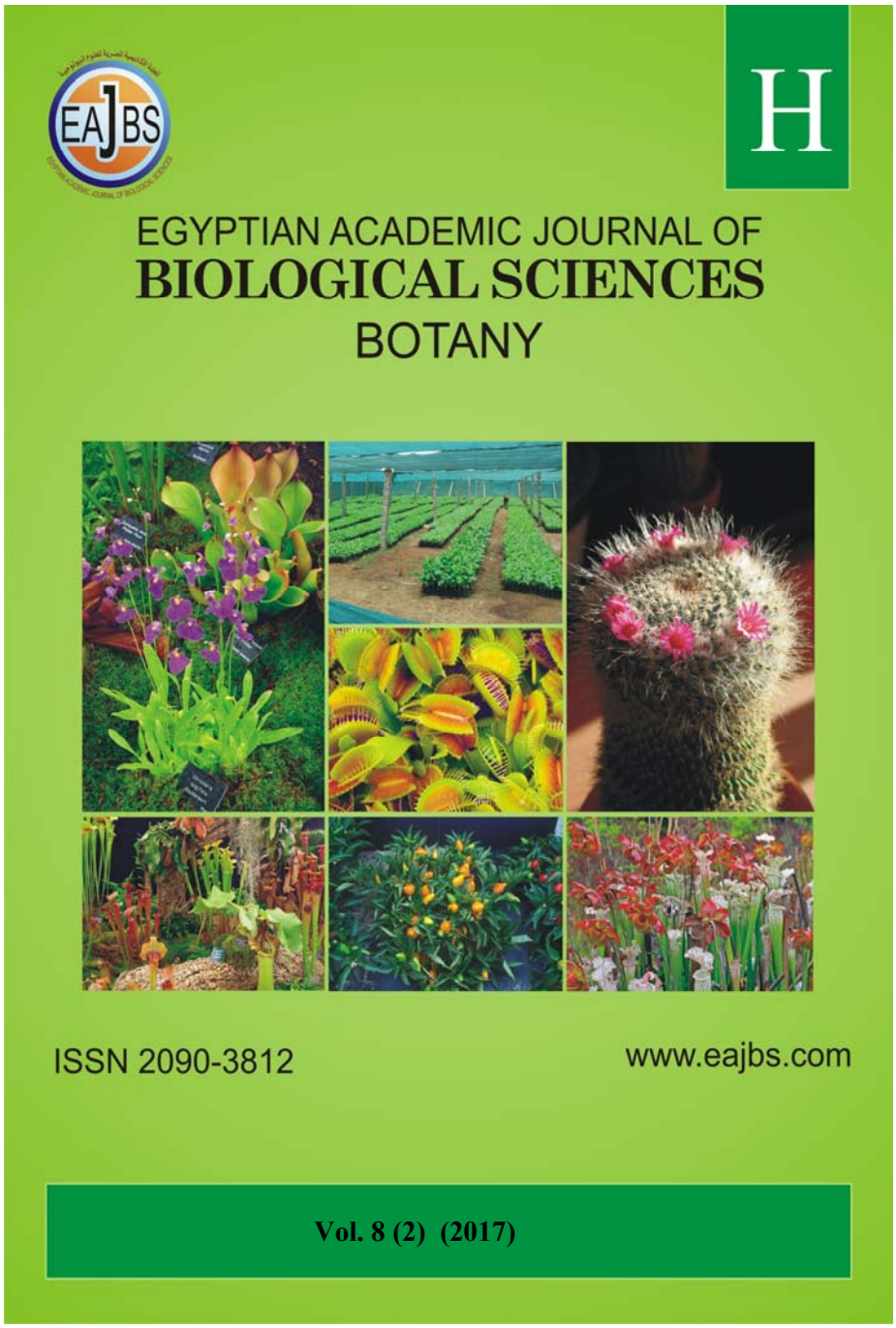

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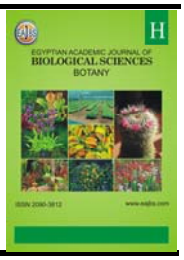

Quantitave, Qualitative and Antifungal Activities of Essential Oils Extract from Two Eucalyptus Species (E. *sideroxylon and E. gomphocephala*).

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ABSTRACT

In this study, the average yields of essential oils were determined for both eucalyptus species. A very important difference was found between the two returns; for the *E. sideroxylon*, the yield is 7 times more than that recorded in the species *E.gomphocephala*. This finding of essential oil (EO) in *E. sideroxylon* has been correlated to the large oil pockets founded on both sides of the *mesophyll* leaf as well as the shape. As for *E. gomphocephala*, the examination of the histological sections by *photonic microscopy* showed that oil pockets are not visualized for this species, which confirms the low yield. The chromatographic analysis of the EO has made it possible to separate 102 compounds, of which five have been identified and which have about 94.58%. Among these compounds, the cineole is the majority compound with a content of 79%. Other metabolites have been found at relatively high levels, namely myrcene, α-terpineol, terpinolene and spathulenol. The quantitative and qualitative difference in essential oil between the two species studied was confirmed by the sensitivity of the different phytopathogenic fungi to the essential oils of Eucalyptus which differs from one species to another. Indeed, the EO of *E. sideroxylon* revealed very strong inhibitory activity on *Fusarium* and *Penicillium*. Nevertheless, the EO of *E. gomphocephala* has no effect on the strains of phytopathogenic fungi tested.

INTRODUCTION

Eucalyptus represents a group of trees and shrubs and includes over 600 species in the plant family Myrtaceae. It is very ecologically important in its original continent (Australia), where it plays a dominant role in the vegetation. Today, *Eucalyptus* is one of the world's most important and widely planted genera, exploited economically for its richness in essential oils (Eos) and

compounds which have been used for medicinal and pharmaceutical purposes (Tsiri et al.; 2003.Ghisalberti et al.; 1996). Traditionally, eucalyptus is used for insect repellent, respiratory infections, and mouthwash. These properties are possible thanks to its leaves, which contain several active substances, including eucalyptol and myrtol. Because of its expectorant and antiseptic virtues, eucalyptus is thus an excellent remedy for the inflammation and the infection of the respiratory tracts, particularly in the case of colds, bronchitis, sinusitis or even influenza. Its benefits are now recognized by the World Health Organization (WHO). Its main use is as a percutaneous penetration agent, antibacterial and expectorant (Tsiri et al.; 2003), anti-inflammatory (Juergens et al.; 1998 or antihypertensive agent (Lahlou et al.; 2002). Its main component, terpenoid 1,8- , is commonly known as eucalyptol. Eucalyptus also exhibit spasmolytic (Sadraei et al.; 2001) and antimicrobial (Filipowicz et al.; 2003) properties due to the monoterpene β-pinene hydrocarbon. The eucalyptol content in EOs varies in the different species of eucalyptus, between 25 and 90% (Giamakis et al.; 2001.Tsiri et al.; 2003). However, the eucalyptus content in eucalyptol in Eos varies widely from one species ranging from 25 to 90% (Giamakis et al.; 2001. Tsiri et al.; 2003).

In the light of this data, our work focuses on the origin of the differences in synthesis or the accumulation of essential oils. The foliar characteristics of the two species of eucalyptus introduced in Morocco and to compare the qualitative and quantitative compositions of the main constituents of the essential oils extracted from each species were studied.

MATERIALS AND METHODS

Plant material:

Leaves were collected from two species; *Eucalyptus gomphocephala* and *Eucalyptus sideroxylon* grown in the garden of the Faculty of Sciences and Techniques of Marrakech (FSTM). They were collected in February 2014. Both tree species were identified in consultation with the engineers of Regional Directorate of Water and Forests and the Fight against Desertification (RDWFFD) of the High Atlas - Marrakech. The identification was carried out on the basis of several criteria such as fruits, leaves, inflorescences, and the nature of the bark and the height of the plants.

Microscopy

The freshly harvested leaves were cleaned and rinsed with water, dried in sunlight, and cut transversally in very thin layers. The cuts were disinfested with 12% (v/v) sodium hypochlorite (NaOCl) for 30min. Then they were rinsed with distilled water and the resulting sections were stained with green carmine dye for 15 min. A second rinse was made with distilled water for 1 min. The cuts are mounted immediately between the blade and the slide in a drop of glycerin.

Extraction of the essential oils

The leaves of two Eucalyptus species are dried for 5 days and were dried away from light and moisture at room temperature. This is an important step that influences the yield and quality of essential oils. Dried plant material (160 g) was subjected to vapor distillation (Fig. 1), with 3 Liters of water for 4 hours and 30min, in the steam-distillation apparatus with a water-cooled oil receiver to reduce hydro-distillation overheating artefacts).

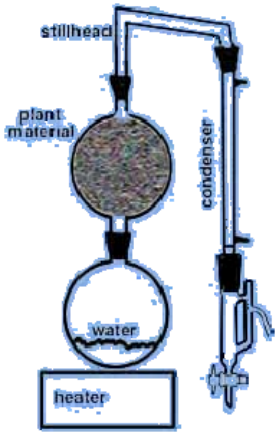

Fig. 1: steam-distillation apparatus

The essential oils are obtained after liquid-liquid separation in a decanter by addition of diethyl ether. The essential oils were collected and dried over anhydrous sodium sulphate than evaporated using a rotary evaporator at 30°C under reduced pressure and stored at 4°C. in the dark until analysis.The obtained oil was subjected to gas chromatography (Shimadzu. GC -2010 Plus) equipped by Rtx-5MS columns (30m x 0.32mm, 0.25µ) containing 5% diphenyl/95% dimethlpolysiloxane using helium as a carrier gas at a flow rate of 3 ml/min. The column temperature was held at 60°C for 5 min following injection, then ramped at 10°C/min to 180°C and held for a further 4 min than ramped at 15°C/min to 290°C during 10min.

Essential oil preparation and Antifungal activity

The fungi employed for assays of antifungal activity are the phytopathogenic genus (*Penicillium itlicum,Botrytis cinerea* and *Fusarium oxysporum*). TheFungal Strains were maintained on sterile malt extract agar (MEA) at 28°C in order to develop a necessary material to antifungal test.

The Essential Oil (EO) was previously dissolved in ethanol (1/3 v/v) before being added to the medium with stirring at the 1/100, 1/300 or 1/1000 dilutions. Nystatin (Sigma), dissolved in methanol (1mg/ml), was added in medium culture, at different concentrations; 5µg/ml, 10µg/ml and 15µg/ml, and served as a positive antifungal control. Essential oils of *Eucalyptus or* positive antifungal control were maintained at 40 °C in Petri dishes at the rate of 15 ml per dish.

After 7 days of young culture, agar-mycelialdisc fragment (diameter, 8mm) tacked from *Penicillium itlicum,Botrytis cinerea* and *Fusarium oxysporum* medium were inoculated into the center of the Petri dish containing different Essential Oil concentrations, or a positive antifungal control. The fungi culture in same conditions without EO or positive antifungal is used as control. After inoculation, the plates were incubated at 28 °C in the dark during six days. Mycelia growth was determined every 24 hours with measure of perpendicular diameter of mycelia. All treatments were replicated 4 times. The percent difference of growth inhibition between the treatment and control groups were calculated using the following formula:

$$\% \text{ Inhibition} = C - T/C \times 100$$

C: average of the four replicates of hyphal extension (mm) in control plates

T: average of 4 replicates of hyphal extension (mm) in medium culture treated with concentration (c) of essential oils or of positive antifungal control.

RESULTS AND DISCUSSION

Microscopic characters.

The upper and lower epidermis is composed of clear polygonal epidermal cells with thick cutaneous outer walls. Both epidermis have hollow stomata. The mesophyll (chlorenchyma) differentiated into palisade and spongy parenchyma regions, two palisade regions with 3 to 4 rows of cells each, each facing each epidermis.

The examination of the cross-sections revealed different structures in the two species studied. Concerning *Eucalyptus sideroxylon*, we have seen the disposition towards the two faces of the large, subglobular internal glands (Fig. 5), doubled with secretory epithelium and containing eucalyptus yellow oil (Fig. 7 c), as well as the form of the nervures: main vein, secondary and cuticle structure. (Figs. 2 and 3). A structure of the xylem and phloem vessels within the main rib (Fig. 4) and calcium oxalate crystals are shown in Figure 6.

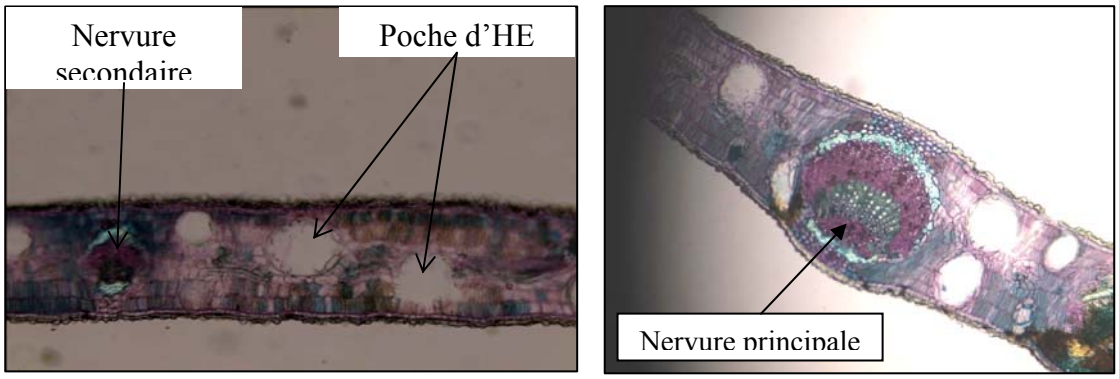

Fig. 2: Transverse section of the *E. sideroxylon* *leaf*. (X100)

Fig.3: Cross section of the *E. sideroxylon* leaf showing the main vein. (X100)

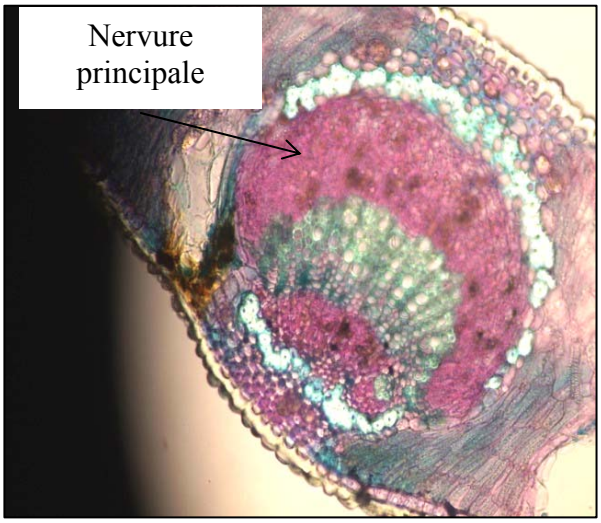

Fig. 4: Cross-section of the main vein of *E. Sideroxylon* from the epidermis to xylem. (X200)

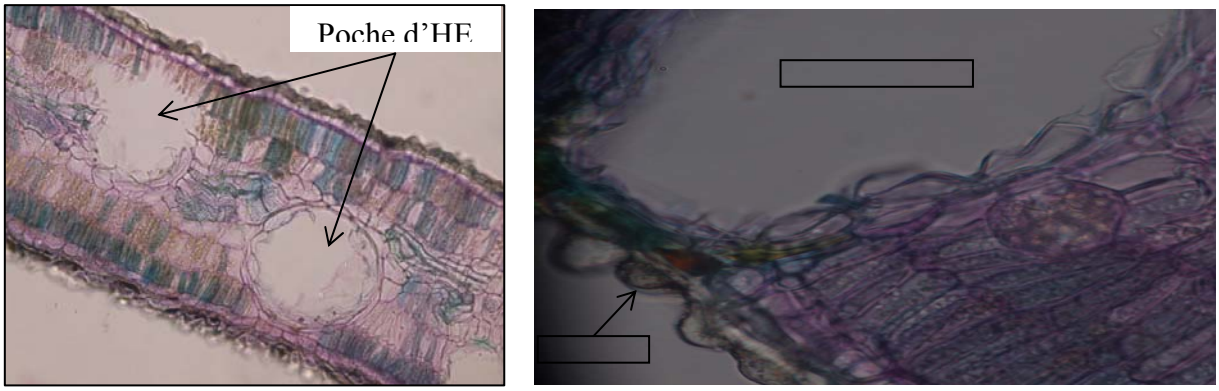

Fig. 5: Cross-section of cuticle and EO pocket of *E. sideroxlon* (a: X200) (b: X400)

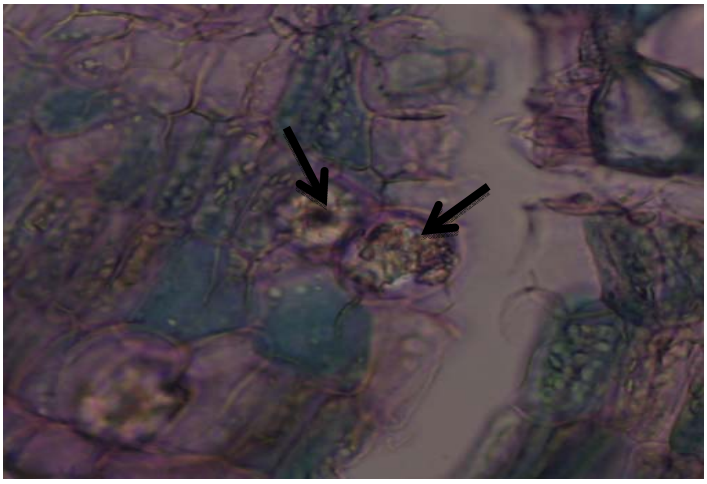

Fig. 6: Cross-section of the E*. sideroxylon* leaf showing calcium crystals (X400

The microscopic examination of the histological sections of *Eucalyptus gomphocephala* (Fig. 6) shows the main vein and the secondary veins but the essential oil glands are not visualized in leaves for this specie.

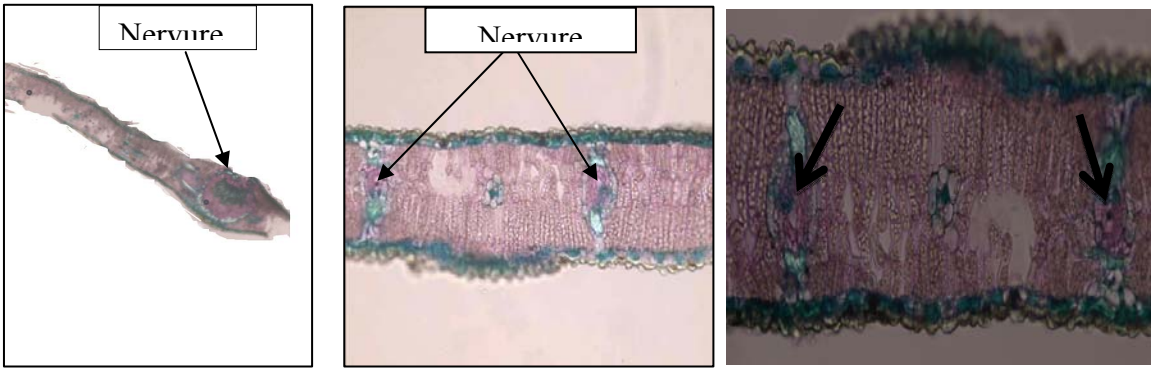

Fig. 7: Transverse section *of E. gomphocephala* leaf (a = X40), (b = X 100) and secondary vein (**c** = X200).

Result of the macroscopic study of the studied Eucalyptus species

The macroscopic study of the eucalyptus trees observed allowed for the determination of the species *E.gomphocephala* and *E. sideroxylon*. Macroscopic characters of fresh leaves were recorded such as length and width, type of leaf base, presence or absence of petiole and characters of fruit and of inflorescence.*E. gomphocephala* adult leaves were alternate, petiolate, whole, lanceolate, slippery, and greenish-gray, measuring at 16 centimeters long and 2.5 cm wide, whereas the *E. sideroxylon*, leaves were shorter (11cm) (Table 1).

Table 1: Morphological and macroscopic characteristics of two species of Eucalyptus

organs			***E. gomphocephala***	***E. sideroxylon***
Fruits	Length (cm)		1.2	1
	Width (cm):	Base	0.3	0.6
		Peak	0.9-1	0.7
	Peduncle: (cm)		1.9	1.3
	Length Width		0.6-1	-
	Disc (cm^2)		3.14	3.76
	Lodge :		4 WrinkledLodges	5 à 6
Leaves	Juvenile:(cm)		Length	5.65
			Width	2.25
			Petiole:	0.6
	Adult: (cm)		Length	16
			Width	2.5
			Petiole	3
Inflorescences			Axillary umbels of 5 to 7 flowers with flattened peduncle. Buttons with a hemispherical seal larger than the calyx.	Axillaria, in umbel of 3 to 7 flowers. Buttons with tapered or slightly rostric seal.
			Axillary umbels of 5 to 7 flowers with flattened peduncle. Buttons with a hemispherical seal larger than the calyx.	Axillaria, in umbel of 3 to 7 flowers. Buttons with tapered or slightly rostric seal.
Flowers			-	yellowish
Crowned			2	3
Bark			Persistent, fibrousreticulate.	Dark reddish brown (rust color) persistent and hard.
Height (m)			-	16

Phytochemical study:

Essential Oil Yield :

The essential oils isolated by water steam distillation of the leaves harvested at adult stage of growth were expressed per dry weight had a purple yellow color. The EO yields of *E. sideroxylon* and *E. gomphocephala* were 3.60% and 0.5 % (w/w) respectively (table 2), thus, the Essential Oil content is 7 fold greater in *E. sideroxylon* than *E. gomphocephala*.

The essential oil production depends not only on the growth stage and on environmental causes but in addition on genetic factors which could be responsible of anatomical and histological differences between plant species. Plants that accumulate essential oils differentiated the specialized glands in which the oils are stored.

Table 2: Yield of the Essential oil of the species studied (in% relative to the dry matter)

Species of Eucalyptus	EO-Yield (%)
E. sideroxylon	3.60
E. gomphocephala	0.5

Composition of the essential oils of the species studied.

Results of analysis by GC chromatography:

a) *E. sideroxylon*

Chromatography analysis of *E. sideroxylon* essential oils led to the separation of 102 compounds, five of which have been identified (Fig. 8) at about 94.58% of total (Table 3). Essential oil exhibited *1, 8-cineole* as major components (80%) (Table3).Myrcene, α-terpineol, terpinolene and spathulenol have been found at relatively high concentrations. The others compounds are in the form of traces.

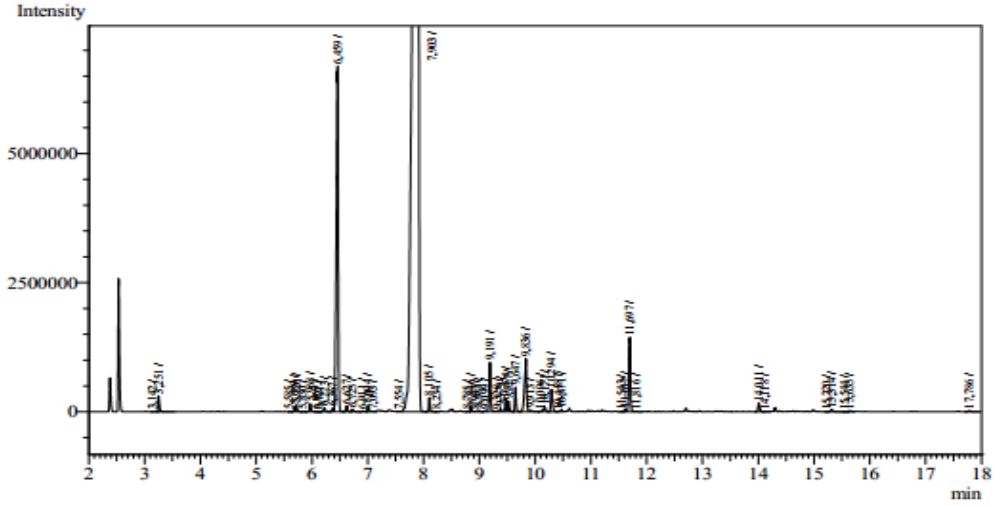

Fig. 8: Chromatographic profile of EOs of *E. sideroxylon* leaves

Table 3:Chemical composition of essential oils of *E.sideroxylon*.

Pics	RT	Concentration %	Compounds
12	6,46	10,14	Myrcene
22	7,90	79,67	1.8 cineole
33	9,19	1,16	a-terpinéol
39	9,84	1,79	terpinolene
59	11,70	1,84	Spathulénol

b) E. *gomphocephala*

The chromatographic analysis of Essential Oil in *E. gomphocephala* leaves allowed to separate 62 compounds with nine were identified and represented74.63% of the total (Fig.9).

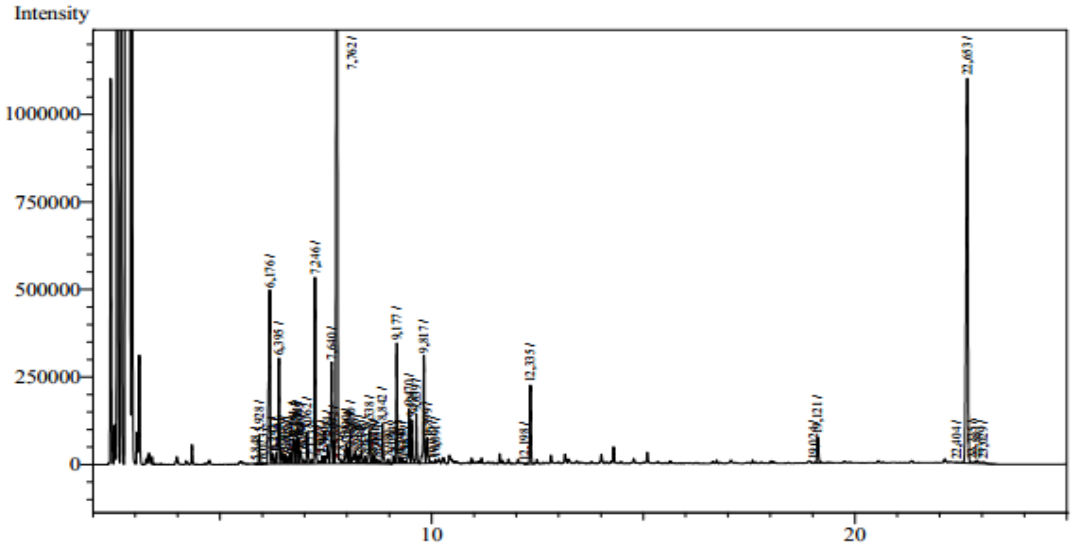

Fig. 9: Chromatographic profile of EOs of *E. gomphocephala* leaves

Among the different components of oil, 1,8-cineole is the most important one, representing a rate of 28.31 (Table 4).

These results are consistent with those previously determined and showed that Eucalyptus species are known for their high content of 1, 8-cineole (Pino et al. 2002; Su et al. 2006; Elaissi et al. 2012).

Table 4: Chemical composition of essential oils E. *gomphocephala*

Pics	TR	Concentration %	Compounds
4	6,18	6,87	Sabinene
7	6,40	3,31	X
20	7,25	5,39	α terpinene
25	7,64	3,34	Para-cymene
27	7,76	28,31	1.8 cineol
43	9,18	3,48	α pinene
50	9,82	3,40	Terpinolene
55	12,34	2,27	Isoborneol
59	22,65	18,26	X

The previous studies already reported that eucalyptus oil is a mixture of different compounds primarily comprised of a variety of monoterpenes and sesquiterpenes ((Brooker and Kleinig, 2006).

Inhibitory activities of the eucalyptus oil on plant pathogenic fungi

The antiphytopathogenic tests of essential oils extracted from two eucalyptus species were evaluated to observe the consequential growth of three phytopathogenic fungi, *Penicillium itlicum*, *Fusarium oxysporum* and *Botrytis cinerea*, following agar disk-diffusion method. Different concentration of oil (previously dissolved in ethanol (1/3 v/v)) such as 1/100 , 1/300 and 1/1000, were taken to evaluate the effect. The control corresponded to inoculating culture disc on the medium without adding any oil.

The obtained results showed that the three species of fungi studied respond differently depending on the origin of the oil used and its concentrations (Figure 10). The inhibition rate of the growth of mycelium exerted by the essential oil of *E. sideroxylon* (Figure 10) is significantly greater than that exerted by the essential oil of *E. gomphocephala*(Figure 10). Both species *penicillium itlicum* and *Fusarium oxysporum* are more sensitive to the essential oilof *E. sideroxylon* than *Botrytis* cinerea with 100% inhibition under both low concentrations (1/100 and 1/300) not exceed 40% under the effect of EO d *E.gomphocephala*.

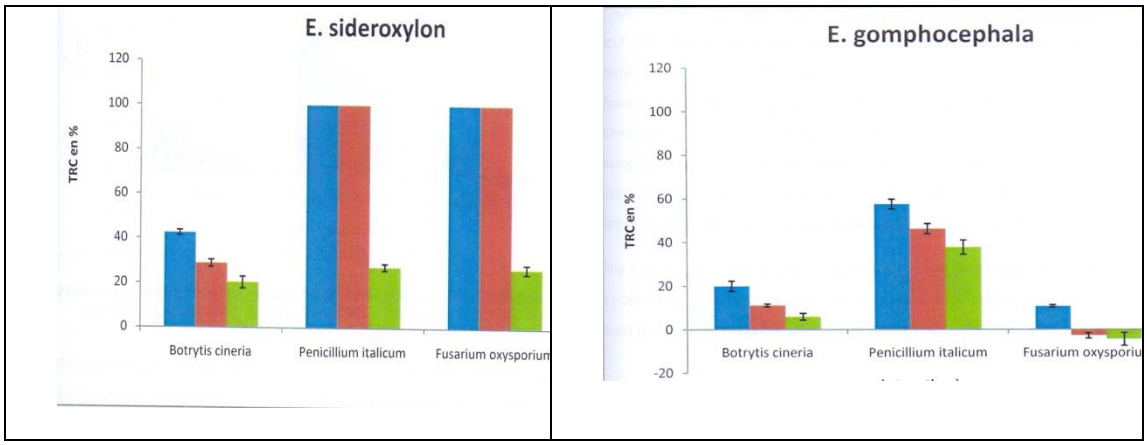

Fig.10: Inhibition rate of the mycelium growth of three species of fungi by two Eucalyptus species essential oils.

The difference in antifungal effect of the two species EOs can be correlated to 1,8-cineole which was found to be the predominant component (79.7%) of the *E. sideroxylon* essential oil.Indeed, previous studies (Hmiri et al., 2011) have shown that pure 1,8-cineole caused inhibition of mycelial growth.These findings are in agreement with those of Marei et al. (2012), whichindicatedthatthe1,8-cineole exhibited pronounced antifungal activity against the four tested fungi. Eucalyptus oils inhibit fungus growth by reducing mycelial growth and spore production and germination (Oluma and Garba, 2004). It has been reported that commercial fungicides inhibit the production and activity of cellulase (Milling and Richardson, 1995).

In summary, the results of this study revealed that Eucalyptol (1,8-cineole) is the major chemical component of the oils obtained from leaves of *Eucalyptus sideroxylon* which incite a significant antifungal effect.

CONCLUSION

The object of this study was to investigate the yields of essential oils in *eucalyptus sideroxylon* and *Eucalyptus gomphocephala* and their antifungal effect related to morphological and histological characters. The microscopic analysis of the

histological section of two species leaves revealed a significant difference at the anatomical, histological level and in oil essential yield. The essential oil glands are not visualized in *Eucalyptus gomphocephala* while these sub-dermal secretory cavities were evident lyembedded within the leaves of *Eucalyptus sideroxylon.* Consequently, essential oils yield is sevenfold in this specie than in *E.gomphocephala.* Essential oils toxicity was investigated against fungi and showed that EO of *E. sideroxylon* exhibited a significantly ($p \leq 0.05$) higher inhibitory effect on *Penicillium itlicum,Botrytiscinerea* and *Fusarium oxysporum*.

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ARABIC SUMMARY

الأنشطة المضادة للفطريات في الزيوت الأساسية المستخلَصة من نوعين من أشجار الأوكاليبتوس (E.*sideroxylon*و*E. gomphocephala*).

ميمون الكاوى*، سكينة زروق**، وحفيظة بوعمامة**

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تم تحديد متوسط مردود الزيوت الأساسية لدى نوعين من أشجار الأوكاليبتوس *E. Sideroxylon* و*E.gomphocephala*. النوعان يختلفان بشكل كبير من ناحية مردود الزيت المستخرج. مردود الزيت لنوع *E.Sideroxylon* أكبر بسبع مرات مقارنة بنوع *E.gomphocephala*. الكمية المهمة من الزيوت عند *E. sideroxylon*تم ربطها للشكل و الحجم الكبير للجيوب الزيتية المتواجدة على جانبي النسيج الأوسط لورقة النبتة (الميزوفيل). أظهر فحص المقاطع النسيجية لورقة *E.gomphocephala*بواسطة المجهر الضوئي، عدم إمكانية مشاهدة الجيوب الزيتية بشكل بارز، الشيء الذي يعلل ضعف مردود الزيت عند هذا النوع. كما أمكن التحليل الكروماتوغرافي للزيوت الأساسية من فصل ١٠٢ مكون، تم تحديد خمسة مكونات والتي تشكل ٩٤.٥٨% من المحتوى الكلي. من أبرز هذه المكونات السينيول cineole الذي يشكل ٧٩%. هناك مكونات أخرى لها محتويات مرتفعة نسبيا في الزيت ك myrcene، α-terpineol، terpinolene و spathulenol. الإختلاف الكمي و النوعي للزيت الأساسية لكل من النوعين النباتيين قيد الدراسة تم التحقق منه عن طريق تأثيرالزيت المستخلصة على مجموعة من الفطريات الممرضة للنباتات، هذا التأثير على الفطريات يختلف باختلاف نوع النبات الذي استُخلصت منه الزيت. في الواقع، الزيوت الأساسية ل *E. Sideroxylon* لها تأثير قوي في كبح نمو الفطريات من نوع Fusarium وPenicillium. بالمقابل، الزيوت الأساسية المستخلصة من E. Gomphocephala لا تأثير لديها على الفطريات المُختبَرة