

Studying the effect of the concentrations of *C. eragrostidis* filtrates on treated tomato plants, three days before inoculation on the activity of GPOX, CAT and PAL, there was surface response in a quadratic model, linear and quadratic, respectively, when the samples were collected from the 3rd leaf (treated) (Figure 3 I, II, and III). For the GPOX activity, there was an interaction between the time of harvest after inoculation with *A. solani* and the concentrations of *C. eragrostidis* filtrates applied to the 3rd leaf. There was maximum activity of 96 hpi (concentration of 20%) with two fold increase of enzyme activity compared to control plants (Figure 3 IA).

CAT activity increased proportionally to time after pathogen inoculation; the highest peak of the dependent variable was observed at 96 hpi (Figure 3 IIA). For PAL activity, there was an interaction between the application of *C. eragrostidis* filtrates and the time of collecting the tomato leaves after inoculation with the pathogen. There was maximum enzyme activity of 35 hpi at a concentration of 10%. There was three times increase in the enzyme activity compared to the estimated value of the control plants in the same period (Figure 3 IIIA).

When analyzing untreated plants with filtrate of *C. eragrostidis*, but inoculated with *A. solani*, there was an interaction between the filtrate concentration and collection time in GPOX and PAL activity. However, there was no difference between the filtrate concentration in the CAT activity, where the best fit of regression models was linear, linear and quadratic, respectively (Figure 3 I, II and III B). A dose-dependence was observed with GPOX activity systemically, with three times increase at 20% concentration of *C. eragrostidis* filtrate and 72 hpi with *A. solani* (Figure 3 IB).

For CAT, there was no effect of the concentration on enzyme activity, but time influenced their response, with greater activity from 24 hpi in treated leaves (Figure 3 IIA). For PAL, it was observed an increase in enzyme activity systemically and in proportion to time and filtrate concentration of *C. eragrostidis*; there was duplication of enzyme activity at 96 hpi when 20% of the filtrate was applied (Figure 3 IIIB).

The increase of GPOX and PAL promoted by the filtrate of *C. eragrostidis* can justify the reduction of tomato early blight observed in treated plants (Figure 1A). It may be inferred that the increase of these enzymes can be related to the recognition of elicitor molecules existing in the filtrate by a receptor on the host, both locally and systemically. This is because in both, there was an increase of enzyme activity when the filtrates were applied compared to plants that were untreated but inoculated with the pathogen (Figure 3 IA and IB). Both enzymes, GPOX and PAL have been reported in defense of plants against pathogen attack and may be activated simultaneously when the plant is exposed to an elicitor agent. Chmielowska et al. (2008) verified that seedlings of bean and lupine when treated with copper, but not inoculated with pathogens had a higher activity of

peroxidase and phenylalanine ammonia-lyase.

The activation of peroxidase is associated with the primary events of the resistance mechanisms induced by elicitors. This is because the enzyme acts on hydrogen peroxide detoxification formed during the oxidative explosion caused after recognition of elicitors during the defense system activation (Van Loon et al., 2006). This generation of hydrogen peroxide in elicited cells increases the peroxidase activity involved in the cross oxidative connection of structural proteins in the cell wall rich in the repetition of proline. It also increases tyrosine that strengthens the plant's cell walls, slowing the phytopathogen colonization process (Kishor et al., 2005).

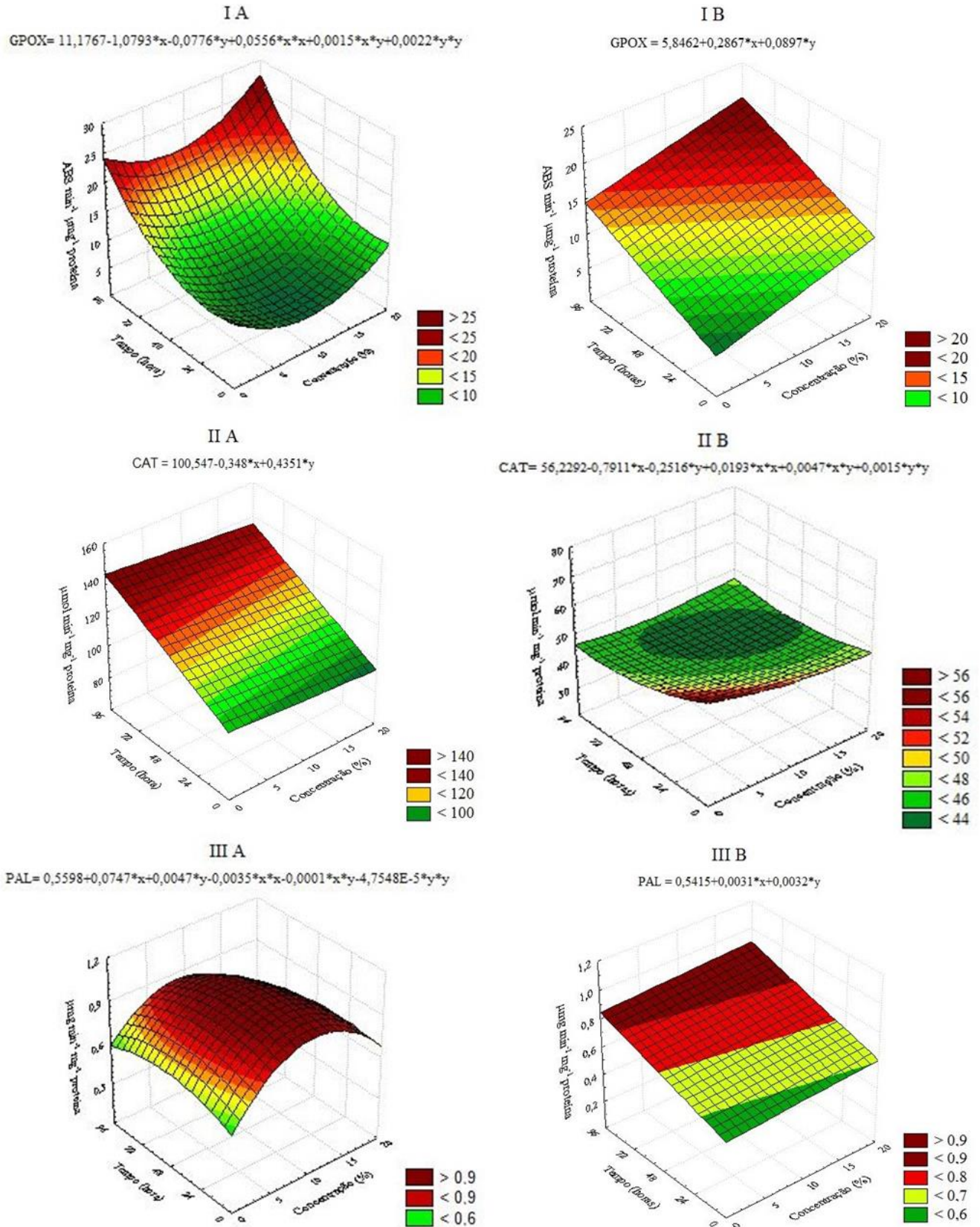
An increase in the PAL activity in plants treated with filtered *C. eragrostidis* may have an essential role in reducing the AUDPC of tomato early blight. According to Umesha (2006), phenylalanine ammonia-lyase is crucial in the protection of tomato plants against the attack of *Clavibacter michiganensis* subsp. *michiganensis*. The enzyme activity is related to the plant resistance to pathogens, essentially, for being involved in the first step of the phenylpropanoids synthesis and its conversion to trans-cinnamic acid, resulting in compounds with antimicrobial activity. This is besides lignin increment, which gives greater resistance to the plants' cell wall (Nakazawa et al., 2001).

In Figures 4 IA, IIA and IIIA, there was a significant effect ( $P < 0.05$ ) in the interaction between filtrate concentration of *P. terrestris* and harvest time for determining guaiacol peroxidase, catalase and phenylalanine ammonia-lyase, with best fit in the quadratic model for the three enzymes when analyzed locally (3rd leaf).

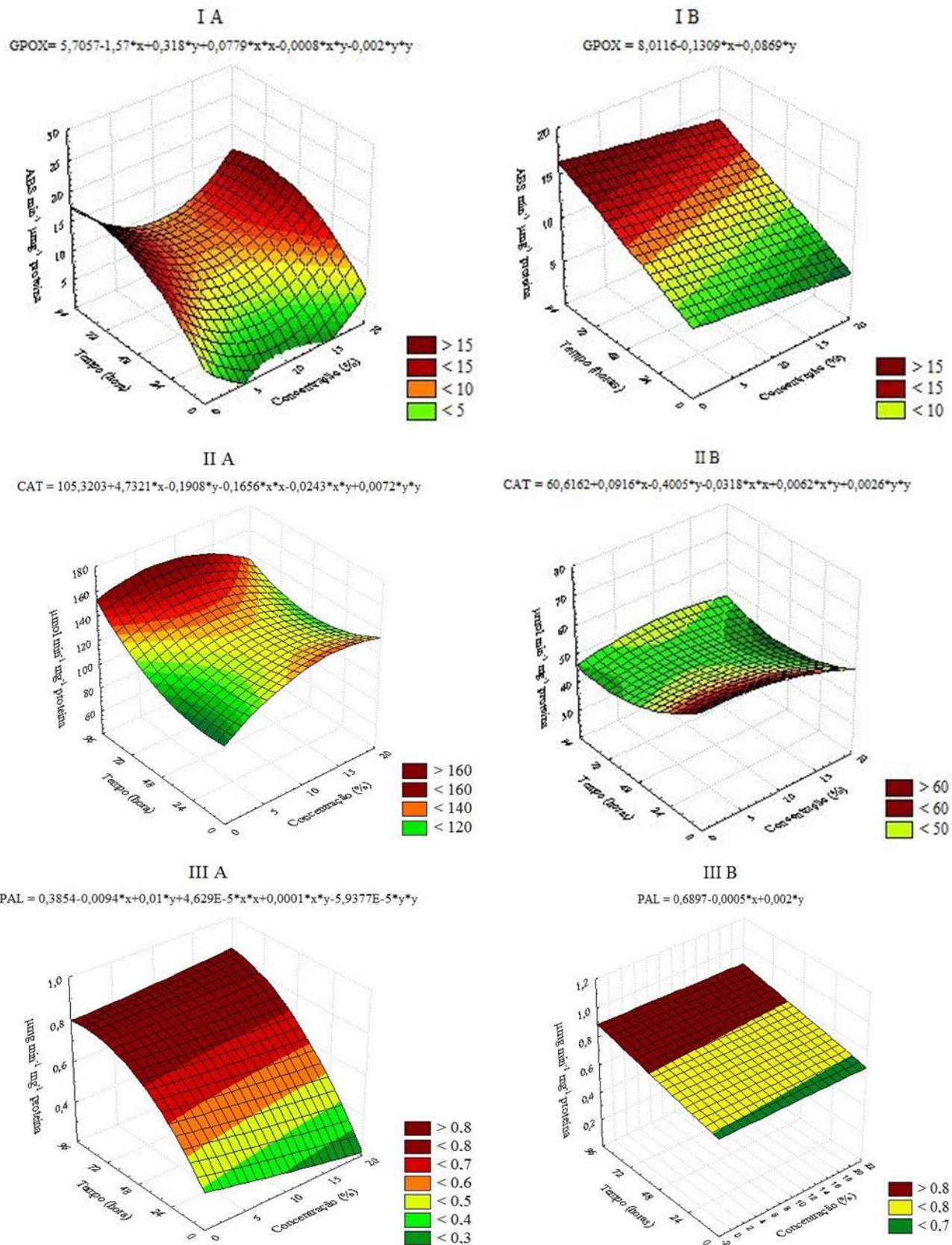
GPOX activity was reduced in the gap between 5 and 15% of the filtrate and opposite (Figure 4 IA). However, there was an increase of CAT within the same interval of concentrations; 96 hpi increase in 10 % concentration, where there was 3-fold increase in enzyme activity. PAL was not influenced by the application of *P. terrestris* filtrate concentrations; however enzyme activity increased with time after inoculation with *A. solani* (Figure 4 IA).

For the GPOX activity in 4th leaf (untreated), there was maximum increment of the enzyme in plants that were not treated with *P. terrestris* filtrate, 96 h after inoculation (Figure 4 IB). CAT activity increased at 72 hpi when 15% was applied, with 2-fold increase in GPOX activity (Figure 4 IIB). For PAL quantification in the untreated leaves, the greatest activity was observed at 96 hpi independent of the filtrate concentration (Figure 4 IIIB).

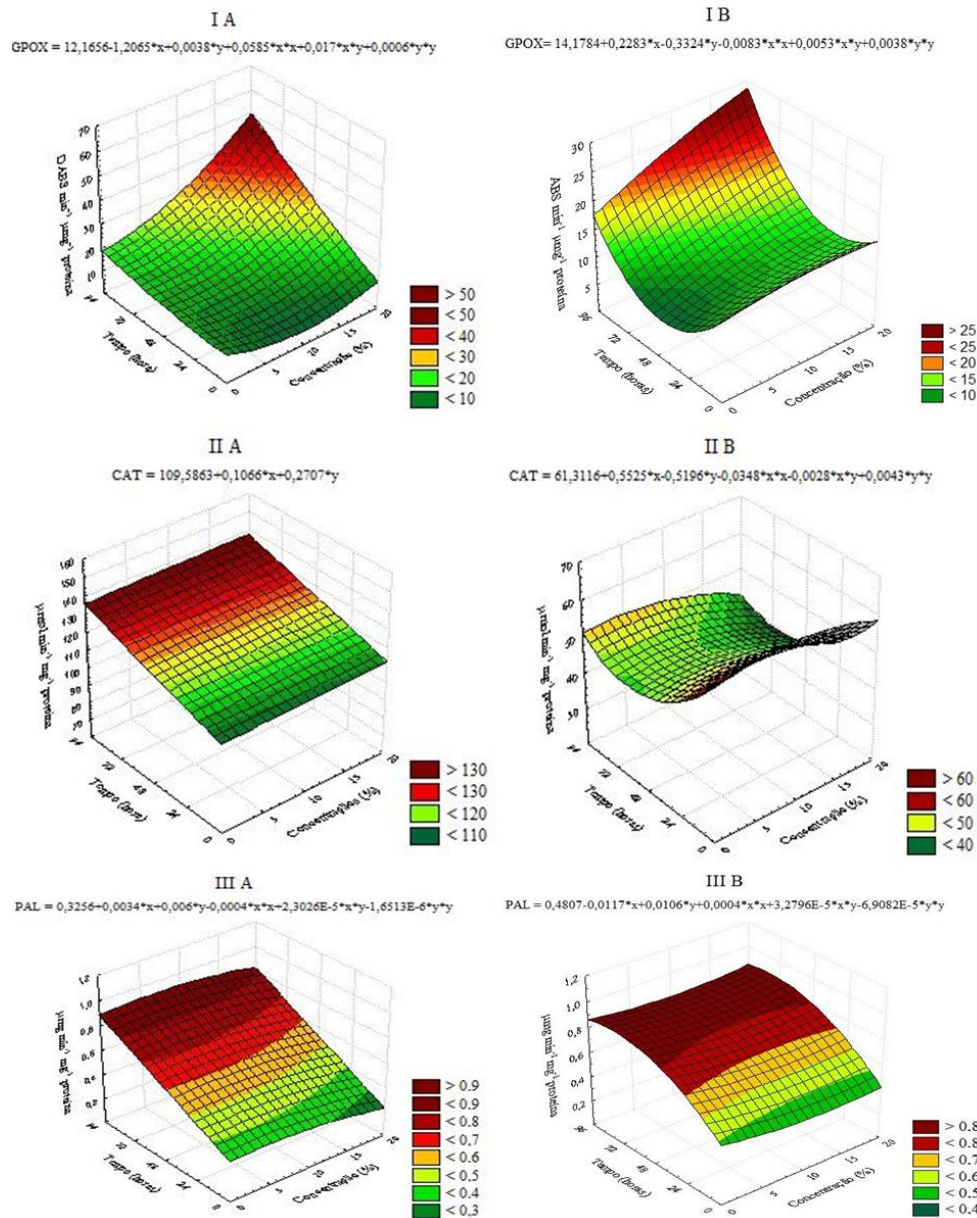
The reduction of AUDPC in tomato early blight (Figures 1A and B) might be correlated to an increase of CAT and PAL activity in plants treated with filtrates of *P. terrestris*. Cavalcanti et al. (2006) observed that tomato plants, from the group Santa Cruz Kada, previously induced with an elicitor made of necrotic tissue extract with *Xanthomonas vesicatoria*, presented less bacterial spot severity caused by *X. vesicatoria*. This protection was related to the



**Figure 3.** Activity of guaiacol peroxidase (I), catalase (II) and phenylalanine ammonia-lyase in leaves (III) treated - local (A) and untreated - systemic (B) with filtrates concentration of *Curvularia eragrostidis* 72 h before inoculation of *Alternaria solani* and harvested at 0, 24, 48, 72 and 96 hpi, Maringa, 2013.



**Figure 4.** Activity of guaiacol peroxidase (I), catalase (II) and phenylalanine ammonia-lyase in leaves (III) treated - local (A) and untreated - systemic (B) with filtrates concentration of *Pseudobotrytis terrestris* 72 h before inoculation of *Alternaria solani* and harvested at 0, 24, 48, 72 and 96 hpi, Maringa, 2013.



**Figure 5.** Activity of guaiacol peroxidase (I), catalase (II) and phenylalanine ammonia-lyase in leaves (III) treated - local (A) and untreated - systemic (B) with filtrates concentration of *Memnoniella echinata* 72 h before inoculation of *Alternaria solani* and harvested at 0, 24, 48, 72 and 96 hpi, Maringa, 2013.

increase in catalase activity from 24 h with peak at 72 hours after the treatments. This similarly occurred with the application of *P. terrestris* filtrate in the interaction of *A. solani* and *S. lycopersicum* (Figure 4 IA).

The enzyme catalase acts in the dismutation of two  $H_2O_2$  molecules into water and oxygen (Sharma et al., 2012); thus, the hydrogen peroxide accumulation can be estimated indirectly by the action of this enzyme.  $H_2O_2$  has been applied in various stress conditions, including induction through biotic and abiotic inducers where CAT rapidly degrades  $H_2O_2$  in an efficient manner (Mallick and

Mohn, 2000). This detoxification is dependent on the intensity, duration and type of elicitation (Han et al., 2009).

The equation model of quadratic regression had the best fit for the interaction between the filtrate concentration of *M. echinata* and time of harvest after inoculation with *A. solani* for GPOX activity, using the surface response method and the linear method for CAT and PAL, when determined locally (Figure 5 IA, IIA and IIIA).

GPOX increased according to the filtrate concentration and the time after inoculation, with 3-fold increase in

enzyme activity after 96 hpi at a 20% concentration in relation to treatment with control plants (Figure 5 IA). CAT activity increased linearly with time of harvest. However, it was not influenced by concentration (Figure 5 IIA). While the application of *M. echinata* filtrate reduced PAL activity in 3rd leaf by increasing the filtrate concentration and the harvesting time after pathogen inoculation (Figure 5 IIIA).

Systemically, the enzymes guaiacol peroxidase, catalase and phenylalanine ammonia-lyase were adjusted to the quadratic model (Figure 5 IB, IIB and IIIB). GPOX activity increased with concentration and harvesting time after inoculation with 2-fold increase by applying the filtrate at 20% and 96 hpi, compared to activity of leaves collected from untreated plants at the same period (Figure 5 IB). The CAT was affected with the time of harvest and there was reduction in the activity with *A. solani* infection, independent of the filtrate concentration of *M. echinata* (Figure 5 IIB).

The application of concentrations of *M. echinata* filtrate promoted greater GPOX activity; this indicates a possible reduction of tomato early blight leads to increase in the activity of enzymes. The increase in peroxidase activity may also be associated with an increase of structural proteins synthesis, which strengthens the cell wall, acting as a barrier to pathogen (Van Loon et al. 2006).

By applying filtrates of *C. inaequalis*, *C. eragrostidis*, *P. terrestris* and *M. echinata*, it can be observed that they are capable of reducing AUDPC of tomato early blight through the induction of distinct biochemical mechanisms. The filtrates of these fungi acted as elicitors agents, being recognized by receptors present in tomato plants, activating its defensive enzymes with increased GPOX, CAT and PAL. The plants' multicomponent defense response is directly related to interactions between the elicitor and receiver, with amplitude, duration and quality dependent on the specific signaling generated (Brencic and Winans, 2005). The knowledge of this variation may explain the distinct behavior observed in each filtrate from the species of saprobes fungi presented in this work.

The peaks of activity from GPOX and CAT simultaneously are noticed when the filtrates of saprobes are applied on the tomato plants. Interestingly, GPOX and CAT showed opposing activities to each other, that is, when there was an increase of GPOX activity the CAT activity was reduced and vice versa. Such response can be observed clearly in plants treated with *P. terrestris*, in which there was a reduction in the peroxidase activity between the concentrations of 5 and 15%; the catalase increased between the same concentrations. Because the enzymes compete for the same substrate, the H<sub>2</sub>O<sub>2</sub> generated during oxidative explosion is caused by stress (Sen et al., 2003).

When considering the enzymes activation related to pathogenesis, it may be observed that the filtrate of *M. echinata* induced GPOX activity in untreated leaves and

*C. eragrostidis* increased the activity of PAL and GPOX, systemically. The potential to activate the defense responses in parts of the plants that were untreated is a characteristic of great importance for resistance inducer, because it prepares the plant tissue that has not received the treatment for a possible pathogen attack. Systemic activation is normally regulated by a signaling cascade mediated by H<sub>2</sub>O<sub>2</sub>, salicylic acid, MAPKs and/or other molecules after recognition of elicitor molecule by receptors present in the cell membrane of the plant cell (Conrath et al., 2006).

In conclusion, the filtrates from *C. eragrostidis*, *C. inaequalis*, *M. echinata* and *P. terrestris* promoted reduction of AUDPC in tomato early blight in greenhouse conditions, when applied three days before inoculation locally and systemically. New studies may be performed to identify elicitor molecule(s) and can be used in the future in the management of plant diseases.

### Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

## Eugenol and linalool: Comparison of their antibacterial and antifungal activities

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Antimicrobial resistance is one of the greatest threats to human health. Alternatives to antimicrobials are needed to combat the rise of bacterial resistance. Essential oils (EOs) and their components are potential sources of new antimicrobials. The present study was conducted to evaluate the antibacterial and antifungal activities of two components of EOs. The antimicrobial mechanisms of eugenol and linalool were investigated against five bacterial strains and four *Candida* strains. Broth macrodilution method was used to compare the antibacterial and anticandidal activities of the two compounds. They exhibited antimicrobial activity against all tested strains. Germ tube formation by *Candida albicans* was investigated and it was found that it was completely inhibited at sub-MICs of eugenol while linalool showed minor activity compared to eugenol. Time kill kinetic studies indicated that eugenol was highly toxic to all bacterial and fungal strains within 2.5 h of exposure. Absorbance of intracellular constituents was measured at 260 nm. Only eugenol was highly effective toward lysis and cellular content leakage compared to control drugs. In addition, scanning electron microscopy (SEM) was used to characterize the effect of the two components on cell morphology and showed that both compounds induced cellular deformity of nearly all tested cells. Also, it was found that only eugenol inhibited the Beta-lactamase production and urease activity and it diminished bacterial motility of all tested bacterial strains. These results indicate that eugenol and linalool are effective antimicrobial agents and both antibacterial and antifungal activities of linalool were much weaker than that of eugenol.

**Key words:** Antimicrobial resistance, antimicrobials, germ tube formation, time kill kinetic, cellular deformity.

### INTRODUCTION

During the past few decades, the incidence of both community-acquired and nosocomial bacterial and fungal infections has significantly raised, increasing the number of patients who are at risk especially those with impaired immunity. There has been a worldwide rapid increase in resistance to antimicrobial agents in almost all bacterial

and fungal genera and to all drug classes. The most important factor influencing the spread of antimicrobial resistance is the excessive microbial exposure to antimicrobials that results in selection pressure in microbial population, allowing only the fittest genotype to thrive (Canton and Morosini, 2011; WHO, 2012).

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Currently, high rates of antimicrobial agents use in the community, hospital and agriculture have contributed to fuel this crisis. The cross resistance to two or more antimicrobial agents is often mediated by a single resistance mechanism (Leclercq, 2002). This might be arisen due to plasmids (Tenover, 2006) or emerging mutation of chromosomal DNA (Sanders and Sanders, 1992). The cost of treatment is increased due to antimicrobial resistance. It may result in prolonged hospital stays, higher mortality rates, and creates broader infection control problems (Neu, 1992).

Natural products, either as pure compounds or as plant extracts provide unlimited opportunities for new drugs due to an increasing demand for chemical diversity (Cos et al., 2006). Essential oils (EOs) extracted from plants have been used primarily for flavoring and perfumery (Ben Arfa et al., 2006). It has long been recognized that EOs have antimicrobial properties, and recent studies have demonstrated that these activities are mainly due to the presence of numerous substituted aromatic molecules. Examples of these molecules include eugenol, cinnamaldehyde, and carvacrol (Moleyar and Narasimham, 1992). Currently, there is a trend in food processing to avoid the application of chemical preservatives such as sodium chloride and nitrates. Thus, the use of bioactive compounds derived from EOs as alternative antimicrobial agents is garnering great interest (Gill and Holley, 2004; Tsukiyama et al., 2002). Although, EOs have been empirically used as antimicrobial agents, their spectrum of activity and mechanisms of action remain unknown for most of them.

Linalool (3,7-dimethylocta-1,6-dien-3-ol) is a terpene alcohol that has broad spectrum antimicrobial activity (Alviano et al., 2005). Eugenol (4-allyl-2-methoxyphenol) is the main component of clove oil (phenolic compounds). It is used primarily as a flavoring agent in food and cosmetic products. Eugenol and linalool possesses various biological abilities, including antimicrobial, antioxidant, anti-inflammatory, anticarcinogenic, anti-spasmodic, and antiparasitic activities. They are also effective as antiseptic agents in dentistry. Many studies most focused on the possibility of using clove oil as a replacement for some chemical additives in the preservation of main food categories such as meat and fish (Burt, 2004; Oussalah et al., 2007).

Combinational therapy is essential in the treatment of serious infection and to reduce the risk of resistant microbes (Kamatou et al., 2012). When linalool or menthol is combined with eugenol it showed the highest synergy, indicating that a monoterpenoid phenol combined with a monoterpenoid alcohol is an effective combination (Bassolé et al., 2010). Eugenol shows excellent synergistic activities and decreases MICs of conventional antibiotics as vancomycin, gentamicin and Beta-lactams (Moon et al., 2011). This synergistic effect can be linked to eugenol's ability to damage the membrane of Gram-negative bacteria. Combination between eugenol and

cinnamate, cinnamaldehyde, thymol or carvacrol leads to greater antimicrobial effect (Pei et al., 2009; Rico-Molina et al., 2012). Certain combinations of eugenol and thymol show a synergistic effect thus potentiate their inhibition of *C. albicans* colonization and infectivity (Braga et al., 2007).

The present study evaluates the antibacterial and antifungal activity of eugenol and linalool and investigates the antimicrobial mechanisms of action against some Gram positive, Gram negative and *Candida* species.

## MATERIALS AND METHODS

### Test organisms

In the current study, five standard strains; *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 7839), *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 10145) and *Candida albicans* (ATCC 10231) were used. They were obtained from MIRCIN culture collection of the Faculty of Agriculture, Ain Shams University. Clinical strains of *Proteus mirabilis* and *Candida* (*Candida albicans*, *Candida glabrata* and *Candida krusei*) were obtained from the Department of Microbiology, Faculty of Pharmacy, Minia University. All cultures were maintained in their appropriate agar slants at 4°C and used as stock cultures.

### Antimicrobial agents

Two compounds; Eugenol and linalool (Sigma-Aldrich, Germany) with 98% purity were used in this study.

### Determination of minimum inhibitory concentration (MIC) and minimum bactericidal (MBC) or fungicidal (MFC) concentrations

A broth macrodilution method was used to determine the MIC and MBC or MFC according to the Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeasts (2012, 2002). The Mueller Hinton broth (MHB) (Merck) or RPMI-1640 [Roswell Park Memorial Institute medium (with glutamine, without bicarbonate, and with phenol red as a pH indicator) was 1640, buffered to pH 7.0 with MOPS (morpholine propane sulfonic acid) at 0.165 M] (Sigma-Aldrich) was supplemented with dimethylsulfoxide (DMSO) (Merck) at a 2% concentration in order to enhance sample solubility. Two fold serial dilutions of eugenol and linalool were prepared. The inocula of the microbial strains were prepared from overnight broth cultures and suspensions then adjusted to the turbidity of a 0.5 McFarland standard. Standardized suspension of the test organism was transferred into each tube. Controls without the test compound were prepared.

To determine MBC or MFC, 100 µL of bacterial inoculum was taken aseptically from tubes that had not presented visible turbidity and inoculated in Mueller Hinton agar (MHA) for 20 h at 35°C for bacteria or in MHA supplemented with 2% glucose and 0.5 mg/mL methylene blue for 24 h at 35°C for fungal strains. The MBC/MFC is defined as the lowest concentration of the essential oil at which 99.9% of the initial inoculum was killed. The experiments were repeated three times. To determine the nature of antibacterial or antifungal effect of these compounds, the MBC/MIC or MFC/MIC ratio was used; when the ratio was lower than 4, the compound was

considered as a bactericidal or fungicidal and when the ratio was higher than 4, it was considered as a bacteriostatic or fungistatic (Levison, 2004).

#### Germ tube inhibition assay

*C. albicans* (ATCC 10231) and clinical *C. albicans* strain were cultivated overnight on SDA at 37°C. Cell suspensions were prepared in NYP medium [N-acetylglucosamine (Sigma; 10<sup>-3</sup> mol/L), yeast nitrogen base (Difco; 3.35 g/L), proline (Fluka; 10<sup>-3</sup> mol/L) and NaCl (4.5 g/L), pH 6.7±0.1] and adjusted to 0.2×10<sup>6</sup> c.f.u. ml<sup>-1</sup>. Eugenol and linalool were diluted in DMSO and 10 µl volumes were added to 990 µl of the *Candida* suspensions (final DMSO concentration of 1%, v/v) to obtain 1/2, 1/4 and 1/8 of the MIC values. Untreated eugenol and linalool control suspensions were used. After 3 h incubation with gentle shaking at 37°C, cell suspensions were examined for the presence of germ tubes using a light microscope. Germ tubes were considered positive when they were at least as long as the blastospore (Pinto et al., 2009).

#### Time kill assay

Four concentrations (0.5, 1, 2 and 4 MIC) of each compound were tested against each microbial strain. The time-kill assay was performed with a final inoculum of approximately 5 × 10<sup>5</sup> CFU/ml in a final volume of 30 ml. The final inoculum was adjusted to match the 0.5 McFarland standards. The tubes were continuously shaken on an orbital shaker and incubated at 35°C. 10 µl samples were withdrawn from each tube at 30, 60, 90, 150 and 24 h and streaked on MHA plate. Ten fold dilutions were prepared when necessary and plated onto each MHA plate. Plates were kept in triplicates for each compound and they were incubated for 24 h at 35°C, and colony counts were determined. Time-kill curves were plotted as log<sub>10</sub> CFU/ml versus time functions. The compound concentration and the 3 log<sub>10</sub>-fold reductions in the bacterial count compared with the growth control were determined (Devi et al., 2010).

#### Estimation of the yeast cells or bacterial cytosol release

Bacteria and *Candida* cells were treated with eugenol or linalool then the release of cytosolic material absorbing at 260 nm from these cells was detected (Bennis et al., 2004b). Aliquots of 1.5 ml of cells suspension for 1 h in phosphate buffer saline (PBS) containing various concentrations of test compound ranging from 1.5 to 12 mM were used. Eugenol, linalool, levofloxacin (Sedico, Egypt) against bacterial cells and ketoconazole (Amriya, Egypt) with fungal cells were tested. Microbial cells suspended in PBS were used as control. Correction was made for the absorption of the suspending liquids containing the same concentration of compound after two minutes contact with bacteria or *Candida* cells at 260 nm in Beckman UV spectrophotometer.

#### Scanning electron microscopy (SEM)

Microbial suspensions treated with the MBC or MFC concentrations of eugenol or linalool for 1 h. Treated and untreated (negative control) cells were prefixed in 2.5% glutaraldehyde for 1 h at 4°C. After each fixation, the cells were rinsed twice with PBS. The cells were then dried through an ethanol series. The samples were gold coated by cathodic spraying (Edwards S 150 B). Finally, scanning electron microscopy examinations were done on a JSM-840 SEM (JEOL Ltd., Tokyo, Japan) (Benyahya et al., 1992).

#### Detection of β-lactamases production

All the bacterial strains were screened for β-lactamase production using two methods:

1. Iodometric method.
2. Acidometric method.

##### Iodometric method

Beta-lactamase production was tested by an iodometric method (Catlin, 1975) as follows: Sterile potassium or sodium penicillin G powder (6 lac units) was dissolved in potassium phosphate buffer pH 6.0, 0.05 M at a concentration of 6000 µg/ml (freshly prepared). Starch solution was prepared by adding 1 g of soluble starch to 100 ml distilled water and slowly heat to boiling to obtain a clear solution. Iodine solution was prepared by dissolving 2.03 g of iodine and 53.2 g of potassium iodide in 100 ml distilled water. One hundred microliters of the penicillin solution was dispensed. Several colonies of the microorganism were suspended in 0.9% saline solution to get dense suspension. Two drops of starch were added and then the mixture was kept at room temperature for 30 to 60 min. One drop of iodine was added which turn the solution blue. Disappearance of the blue colour in 10 minutes indicates that the organism is β-lactamase producer. Penicillin alone without any culture suspension was used as negative control.

##### Acidometric method

Acidimetric method used to assay enzyme β-lactamase using benzylpenicillin as substrate (Koneman, 2006). A single colony was resuspended and mixed with the indicator solution. One hundred µl of 1% phenol red solution was added to 1 ml of sterile distilled water for preparation of indicator solution. After mixing, this solution was added to a vial of one million units of sodium benzylpenicillin (Crystapen, Glaxo). Since this solution was at an acidic pH due to citrate buffer in the penicillin, a solution of 1 N sodium hydroxide was added drop-wise until the development of violet colour (pH 8.5). To get a dense suspension, several colonies were suspended 0.9% Sodium Chloride solution then added to 150 µl of penicillin phenol red solution. The color development observed within 1 h. The solution turned yellow if β-lactamase enzyme was produced.

#### Effect of sub-MIC and MIC levels of eugenol and lialool on bacterial urease activity

Urease activity was determined according to the method described by Derakhshan et al. (2008). Urea broth containing sub-MIC and MIC levels of eugenol and linalool was used for cultivation of bacterial strains overnight. Untreated cultures were used as negative control. All tubes were incubated at 37°C for 24 h. Suspensions were centrifuged at 5000×g for 3 min and the supernatants were recovered. Color intensity was measured at 560 nm using a universal microplate reader (Biotek, China). The samples were performed in triplicate.

#### Motility test

The motilities of the bacterial cells were determined according to Wojnicz and Tichaczek-Goska (2013). Bacterial strains were incubated for 24, 48 and 72 h at 37°C with and without eugenol and linalool. Suspensions were centrifuged at 2500 rpm for 2 min. Separated bacterial cells were washed three times with PBS then inoculated into motility agar tubes. Controls without the test

**Table 1.** Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum fungicidal concentration (MFC) values against tested strains\*.

Strain	Eugenol			Linalool		
	MIC (mM)	MBC or MFC	MBC/MIC or MFC/MIC	MIC (mM)	MBC or MFC	MBC/MIC or MFC/MIC
<i>S. aureus</i> (ATCC 6538)	6.25	25	4	100	100	1
<i>E. coli</i> (ATCC 7839)	25	25	1	50	50	1
<i>K. pneumoniae</i> (ATCC 10031)	12.5	50	4	12.5	50	4
<i>P. mirabilis</i>	12.5	25	2	25	50	2
<i>P. aureginosa</i> (ATCC 10145)	50	100	2	50	100	2
<i>C. albicans</i> (ATCC 10231)	25	50	2	25	25	1
<i>C. albicans</i>	25	25	1	25	50	2
<i>C. glabrata</i>	12.5	12.5	1	25	50	2
<i>C. krusei</i>	12.5	12.5	1	25	25	1

\*Tests were performed in triplicate and modal values were represented.

compounds were used.

## RESULTS AND DISCUSSION

### Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of eugenol and linalool

In order to achieve precisely the antimicrobial properties of eugenol and linalool compounds, determination of MICs and MBCs were necessarily performed. The assayed compounds showed effectiveness in inhibiting all tested strains. Eugenol showed the highest effect against *S. aureus* (the lowest MIC) compared to the other Gram-negative bacterial strains. Generally, Gram-negative bacteria are more resistant to EOs and other natural extracts with antimicrobial activity than Gram-positive bacteria (Trombetta et al., 2005). This resistance could be attributed to the structure of the cell walls of Gram-negative bacteria, mainly with regard to the presence of lipoproteins and lipopolysaccharides that form a barrier to restrict entry of hydrophobic compounds (Russell, 1995).

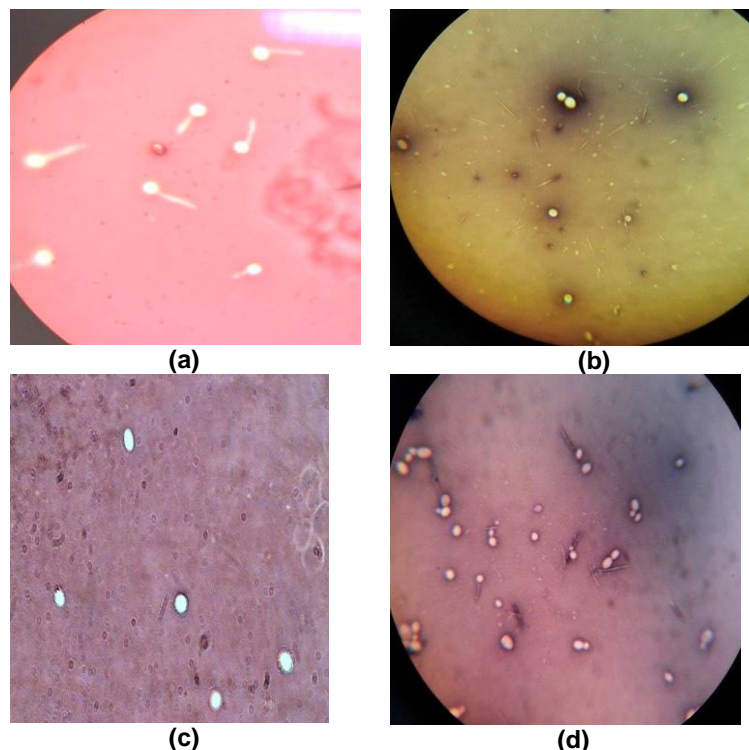
Eugenol and linalool had nearly the same effect against all other tested microorganisms. The MICs of eugenol ranged from 6.25 to 100 mM while for linalool, MICs ranged from 12.5 to 100 mM. For all *Candida* strains and most bacterial strains, MBCs and MFCs were equal to or two-fold greater than the MICs. However, the MBCs of eugenol against *S. aureus* and *K. pneumoniae* and MBCs of linalool against *K. pneumoniae* were four times greater than that of the MICs (Table 1). The ratios of MBC to MIC and MFC to MIC were ranging from 1 to 4. In most cases, MBC and MFC were close to the MIC, indicating a good bactericidal or fungicidal activity against the tested strains. Other authors revealed the antibacterial activity of eugenol against various pathogens such as *E. coli*,

*Bacillus cereus*, *Helicobacter pylori*, *S. aureus*, *S. epidermidis*, *Streptococcus pneumoniae* and *S. pyogenes* (van Zyl et al., 2006; Leite et al., 2007). Previous studies confirmed the antifungal activity for clove oil and eugenol against yeasts and filamentous fungi, such as food-borne fungal species (López et al., 2005) and human pathogenic fungi (Chaieb et al., 2007). Antifungal and antibiofilm activity of linalool against *C. tropicalis* was previously reported (Souza et al., 2016). Researchers found that linalool compound and basil oil which contains high amounts of the monoterpene linalool had antimicrobial activity against *S. aureus*, *B. subtilis*, *E. coli* and *Aspergillus niger* (Hussain et al., 2008). Consistent with our results, Hsu et al. (2013) demonstrated the fungicidal effects of linalool against *C. albicans* clinical isolates and non-*C. albicans* *Candida* spp.

The antimicrobial activity of EOs depends on the composition and percentage content of active constituents in EOs, which have been found to have an important role in slowing down or stopping the bacterial growth or killing the bacteria (Bozin et al., 2006). Eugenol belongs to a class of phenylpropenes. Free hydroxyl groups confer the antimicrobial activity of this class (Laekeman et al., 1990). The antimicrobial activity of eugenol can be attributed to the presence of a double bond in  $\alpha$ ,  $\beta$  positions of the side chain and to a methyl group located in the  $\gamma$  position (Jung and Fahey, 1983). Linalool is one of the most common terpenoids, which are terpenes with added oxygen molecules or that have had their methyl groups moved or removed by specific enzymes (Caballero et al., 2003).

### Effect of eugenol and linalool on germ tube formation by *C. albicans*

Germ tube formation is one of the important mechanisms



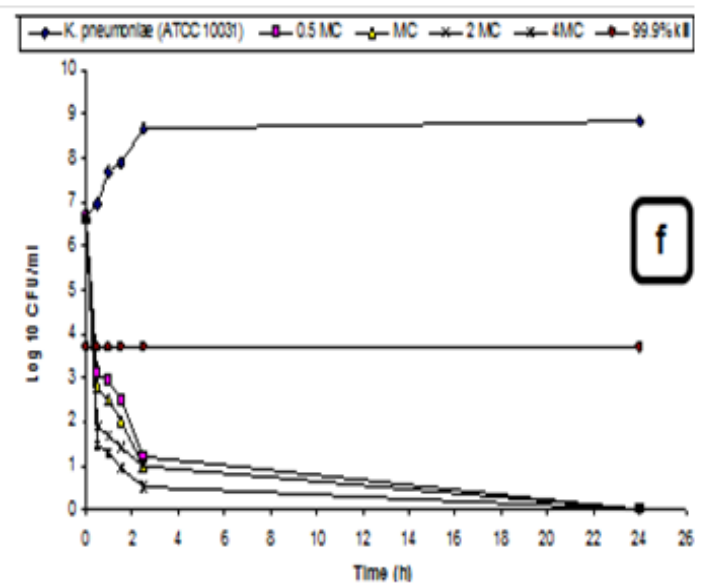
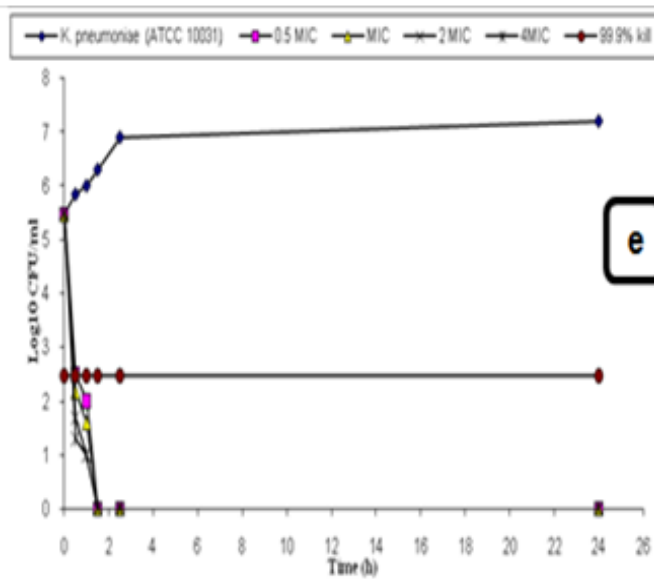
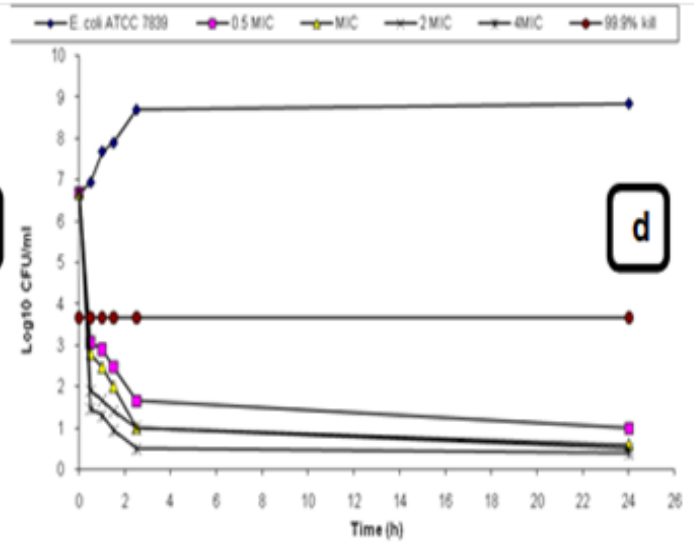
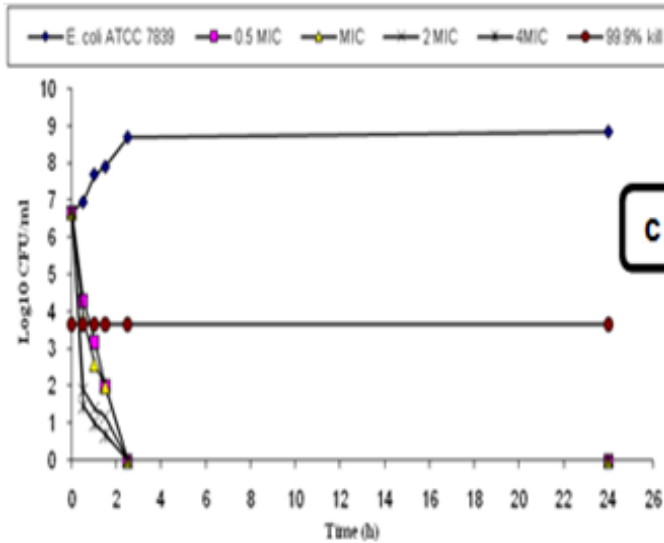
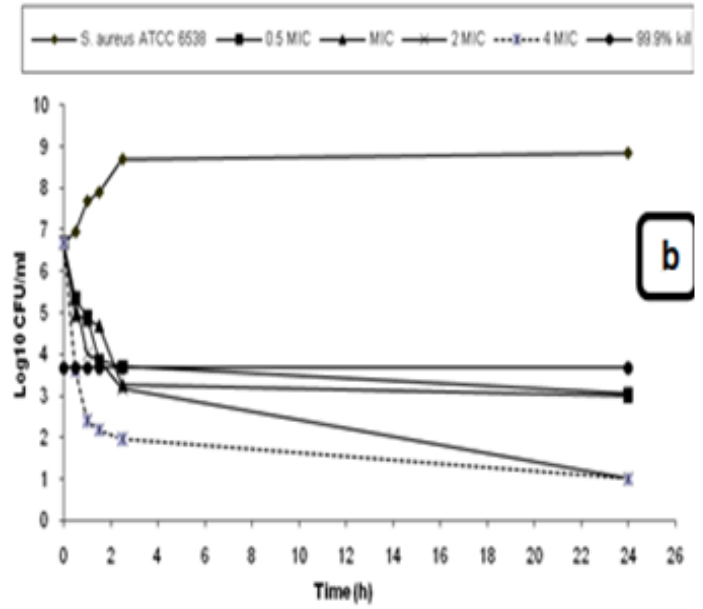
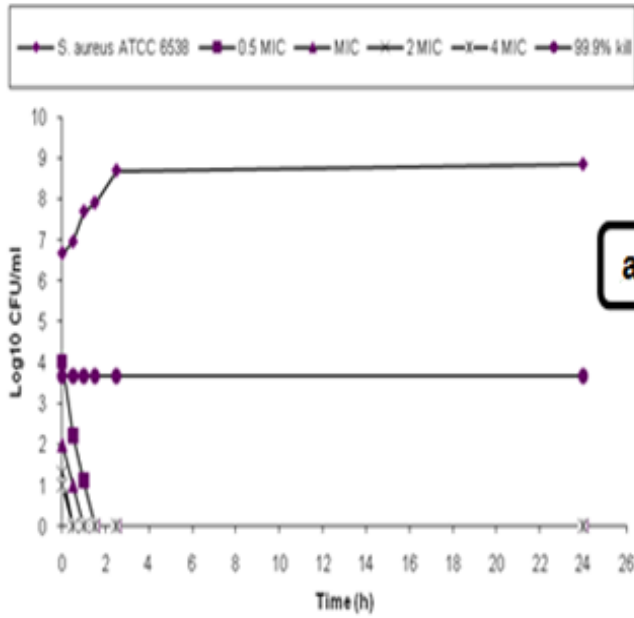
**Figure 1.** Effect of eugenol and linalool on germ tube formation by *Candida albicans* strains. a. Untreated *C. albicans* (ATCC 10231) (Control); b. *C. albicans* (ATCC 10231) treated with eugenol; c. *C. albicans* pathogenic strain treated with eugenol; d. *C. albicans* (ATCC 10231) treated with linalool.

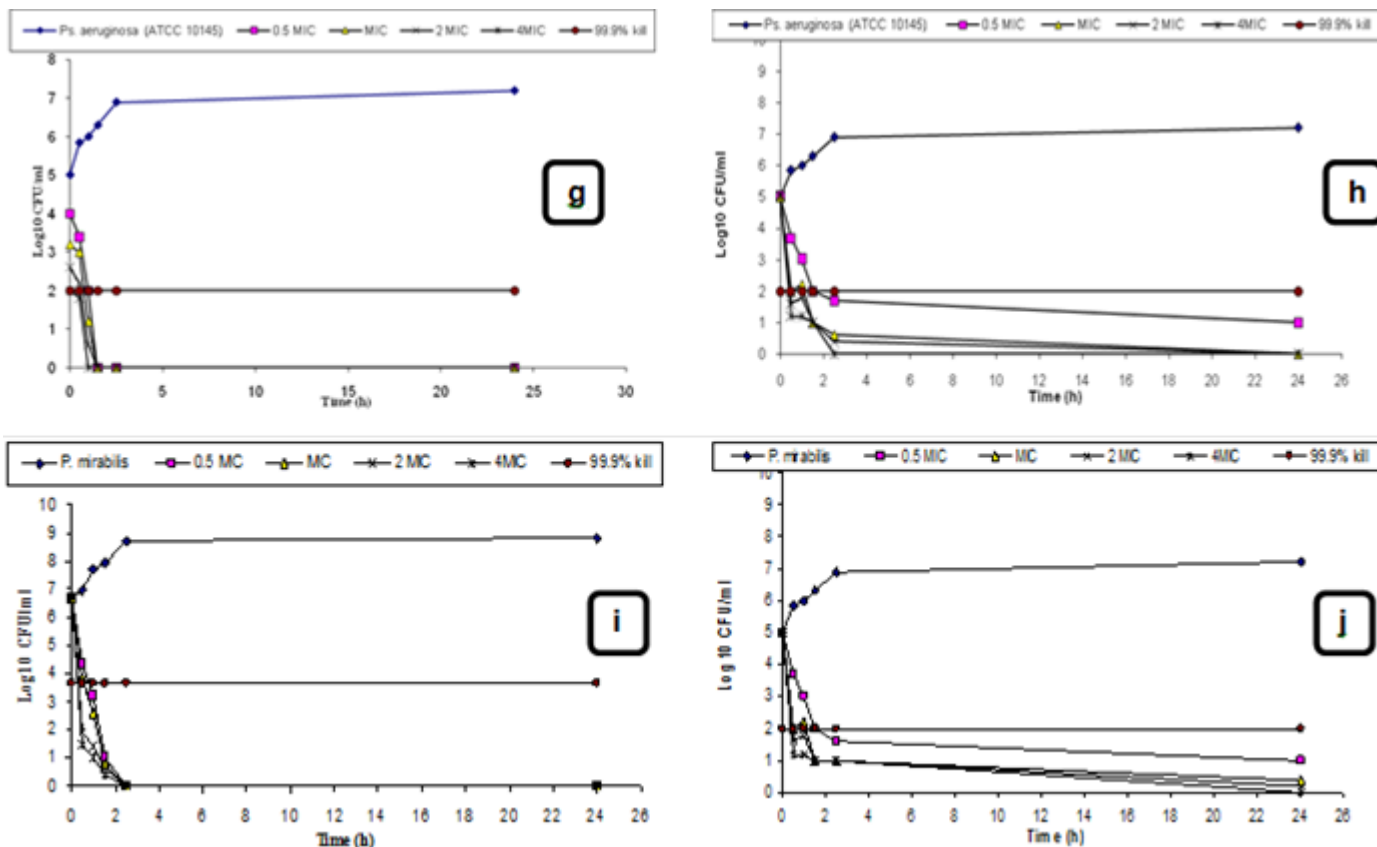
of pathogenicity of *C. albicans* (Pinto et al., 2008). Most antifungal drugs are germ tube formation inhibitors (Ellepola and Samaranayake, 1998). By testing the effect of eugenol and linalool at sub-inhibitory concentrations on the germ tube formation of *Candida albicans* strains, it was found that eugenol completely inhibited serum induced hyphae in *C. albicans* (ATCC 10231) and *C. albicans* clinical strain at Sub-MICs. The inhibitory effect was dose-dependent. Linalool reduced the germ tube production significantly, rendering the cells less pathogenic as compared to control but to lesser extent compared to eugenol (Figure 1). Consistent with this, Pinto et al. (2009) found that eugenol's sub-inhibitory concentrations completely inhibited germ tube formation by *C. albicans*. Chami et al. (2004 and b), revealed the antifungal activity of eugenol in the treatment and prophylaxis of animal models of oral and vaginal candidiasis. Also, Hsu et al. (2013) demonstrated that linalool exhibited dose-dependent inhibitory activity against germ tube formation.

#### Time-kill assay

The results of time kill kinetic studies are illustrated in

Figures 2 and 3. Eugenol was effective against all bacterial and fungal strains. It inhibited most isolates at half MICs either within 1.5 h or 2.5 h exposure, while standard *C. albicans* and *C. krusei* required MICs for inhibition. Linalool showed less activity. It had slower kill rate at 24 h of incubation either at 0.5 MIC in case of *K. pneumoniae* or at MIC as shown by *P. aeruginosa* and *C. albicans*. *C. glabrata* and *P. mirabilis* required two fold higher concentration and four fold MICs respectively. Also, linalool showed no activity against *S.aureus* and *E.coli*. Only MIC was fungicidal to *C.krusei* at 2.5 h of incubation. Kill curve assays of *C. albicans* standard strain was performed by Hsu et al. (2013) using the broth macrodilution method. Linalool showed its fungicidal effect at MIC, and the fungicidal endpoint was achieved within 1 h. Zore et al. (2011) demonstrated that MFC of linalool and eugenol killed 99.9% of *C. albicans* inoculum within 7.5 min of exposure. Recent investigations about the anti-candidal action of terpenoids showed that they modulate mevalonate pathway (MP), alter cellular levels of intermediate molecules and associated functions in eukaryotic cells (Mo and Elson, 2004). Other researchers reported that terpenoids destabilize membrane and modulate its associated functions like permeability, cell signaling, etc., leading to cell death and ascribed the





**Figure 2.** Time-kill curves with different concentrations of eugenol (Left panels) and linalool (Right panels) for bacterial species: (a and b) *S. aureus* (c and d) *E. coli* (e and f) *K. pneumoniae* (g and h) *P. aeruginosa* and (i and j) *P. mirabilis*.

antibacterial activity of linalool to its membrane destabilizing activity, sensitization of bacteria and enhancement of drug sensitivity (Trombetta et al., 2005). Regarding eugenol, hydroxyl group contributes to its inhibitory effect at sub-lethal concentrations as it binds to and affect the properties of proteins, thus inhibits the activity of some enzymes such as ATPase which may be important for cell killing at high eugenol concentrations because energy generation needed for cell recovery is impaired (Gill and Holley, 2006b).

#### Effect of eugenol and linalool on release of cytosolic material

To confirm the results of kill kinetics and for further understanding the mechanism of antimicrobial action of eugenol and linalool we evaluated the lysis of microbial cells, by treatment with different concentrations of the two compounds compared with levofloxacin and ketoconazole and measure the release of substances absorbing at 260 nm. The evaluation of the loss of cell contents contributes to demonstrate the severity of the cell membrane damage.

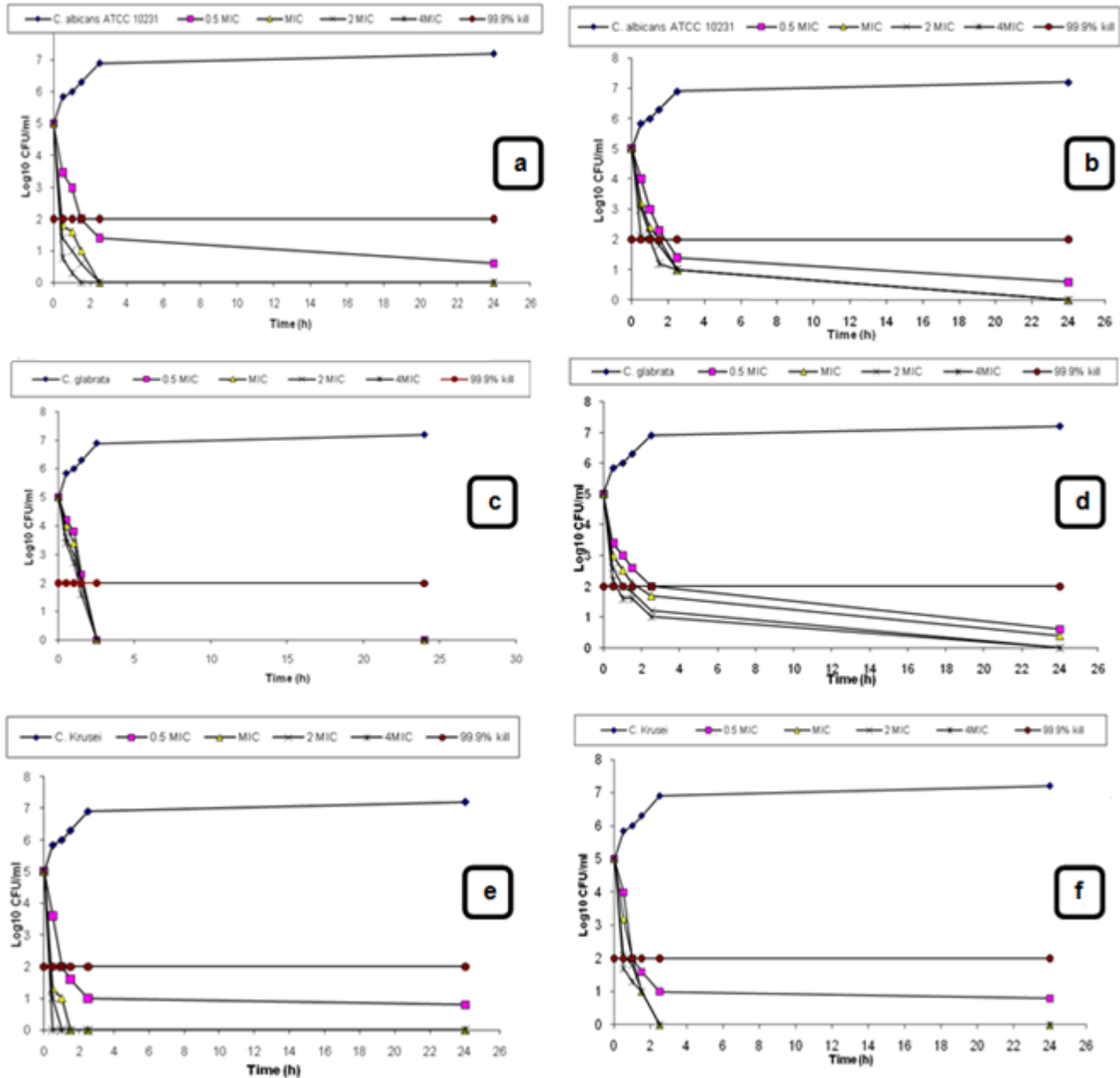
Our study shows that no significant absorbance was

obtained on treatment with linalool. Concerning *C. glabrata*, slight increase obtained but still much lesser compared to eugenol and ketoconazole. Figures 4 and 5 show that the absorbance and accordingly the intracellular constituents release was increased in linear pattern with the increase of eugenol concentration. With all bacterial strains and at all tested concentrations, eugenol showed higher absorbance than levofloxacin. On the other hand, release of cellular constituents obtained with ketoconazole was higher than eugenol after 6 mM with standard *C. albicans* strain and at 12 mM with *C. glabrata* strain. Also, sudden increase was obtained with *S. aureus* after 1.5 mM. These results can be attributed to the eugenol's ability to disrupt the membrane and allowing the leakage of cellular content.

A study performed by Oyedemi et al. (2009) on some Gram-negative and Gram-positive bacteria revealed that eugenol induced cell lysis by damaging the cell wall and membrane caused leakage of protein and lipid contents after 120 min of exposure.

Also, it was reported that eugenol's action on membranes occurs mainly by a non-specific permeabilization which increase the transport of potassium and ATP out of the cells (Gill and Holley, 2006a).





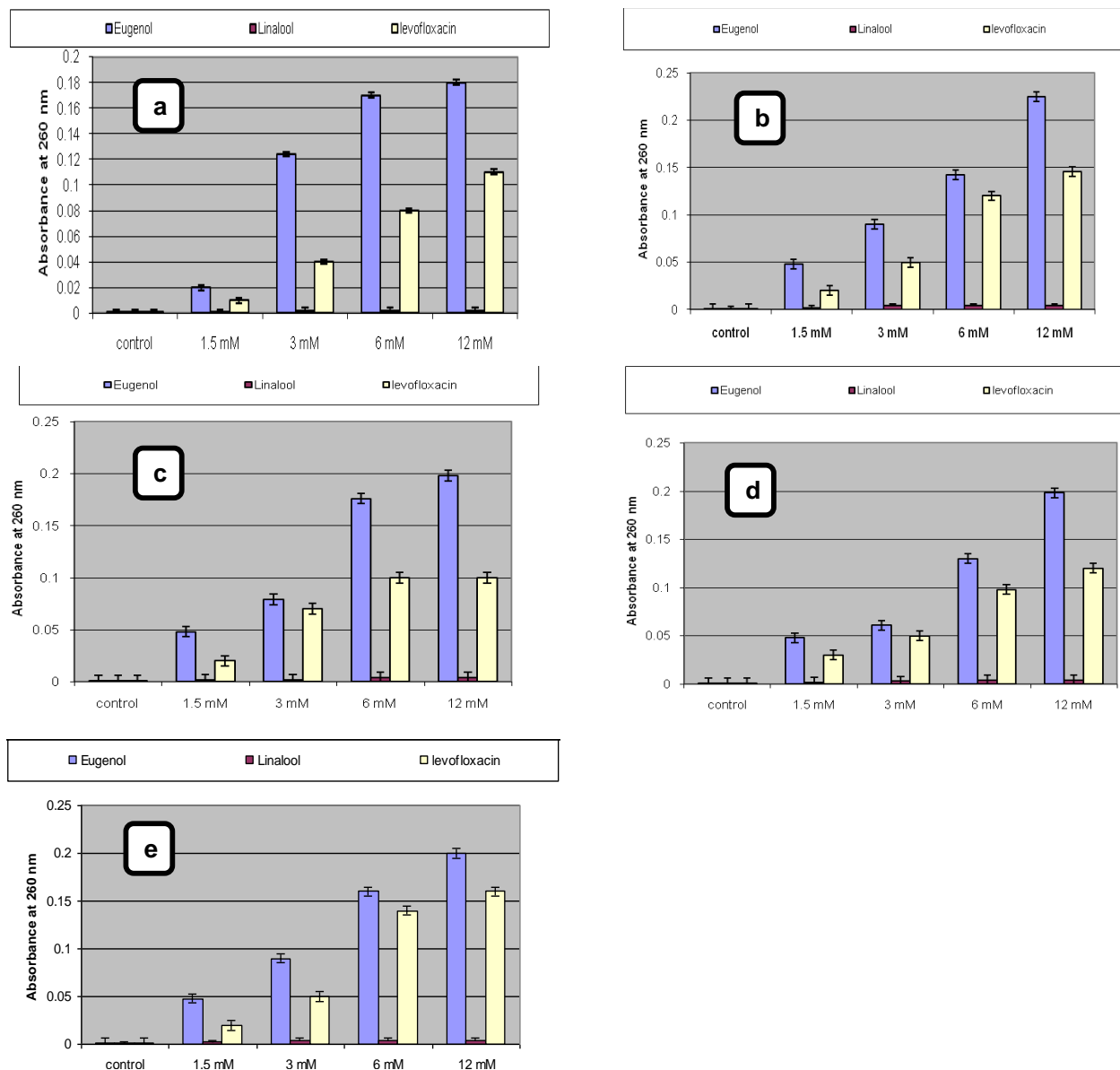
**Figure 3.** Time-kill curves with different concentrations of eugenol (Left panels) and linalool (Right panels) for *Candida* species: (a and b) standard *C. albicans* strain (c and d) *C. glabrata* and (e and f) *C. Krusei*.

### Effect of the tested compounds on the morphology of microorganisms using scanning electron microscopy (SEM)

We studied the action of eugenol and linalool on the cell morphology using SEM. Linalool treated *S. aureus* cells showed disrupted membranes and appeared as a mass of cells (Figure 6b). While in case of *P. aeruginosa* treated cells no significant changes were obtained compared to the non treated control (Figure 6f). Concerning *E. coli* cells treated with eugenol visualized

by SEM, significant deformities, irregularities and holes in the envelope were observed (Figure 6d).

Regarding *C. albicans* (ATCC 10231), Figure 7a demonstrated that the action of linalool on cells appeared as cell wall deformity and holes. While in case of eugenol treated cells, the cell wall is different compared to that of the control cells as treated ones shows many pores on the membranes (Figure 7b). SEM observations revealed that both compounds affect not only the membrane but all the envelope of bacterial and fungal cells. Bennis et al. (2004a) studied the effect of eugenol on *Saccharomyces*

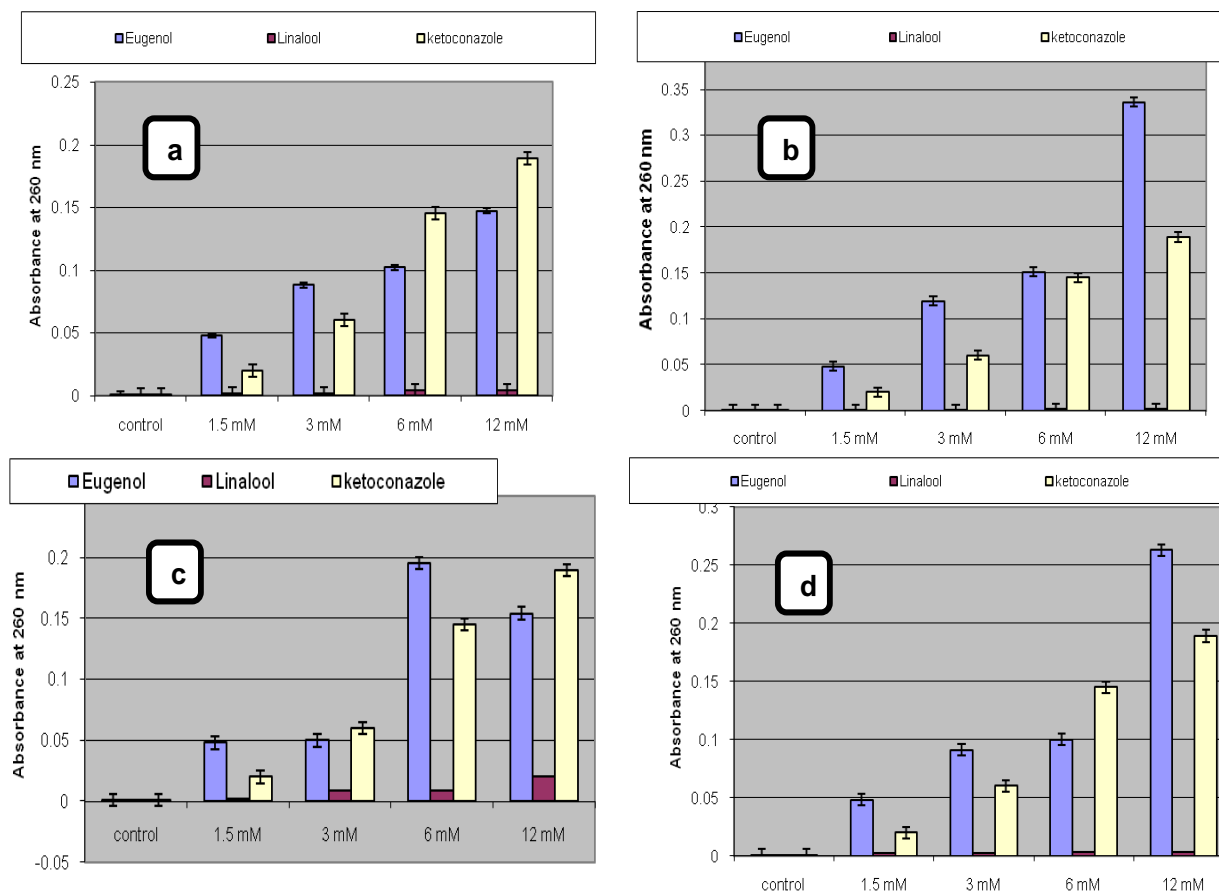


**Figure 4.** The effect of eugenol, linalool and levofloxacin on the release of 260 nm absorbing material from bacterial strains: (a) *S. aureus* (b) *E. coli* (c) *K. pneumoniae* (d) *P. aeruginosa* (e) *P. mirabilis*.

*cerevisiae*, *B. subtilis* and *E. coli* cells. Eugenol induced important morphological damages in *S. cerevisiae*. Also, the difference in the eugenol's action on the *B. subtilis* and *E. coli* was suggested to be the result of the difference in structure between Gram-positive and Gram-negative bacteria. Nazzaro et al. (2013) reported that rod shaped bacterial cells are more sensitive to EOs than coccoid cells. Results obtained by Di Pasqua et al. (2007) revealed alterations in the composition of the fatty acids and the morphology of the cells when treated with eugenol. They suggest that eugenol may disrupt the *E. coli* membrane and allow the leakage of intracellular contents.

### Screening of $\beta$ -lactamase production, urease production and motility

Other possible antibacterial mechanisms of eugenol and linalool were investigated. Two methods were used for detection of  $\beta$ -lactamase production in all strains; iodometric and acidometric methods. Both methods showed that linalool had no significant activity on  $\beta$ -lactamase producing strains, while  $\beta$ -lactamase production was inhibited on treatment with eugenol (Table 2). Previous report of Dhara and Tripathi (2013) demonstrated that eugenol and cinnamaldehyde possess strong antibacterial activity against extended-spectrum  $\beta$ -



**Figure 5.** The effect of eugenol, linalool and ketoconazole on the release of 260 nm absorbing material from *Candida* strains: (a) *C. albicans* (ATCC 10231) (b) *C. albicans* clinical strain (c) *C. glabrata* (d) *C. krusi*.

lactamase (ESBL) positive strains. These compounds are hydrogen bonded with catalytic and crucial amino acid residues of ESBL proteins.

Bacteria need urease in order to obtain nitrogen for their growth. Urease inhibitors are potential alternatives for the prevention of *K. pneumoniae* from colonizing the gastrointestinal tract (Maroncle et al., 2006). Our data showed that eugenol inhibited urease production and arrest motility of all treated cells. On the other hand, linalool failed to inhibit bacterial urease enzyme and motility as summarized in Table 2.

The effect of terpenes on *Proteus* spp. swarming behavior was indirectly reported by Mansouri et al. (2005). Echeverrigaray et al. (2008) evaluated the effect of 17 monoterpenes on *P. mirabilis* swarming and they found that 8 compounds inhibited swarming significantly. They revealed that linalool had no activity on the *P. mirabilis* swarming. The monoterpenes antimicrobial activity increases with the presence of an oxygen containing functional group (Naigre et al., 1996). Effect of other EOs on bacterial motility was studied. Gabel and Berg (2003) reported that carvacrol inhibited flagellin.

Synthesis and decreased the proton motive force

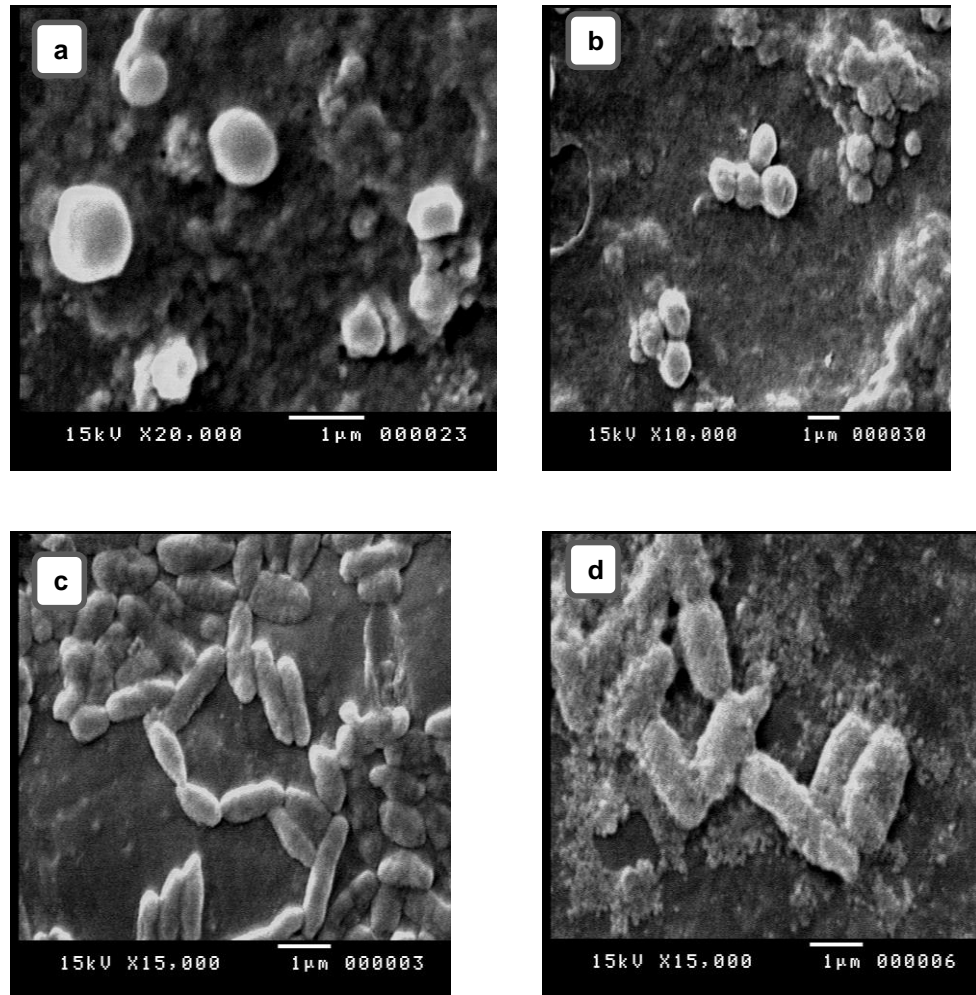
required for flagellar movement of flagellated cells.

## Conclusion

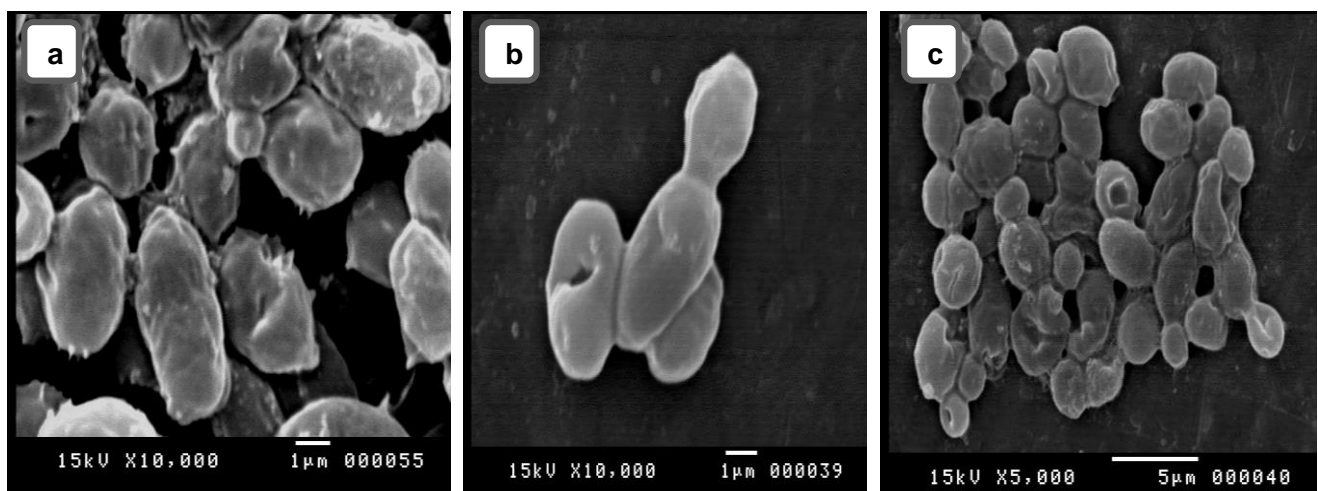
In conclusion, our results indicate that eugenol and linalool exhibit promising antimicrobial activities against Gram-positive, Gram-negative and *Candida* pathogenic strains and could be used as natural alternatives for application in medical field. It was concluded that the antibacterial activity of linalool was much weaker than the antifungal activity. Also, the antimicrobial activity of eugenol is more powerful than linalool. These results suggest that a number of the components of the EOs such as eugenol and linalool may have potential clinical applications in treating microbial infections alone or in combination with other EO constituents or with conventional antibiotics.

## Conflict of Interests

The authors have not declared any conflict of interests.



**Figure 6.** Scanning electron microscopy images showing: (a) *S. aureus* untreated cells (Control), (b) *S. aureus* cells treated by linalool, (c) *E. coli* untreated cells (Control), (d) *E. coli* cells treated by eugenol, (e) *P. aeruginosa* untreated cells (Control) and (f) *P. aeruginosa* treated by linalool.



**Figure 7.** Scanning electron microscopy images of *C. albicans* (ATCC 10231) cells treated by (a) linalool and (b and c) eugenol.

**Table 2.** Effect of eugenol and linalool on  $\beta$ - lactamase production, urease production and motility of the tested strains.

Strains	$\beta$ - lactamase production		Urease production		Motility	
	Eugenol	Linalool	Eugenol	Linalool	Eugenol	Linalool
<i>S. aureus</i>	-	+	-	+	NT	NT
<i>E. coli</i>	-	+	NT	NT	-	+
<i>K. pneumoniae</i>	-	+	-	+	NT	NT
<i>P. aeruginosa</i>	-	+	-	+	-	+
<i>P. mirabilis</i>	-	+	-	+	-	+

-, Negative; +, positive; NT, not tested.

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