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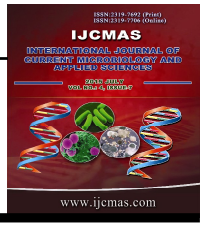

Original Research Article

Antifungal potential of *Syzygium aromaticum* and *Zanthoxylum xanthoxyloides* essential oils against *Phaeoramularia angolensis*, causing phaeoramularia leaf and fruit Spot disease on citrus

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K e y w o r d s

Essential oil, Z. xanthoxyloides, *S. aromaticum*, chemical composition, *P. angolensis*, antifungal activity

A B S T R A C T

The *Phaeoramularia* leaf and fruit spot disease caused by *Phaeoramularia angolensis* (*P. angolensis*) is the most damaging disease of citrus plant in tropical Africa. In Cameroon, fungicides application have shown a great inhibitory effect on the fungus but are not without harmful effects on man and the environment. Thus this study were conducted to determine the chemical composition and antifungal activity of essential oils (EOs) from fruits and dried buds of *Zanthoxylum xanthoxyloides* (*Z. xanthoxyloides*) and *Syzigium aromaticum* (*S. aromaticum;* Clove), from Cameroon against *P. angolensis*. EOs, obtained by hydrodistillation was analysed by Gas Chrommatography (GC) and Gas Chromatography coupled with Mass Spectrometry (GC/MS). The antifungal activity was tested by Agar Dilution Technique. The hydrodistillation yielded 10,54% and 1,89% EOs for *S. aromaticum* and Z. *xanthozyloides* respectively. Eugenol (83.02%) and eugenyl acetate (9.15%) were the main components from clove oil while α-citronelol (25.83%) and trans-geraniol (16.49%) were mostly founded in the *Z. xanthozyloides* oil. Mycelia growth inhibition was observed for all tested concentrations for each oil, whereas total inhibition was obtained only at higher concentrations. The minimal inhibitory concentration (MIC) was obtained at 600 and 1400 ppm for *S. aromaticum* and *Z. xanthoxyloides* essential oils respectively. These EOs could thus constitute an alternative to synthetic fungicides usually used for citrus fruit crops.

Introduction

The genus *Citrus* of the family *Rutaceae* includes 17 species distributed throughout the tropical and temperate regions (Davies and Albrigo, 1994; Shaw, 1977). Some

common species such as *Citrus sinensis* (L.) Osb., *Citrus reticulata* Blanco, *Citrus paradisi* Macf., *Citrus maxima* (Burm.) Merr., *Citrus limon* (L.) Burm. F., *Citrus aurantifolia* (Christm.) Swing. *Citrus aurantium* (L.) and *Citrus medica* (L.) are grown in Cameroon by different ethnic groups both for economical purpose and for their nutritional values.

In fact, Citrus fruits belong to a very interesting food group due to their high vitamins, fibre and rock salt contents. The consumption of Citrus fruits is a primordial guarantee to good health (Jazet *et al*., 2009). In addition, essential oils obtained from Citrus are of great commercial interest due to their odorous properties, pharmaceutical, and organoleptic interest.

They thus constitute the raw materials for perfumery, cosmetic, food, chemical and pharmaceutical industry. Incomes generated by the sales of citrus fruits and their EOs could therefore contribute to the revival of the economies of the countries (Economos and Clay, 1999).

In Cameroon, efforts has been made to develop this crop but they are hindered by many obstacles amongst which the use of non-selected seedling (Ndo, 2011), the poverty of farmers mostly smallholders, that limits the access for chemical inputs leading to fungi and insects attacks (Dury, 1999; Kuate *et al.*, 2006; Ndo, 2011). Indeed, for a few decades, a pathogenic fungus, *Phaeoramularia angolensis* has inflicted heavy losses on the production of citrus fruits harvests leading to over 80% loss of total harvest in certain areas of the country with high disease incidence (Kuate *et al*., 1994; Kuate, 1997; Jazet *et al*., 2009).

To control the disease, a wide range of organic and systemic pesticides have been proposed over time namely: benomyl, folpet, captane, dichlofluanide, mancozebe,penconazole, carbendazime, methidation, chlorpyrifos, dimethoate, fenarimol, cypermethrine (Cabras and Angioni, 2000; Cuthberson and Murchie, 2003; Edder *et al*., 2004; Akiyama *et al*., 2005).

In spite of the effectiveness of these synthetic chemicals, less than 85 tons of citrus fruits are produce in Cameroon annually, failing to satisfy the national demand that is estimated to 150 000 tons per year (FAOSTAT, 2011). Besides, the use of such chemicals has significant drawbacks including cost, handling hazards, and threats to the environment (Blasco *et al*., 2005; Ortelli *et al*., 2005; Jawich, 2006). In addition, the application of higher concentration of chemicals in an attempt to overcome this problem increases the risk of high level toxic residues in the product, which is particularly serious because fruit and vegetables are consumed in a relatively short time after harvest (Mansoori and Banihashemi, 1982; Maleki et al., 2011). These problems have caused research to be directed towards plant-derived natural fungicides which are relatively less damaging for pest and disease control in agriculture (Costa *et al.*,2000). Previous in vitro and in vivo investigations suggested that the essential oils could be used as effective antifungal agents Adam *et al* ., 1998). However, there are only limited data available on the antifungal activity of essential oils against human and plant fungal pathogens (Vidyasagar *et al*., 2013; Nana *et al.*, 2015). Nevertheless, as disease control agents, essential oils have been used since they are known for their broad spectrum; particularly their antioxydant (Bouzouita *et al.*,2008; Jazet *et al*., 2008), insecticidal (Erler *et al*., 2006; Cheng *et al*., 2009) cyto-toxic (Bakarnga-Via *et al*., 2014) and antimicrobial (Magina *et al*., 2009 Sirrirat and Kittipot; 2010; Xing *et al*., 2011;

Senthilkumar and Venkatesalu, 2013) properties.

Z. xanthoxyloides Lam and *S. aromaticum* (L.) Merr. & L. M. Perry, belonging for the family of Rutacee and myrtaceae respectively, are used in Cameroon as food additives and medcine. Previous research papers reported the antimicrobial potential of their essential oils toward both human and phytopathogens (Ngono *et al.,* 2000; Tatsadjeu *et al*., 2003; Sirirat and Kittipot, 2010; Xing *et al.*,2011) but their effects onthe inhibition potential of essential oils from these plants has notyet been reported against *P. angolensis*. Therefore, the present study aimed to evaluate the phytochemical composition and *in vitro* antifungal activities of these essential oils against *P. angolensis*, causative agent of citrus cercosporiosis.

Materials and Methods

Fungal material

The strain CMR5 of *P.angolensis* from *Citrus sinensis* var Valencia late was used in this study. The fungus was maintained in the culture collection in Regional Laboratory of Biological control and Apply Microbiology of the national Institute of Agricultural Research for Development (IARD) Yaounde-Cameroon. It was grown on Potatoes Dextrose Agar (PDA) (Difco, Detroit, MI) at 22°C for 15 days and stored at 4°C until used.

Sample collection and essential oils extraction

Fresh fruits of *Z. xanthoxyloides* and *S. aromaticum* were harvested in Bangou and Kribi (West and South region Cameroon respectively) in mid April 2011 and identified at the National Herbarium of Cameroon, where voucher specimens are deposited under the Registrations numbers: 506167HNC and 21793SRFCAM for *S. aromaticum* and *Z. Xanthoxyloides* respectively. The samples were then dried at room temperature (28^{o}C) for two weeks and each were ground using a grinder. 100 g of powder form each sample were subjected to hydrodistillation for 3h using a Clevenger type apparatus. The isolated essential oils were dried on anhydrous sodium sulfate column (Na_2SO4), and then stored in dark glass container at 4°C until the use**.**

Chemical analyses

The essential oil obtained was analyzed by gas chromatography and gas chromatography coupled with mass spectrometry.

Gas Chromatography

Essential oil (10 µl) was dissolved in pentane (100 µl) and 2 µl of the solution was injected into a Varian CP-3380 GC with flame ionization detector (FID) and fitted with a fused silica capillary column (30 m x 0.25 mm coated with DB5, film thickness 0.25 µm); temperature program 60°-220°C at 3°C/min, injector temperature 200°C, detector temperature 220°C. Nitrogen gas was used as a carrier gas at a constant flow rate of 1 ml/min. the linear retention indices of the components were determined relative to the retention times of a series of *n*- alkanes and the percentage compositions were obtained from electronic integration measurements without taking into account relative response factors.

Gas chromatography/ Mass spectrometry

GC/MS analyses were performed using a Hewlett–Packard GC 5890 series II equipped with a HP5 (5%-phenyl-methylpolysiloxane) fused silica column (30 m x 0.25 mm; film thickness 0.25 µm) interfaced with a quadrupole detector

(Model 5972) applying the same temperature program as for the GC/FID analyses; injector temperature, 220°C; MS transfer line temperature, 250°C; carrier gas, helium at a flow rate of 0.6 mL/min; injection type, split, 1:10 (1 µL 10:100 CH_2Cl_2 solution); ionization voltage, 70 eV; electronmultiplier 1460 eV; scan range 35-300 amu; scan rate, 2.96 scan/s.

Identification of the components

The identification of the constituents was based on comparison of their relative retention indices with either those of authentic samples or with published data in the literature (Adams, 2007) and by matching their mass spectra with those obtained with authentic samples and/or the NBS75K, Wiley 7th NIST 98 EPA/NIH, and FFNSC 2 libraries spectra.

Agar dilution assay

The antifungal activity of the essential oil against *P. angolensis* was evaluated by the agar medium assay as described by Grover and Moore (1962). The PDA (potatoes dextrose agar) medium was autoclaved (121°C, 15 min, 1.1 Pa).

Essential oil was mixed with polysorbate 80 (Tween 80) (10%) in proportions 1/9 v/v.The mixture of EO/Tween 80 obtained was incorporated in the melted PDA medium (45°C), so as to obtain 200, 250, 300, 350, 400, 600, 800, 1000 and 1200 ppm.

The medium thus supplemented was poured in Petri dishes of 90 mm at a rate of 20 ml per dish and allowed to rest for solidification. A mycelia disc of 5mm in diameter taken on a 15 day old preculture of *P. angolensis* was placed directly at the centre of each dish. In controls, Benomyl (3.3 g/l) (positive control) or sterile distilled water mixed with Tween 80 (0.5%, v/v) (negative control) were used instead of essential oils. Three Petri dishes were use for each concentration. The overall dishes were incubated in an inverted position at 22°C in the dark. After 10 days, the mycelia growth was observed with the diameter measured along two perpendicular lines passing across the centre of the dish, with a 10 day regular interval for up to 40 days.

Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of essential oil in which no growth occurred.The inhibition percentage (I%) was calculated according to the equation: I% = (dt – dc/dt) ×100 Where dc = mean diameter of mycelial growth in control dishes and dt = mean diameter for treated mycelium.

Minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC)

Petri dishes with the lowest concentration of essential oil without any mycelia growth until 40 days, were recorded as MIC. MFC was determined by sub-culturing agar plugs from dishes showing no growth on PDA medium without essential oil. The lowest concentration on which no growth occurred were defined as the MFC after 40 days of incubation (Angelini *et al*., 2006).

Data analysis Data from four independent replicate were subjected to one-way ANOVA using SPSS Statistical package (version 17.0). Differences between means were tested using Least Significance Difference (LSD) test and the Pearson correlation was used to study the relationships between incubation time, concentration and inhibition percentages.

Results and Discussion

Essential oils analysis

The quantitative and qualitative analytical results of the essential oils revealed a total of 17 and 14 components in different amount, respectively in *Z. xanthoxyloides* and *S. aromaticum* essential oils (table 1 and Figure 1). The tested essential oil contained high percentages of oxygenated monoterpenes (89.58 % for*S. Aromaticum* and 88.2 % for *Z. xanthoxyloides*). sesqueterpenes hydrocarbons (8.30%), and oxygenated (1.45%) was identified only in the clove oil. Inversely, Hydroarbon monoterpenes was only present in the essential oil of Z. xanthozyloides (6.92%). A few amount of linear compounds was identified in the both essential oils (0.63 % for *S. aromaticum*and 4.83% for *Z. xanthoxyloides*). The major components in clove oil were eugenol (83.02%), eugenyl acetate (9.15%), isocaryophyllene (7.04%), α-caryophyllene (0.85%) and α-bisabolène epoxide (0.78%).While α-citronelol (25.83%), trans-geraniol (16.49%), citronellic acid (8.51%), α-citronellal (6.85%),citronellyl acetate (5.02%), limonene (3.95%), geranyl acetate (3.82%) and p-menthenol (3.69%)were mostly identified in *Z. xanthozyloides* oil. These essential oils also contains some other constituents (<1%) in minor level (Data not shown).

Antifungal activity

The *in vitro* assay showed that all the tested essential oils significantly reduced the mycelial growth of *P. angolensis* as compared to the negative controls ($P<0.05$). The mycelia growth was considerably reduced with increasing concentration of essential oil while the mycelial linear growth increased with incubation time (figures 2 and 3). These observations were highlighted by the Pearson correlation which showed a negative relationship between mycelial growth and increase in concentration ($r = -0.927$ and -0.914; $P<0.01$ respectively for *Z. xanthozyloides* and *S. aromaticum*). In addition, there were no fungal growth at concentrations above 600 ppm and 1400 ppm for *Z. xanthozyloides* and *S. aromaticum* respectively over the 40^{th} day of incubation. In addition, at 3.3 g/l (recommended by the manufacturer) Benomyl totally suppressed the mycelial growth of *P. angolensis*

Inhibition Percentages, MIC and MFC

The inhibition percentages at each concentration for the two EOs revealed an increase as concentration of EOs increases (figure 4). The highest activities being observed at high concetrations. Total inhibition is thus reached at concentrations equal or above 1400 ppm for the *Z. xanthozyloides* oil which seems to be the least effective, the most effective being clove oil which showed no mycelia growth stating at 600 ppm ($p<0.05$) (figure 4). The MIC values obtained by the essential oil of *S. aromaticum* and *Z. xanthozyloides* were respectively 600 ppm and 1400 ppm. After the culture of mycelia dishes coming from inhibited plates on fresh PDA without essential oils, no growth occurred until the 40^{th} day of incubation showing the lethal effect of tested essential oils at these concentrations.

In recent years, environmental issues caused by excessive use of pesticides has been a major topic in debates conducted for both scientists and public (Koul *et al*., 2008). Pesticides reduce biodiversity of flora and fauna, their residues are present in all compartments of agro ecosystems and its consumption in food as vegetables and fruits

represents a real risk to human's health (Price *et al*., 2008). The use of chemicals for pest control in agricultural products, especially in directly consumable foods as fruits is becoming increasingly restricted by various authorities, and by consumers who prefer technologies which are safe to humans and the environment (Ganmor *et al*., 2011). Explicit requirements for food safety and environmental protection have led to the need to create new and safe plant disease control strategies (Adebayo *et al*., 2013). Natural products are a suitable alternative to synthetic pesticides as a mean to reduce negative impacts on human health and the environment (Wegulo *et al*., 2011; Badea and Delian, 2014). In this context, several studies are being conducted to explore the potential of essential oils and plant extracts as antifungal agents (Browers and Locke, 2004; Arıcı *et al*., 2011; Sue *et al*., 2012; Serife and Arif, 2014; Badea and Delian, 2014, Nana *et al*., 2015b) in view of their potential applications as botanical fungicide.

In this study, we investigated the antifungal property and the chemical composition of essential oils from seeds of Z. *xanthozyloides* and *S. aromaticum* against *P. angolensis*, causal agent of Citrus fruits leaf and fruits spot in Cameroon and many other African countries. The hydrodistillation of plant materials afforded essential oils with 10,54% and 1,89% yields for *S. aromaticum* and Z. *xanthozyloides* respectively. These results are different to those previously obtained by Ayoola *et al.* (2008) who obtained 7% yield of EOs from *S. aromaticum* harvested in Nigeria and Jazet. (2008) who, obtained oilsfrom dried fruits of Z. *xanthozyloides* fromBangou (West-Cameroun) with much higher yield (3,88%). Not surprising results since Braga *et al.*(2005) and Bakarnga-Via *et al*. (2014) reported that extraction yield could differ according to the plant species and geographical origin. Phenolic compounds (eugenol and eugenyl-acetate), oxygenated mono (α-citronelol, Trans- geraniol, linalol…) and sesquiterpènes (Farnesol, Bisabolene epoxid…), and their respective hydrocarbons (α-pinène, Myrcène, Limonène and Cubebene, Isocaryophyllene, Caryophyllene) were identified in at least one of the tested essential oils. These results are in agreement with earlier findings of Al-Reza *et al*. (2009) and Cakir *et al*. (2004) who reported that mono and sesquiterpene hydrocarbons and their oxygenated derivatives are the major components of essential oils of plant origin. The identification of eugenol and eugenyl-acetate as mains components in clove oil corroborate with several reports (Eugenia et al., 2009; Xing *et al.*, 2011). Changes in relative percentages of components available in essential oils of Clove and *Z. xanthozyloides* as compared with results obtained by Xing *et al.* (2011) and Jazet (2008) respectively could depend upon the climate, the method of drying the plant samples and the soil type (Braga *et al.*, 2005).

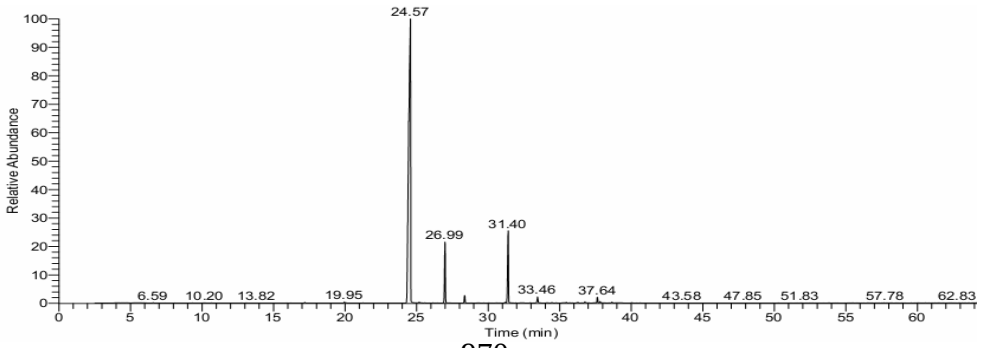
Further survey of the available literature reveals that the activity of the oils would be expected to relate to the respective composition of the plant volatile oils, the structural configuration of the constituent components and their functional groups (Tassou *et al*., 2000; Kim and Park, 2012; Moustafa *et al*., 2013). In addition, Burt. (2004) reported that essential Oils containing a high percentage of phenolic monoterpens compounds such as carvacrol, thymol and eugenol possess strongest antimicrobial properties against foodborne pathogens. The high activity of these secondary metabolites is certainly due to the acidic nature of the hydroxyl group of phenols which facilitates a hydrogen bond with pathogens enzyme active centres (Griffin *et al*., 1999; Roser *et al*., 2013).

Table.1 Main components relative content and Kovat Index of EOs from *S. aromaticum* and *Z. xanthozyloides*

Compounds	*KI	Relative content (%)	
		S. aromaticum	*Z. xanthozyloides.*
Hydrocarbons Monoterpens		**0**	**6.92**
α-pinène	934	-	1.03
Myrcène	985	-	1.06
Limonène	1024	-	3.95
Oxygenated Monoterpens		**89.58**	**88.2**
trans-Rose oxid	1041	-	1.3
p-Methyl-5-octen-2-ol	1059	-	1.48
p-Menthènol	1073	-	3.69
α –Citronellal	1082	-	6.85
Linalol	1091		1.59
α –Citronellol	1160	-	25.83
trans-Geraniol	1185	-	16.49
p- hydroxyphenylpropylène	1192	0.14	-
Acide Citronellique	1254	-	8.51
Citronellol acetate	1274	-	5.02
Eugenol	1280	79.42	-
Acide geranique	1288	-	2.35
geraniol acétate	1302	-	3.82
Eugényl acetate	1429	9.15	-
Hydrocarbons Sesquiterpens		**8.30**	**0**
α-Cubebene	1293	0.1	-
Isocaryophyllene	1332	7.04	-
Caryophyllene	1333	0.11	-
α-Caryophyllene	1362	0.85	
Epizonarene	1425	0.12	-
Oxygenated Sesquiterpens		**1.45**	**0**
α-Bisabolene epoxid	1476	0.78	-
Alloaromadendrene oxid	1522	0.10	-
Isoaromadendrene epoxid	1541	0.12	-
Longipinocarveol	1553	0.16	-
Farnesol	1578	0.17	-
α-Farnesol	1598	0.11	-
Linear Compounds		**0.63**	**4.83**
Undecanol	1420		1.22
(9E)-Hexadecen-1-ol	1574	0.63	-
(Z)-3-Heptadecene,	1480	-	1.69
(9E)-9-Hexadecen-1-ol	1487	-	1.31

*KI: Kovat Index

Fig.1 Gas Chromatography chromatogram of essential oils of *S. aromaticum* (a) and *Z. xanthozyloides* (b)



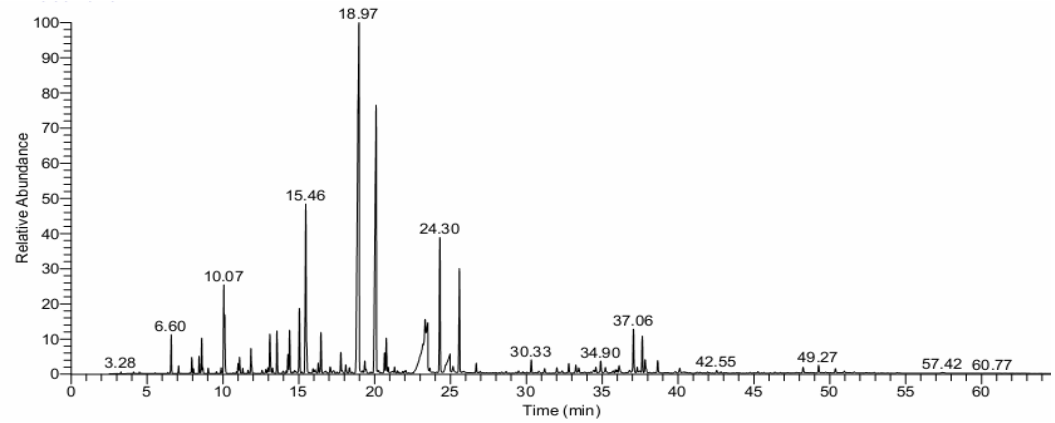


Fig.2 Curves of *P. angolensis* mycelial growth versus incubation time for *S. aromaticum* essential oil

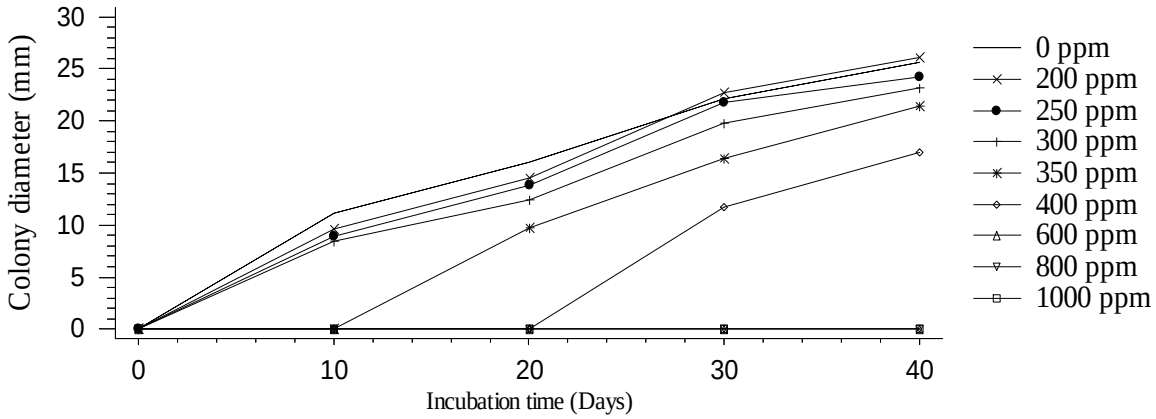


Fig.3 Curves of *P. angolensis* mycelial growth versus incubation time for *Z. xanthozyloides* essential oil

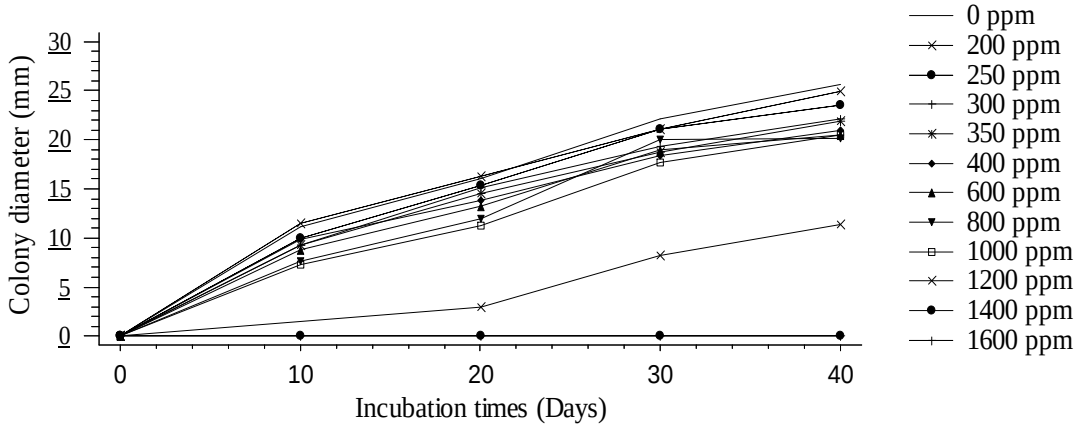
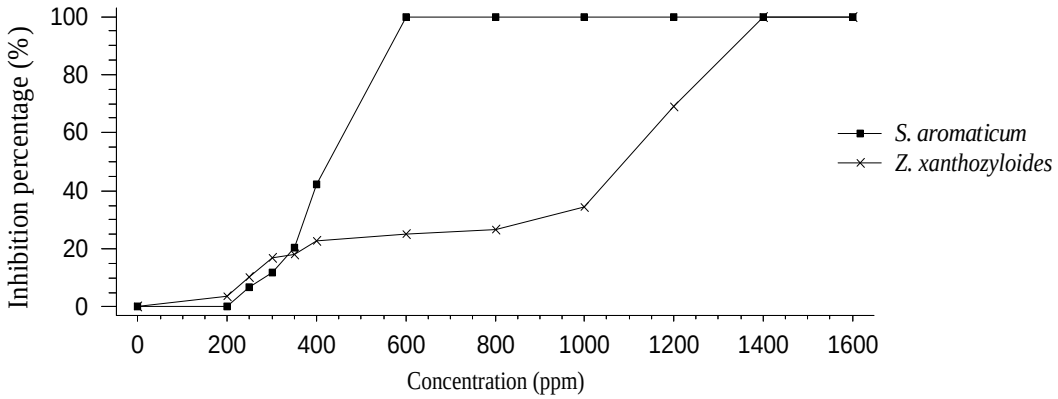


Fig.4 Inhibition percentages of mycelial growth of *P. angolensis* versus concentration of essential oils of *S. aromaticum* and *Z. xanthozyloides*



It has been also assumed that these compounds could disrupt the phospholipid bilayer of the microbial cytoplasmic membrane causing increased permeability and unavailability of the vital intracellular constituents Lambert *et al*., 2001; Vidyasagar *et al*., 2013**).** These results could explain the interesting activity obtained by using clove oil which was made up of 79.42% eugenol. Besides, Viollon and Chaumont (1994) showed that geraniol, and citronellol, found in this study as major components of *Z. xanhozyloides* oil was among the most active terpenoids tested for their antimicrobial property. These findings further highlight the effectiveness of the main components of EOs tested in this study. Therefore the difference in the expressed response of the two EOs against *P. angolensis* could be due in part to the relative amount of these active principles or to synergistic interactions between active molecules or between actives and non-actives compounds (Lee, 2007; Kim and Park, 2012); more often, in a chemical mixture such as EOs a single compound does not exhibit the same inhibitory effects when tested alone as compared to the hold EO (Vidyasagar *et al*., 2013). This is due to other components that often exercise a synergistic effect which surpasses their individual performance against a particular species of microorganisms, maybe by forming a more effective complex. It is also suggested that some phytocompounds without antimicrobial activity could disturb pathogens plasma membrane permeability and thereby facilitate the influx of active principles having their target in the cytoplasm or nucleus (Shiota *et al*., 2004; Sibanda and Okoh, 2007). But the purification of EOs components is needed to conclude about the active components and their related mechanism.

From these results, it can be concluded that essential oil of *Z. xanthozyloides* and *S. aromaticum* which showed an antifungal activity against *P. angolensis* are interesting and could be useful as natural antifungal agent against this fungus which is responsible for heavy losses on *Citrus* fruits. For the practical use of these oils as novel fungal control agents, further investigation is needed on the formulation to improve the fungicidal potency and stability.

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