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Essential oil composition and antifungal activity of *Salvia officinalis* originating from North-East Morocco, against postharvest phytopathogenic fungi in apples

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ABSTRACT

The essential oil of Salvia Officinalis, an endemic medicinal plant from Morocco, have been studied using gas chromatography (GC) and GC-mass spectrometry (GC-MS). 96.3% of the components are detected. and the major components were ,l'α-thujone (22.2%), le 1,8-cinéole (18.4%), le trans-pinocarveol (9.4%), le β-thujone (8.4%) le β-pinène (8.2), le globulol (5.9%) , le α-Humulene (3.7 %) et le trans-caryophyllène (2.6%).The antifungal activity of the essential oil was evaluated in vitro by poison food (PF) technique and the volatile activity assay (VA) against three phytopathogenic causing the deterioration for apple such as Botrytis cinerea, Penicilliumexpansum and Rhizopusstolonifer. The PF technique demonstrated significant inhibition of the mycelial growth of all strains ($p < 0.05$), with the complete inhibition of P. expansum at MIC = 2 µL/mL. Similarly, the VA assay showed that the essential oil strongly inhibits all three fungi. The complete inhibition of the mycelial growth of both P.expansum and R.stolonifer, was observed respectively at MIQ = 80 µL/disc and 160µL/disc. The overall results suggest that S.Officinalis essential oil have a potential as antifungal preservatives for the control of postharvest diseases of apple.

Key words: Antifungal activity, *SalviaOfficinalis*, Essential oil, GC-MS analysis, Apple.

INTRODUCTION

During storage, fruits and vegetables are often subject to varying levels of microbial decay, mainly attributable to pathogenic fungi, which usually infect the host through wounds sustained during harvest handling and/or processing. It is estimated that about 20 - 25% of harvested fruits and vegetables are destroyed by pathogens after postharvest handling, even in developed countries [1].

The use of synthetic chemicals as fungicides is a principal method of controlling the postharvest decay of apples. However, the emergence of strains of pathogens resistant to these fungicides, as well as the growing concern for

human safety and the protection of the environment, compel us to look for alternatives to the use ofsynthetic fungicides to control postharvest diseases [2].

The apple (Malusdomestica) is the third most cultivated fruit crop in the world (5280 Mha) and the third in production (59 059 Mt) after Citrus sinensis (orange) and Musa paradisiaca (banana) in 2004, according to Food and Agricultural Organization statistics [3]. However, the quality of apple deteriorates because of postharvestdiseases, such as blue mold caused by Penicilliumexpansum, Bull's-eye rot caused by Alternaria species and Rhizopus soft rot caused by Rhizopusstolonifer[4]. The survival of these pathogenic fungi in food is a serious problem in developing countries which can lead to spoilage and deteriorate the quality of food products. These fungal agents produce also mycotoxins that can be mutagenic, teratogenic, carcinogenic causing feed refusal and emesis in humans or animals [5]. Recently, many researchers have shown that natural sources such as essential oils could develop as a promising alternative to synthetic fungicides because of their low mammalian toxicity, less environmental effects and wide public acceptance [6].

Salvia officinalis is a sub-shrub of the Lamiaceae family, often grown in gardens as condiment and officinale plant or just for the beauty of its foliage and flowers. It is also called sacred herb. This is a highly branched plant, the square rods, the woody base. The stalked leaves are pale green, velvety, oblong. The flowers on upright flower stalks are grouped in small clusters.It is used most often in the form of infusion. It is then mainly used against sore stomach and digestive problems, It also helps to fight against excess sweat. It is thus often given to postmenopausal women to reduce night sweats. Finally, it would also be effective in relieving inflammation of the upper respiratory tract (nose, mouth and throat) [7, 9-11]. Reports on the essential oil composition of this species have been published by several authors [12-18], Apartfrom some chemical variations, *cis*- and *trans*-thujones.The objective of this study is the study of the antifungal effect of essential oil of *Salvia officinalis* on mycelial growth of three fungi (*Botrytis cinerea*, *Penicilliumexpansum and Rhizopusstolonifer*)responsible for apple rot in storage in fridges.

MATERIALS AND METHODS

2.1. Plant material

The aerial part of *Salvia Officinalis* was harvested inMars 2011 in the wild in the mountain Assoullocated***Taza*** at the Nord-east of ***Morocco***. A voucher specimen wasdeposited in the Herbarium of Faculty of Sciences, Oujda, Morocco. The dried plant material is stored in thelaboratory at room temperature (298 °K).

2.2. Essential oil isolation

The dried vegetal material (100 g) were water-distillated (3 h) using a Clevenger-type apparatus. The essential oilobtained was dried under anhydrous sodium sulfate and stored at 4°C in the dark before analysis. The essential oils average yield was 1.5%.

2.3. Hydrodistillation apparatus and procedure

Often the hydrodistillation was performed by use of Deryng apparatus or Clevenger type apparatus. In this extraction of essential oil of the aerial part of *Salvia Officinalis* was conducted by hydrodistillation using a Clevenger type apparatus fig.1.

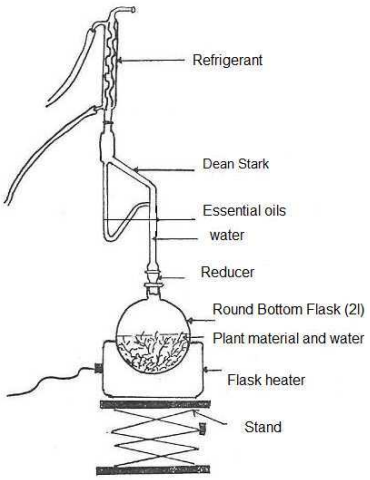

Fig.1.Hydrodistillation by Clevenger apparatus[7-8]

2.4. Characterization and chemical composition of essential oils

Gas chromatography–mass spectrometry (GC-MS) combines the features of gas-liquid chromatography (GC) and mass spectrometry (MS). This makes it possible to identify different substances within a test sample. GC-MS hasmany uses include drug detection, fire investigation, environmental analysis and explosives investigation. It can alsobe used to identify unknown samples. GC-MS can also be used in airport security to detect substances in luggage oron human beings. Additionally, GC-MS can identify trace elements in deteriorated materials, even after the samplefell apart so much that other tests cannot work. GC-MS is the best way for forensic experts to identify substancesbecause it is a specific test. A specific test positively identifies the actual presence of a particular substance in agiven sample.

The chemical components of *SalviaOfficinalis* essential oil was determinate by spectral analysis of gas chromatography and gas chromatography coupled to mass spectrometry (GC-MS), being identified six major components for essential oil studied.

GC analyses were performed using a Perkin-Elmer Autosystem GC apparatus (Walhton, MA, USA) equipped with a single injector and two flame ionization detectors (FID). The apparatus was used for simultaneous sampling to two fused-silica capillary columns (60 m · 0.22 mm, film thickness 0.25 lm) with different stationary phases: Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). Temperature program: 333–503 K at 275 °K/min and then held isothermal 503 °K (30 min). Carrier gas: helium (1 mL/min). Injector and detector temperatures were held at 553 °K. Split injection was conducted with a ratio split of 1:80; electron ionization mass spectra were acquired with a mass range of 35–350 Da. Injected volume: 0.1 lL.

For gas chromatography–mass spectrometry, the oils obtained were investigated using a Perkin-Elmer TurboMassQuadrupole Detector, directly coupled to a Perkin-Elmer Autosystem XL equipped with two fused-silica capillary columns (60 m · 0.22 mm, film thickness 0.25 lm), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). Other GC conditions were the same as described above. Ion source temperature: 423 °K; energy ionization: 70 eV; electronionization mass spectra were acquired with a mass range of 35–350 Da. Oil injected volume: 0.1 lL[7].

Identification of the components was based (i) on the comparison of their GC retention indices (RI) on non-polar and polar columns, determined relative to the retention time of a series of n-alkanes with linear interpolation, with those of authentic compounds or literature data [19] and (ii) on computer matching with commercial mass spectrallibraries[19,20] and comparison of spectra with those of our personal library. Relative amounts of individualcomponents were calculated on the basis of their GC peak areas on the two capillary Rtx-1 and Rtx-Wax columns, without FID response factor correction.

2.5. Fungal strains isolation

Three fungal isolates causing apples rot: *Botrytis cinerea*, *Penicilliumexpansum* and *Rhizopusstolonifer* were isolated directly from rooted apples collected from different rooms in Midelt station (Morocco). All isolated fungal species were transferred to sterilized three replicates 9 cm Petri dishes containing fresh Potato Dextrose agar (PDA) medium in the presence of a quantity of streptomycin to stop the growth of bacteria. The plates were incubated at 25±2°C for 7 days and darkness. The developing fungal colonies were purified and identified up to the species level by microscopic examination through the help of the following references [21]. The isolates collected were maintained on PDA at 4 °C.

2.6. Antifungal activity assay

The antifungal activity of the essential oil of *Salvia Officinalis* against mycelial growth of fungi isolated was undertaken using poisoned food technique (PF) [22] and volatile activity assay (VA) [23] with some modifications.
In PF, the essential oil were dispersed as an emulsion in sterile agar suspension (0.2%) and added to PDA immediately before it was emptied into the glass Petri dishes (90×20 mm in diameter) at a temperature of 40–45°C. The concentrations tested were 0.25 to 2 µL/mL. The controls received the same quantity of sterile agar suspension (0.2%) mixed with PDA. The tested fungi were inoculated with 6 mm mycelial plugs from 7-days-old cultures cut with a sterile cork and incubated for 11 days for B.cinera, 7 days for P.expansum and 60 hours for R.stoloniferat 25±2°C.

In VA assay, the Petri dishes (90×20 mm) were filled with 20 mL of potato dextrose agar (PDA) medium and thenseeded with a mycelial disc (6 mm diameter), cut from the periphery of 7 days old mycelium culture of the testedfungi. The Petri dishes (90×20 mm, which offer 80 mL air spaces after addition of 20 mL agar media) were invertedand sterile filter paper discs (9 mm in diameter) impregnated with different concentrations of essential oil: 10, 20, 40, 80 and 160 µL/disc air are deposited on the inverted lid and incubated for 10 days for B.cinera, 6 days for

P.expansum and 48 hours for R.stoloniferat 25±2°C. For each corresponding control equal amount ofwater was poured on the sterilized paper filter.

In both types of experiments, three replicate plates were inoculated for each treatment (fungi/amount) and theexperiment was conducted three times and the mycelial growth was followed by measuring the diameter followingtwo perpendicular lines passing by the centre of the dish. Fungitoxicity of essential oil was expressed in terms ofpercentage of mycelial growth inhibition (I %) and calculated following the formula of Pandey et al. [24].

$$I\,(\%\,) = \frac{D_{\,t} - D_{\,i}}{D_{\,t}}\,x100$$

Where $D_t$and$D_i$is represent mycelial growth diameter in control and treated Petri plates, respectively.

The measurements were used to determine the Minimum Inhibitory Concentration (MIC) (lowest concentration of the essential oil that will inhibit the visible growth of a microorganism after overnight incubation). Thefungistatic–fungicidal nature of essential oil was tested by observing revival of growth of the inhibited mycelial disc following its transfer to non-treated PDA. A fungicidal effect was where there was no growth, whereas a fungistaticeffect was where temporary inhibition of microbial growth occurred.

2.7. Spore production assay

Fungal spore production was tested using the modified method of Tzortzakis and Economakis[25].The spores of the previously exposed colonies by essential oil vapour were collected by adding 5 mL sterile water containing 0.1% Tween-20 to each Petri dishes and rubbing the surface three times with the sterile L-shaped spreader to free spores. The spore suspensions obtained were filtered through sterilize cheesecloth into a sterile 50 mL glass beaker and homogenized by manual shaking. Spore concentration was estimated using a haemocytometer slide (depth 0.1 mm,0.0025 mm^2). The percentage reduction of spore production was computed by the following equation:

$$I(\%) = \frac{N_t - N_i}{N_t}\,x100$$

WhereN_t andN_i represent the number of spore in control and treated Petri plates, respectively.

2.9. Transfer experiments

In both types of experiments, to make a distinction between fungistatic or fungicidal effects of the Essential Oil on the target organism (minimum fungicidal concentration (MFC) and minimum fungicidal quantity (MFQ)), transfer the discs from the Petri dish where inhibition by essential oil was total in a PDA medium not supplemented by essential oil. It was fungistatic if growth began again and fungicidal if it did not.

2.10. Data analysis

The inhibitory effect of essential oil on mycelial growth was analyzed by an analysis of variance (ANOVA). Mean and standard error of data were calculated using SAS software (SAS for Windows. version 9.0). The separation of means was done by using the Least Significant Difference (LSD) test at $p<0.05$.

RESULTS

3.1. Essential oil composition

The analysis of essential oil composition of *S.Officinalis*was carried out using GC and GC-MS according to the methodology described in experimental part. Thus, 31 components accounting for 96.3% of the total oil were identified by comparison of their EI-mass spectra and their retention indices with those of our ownauthentic compound library (Table 1).

3.2. In vitro antifungal activity

Using the PF technique, the effects of the concentrations of the essential oil of *Salvia Officinalis*onmycelial growth after an incubation period of eleven days for B.cinerea, and seven days for P. expansum and sixty hours for R. stolonifer, at 25 ± 2 °C are summarized in Table 2.

Table 1:Chemical composition of essential oil of *S.Officinalis* from Morocco [7]

Composés	IL	Ir /apol	Ir /pol	HE% apol
Cis-Salvene	849	853	948	0,3
α-Thujene	932	931	1025	0,7
α-Pinene	936	939	1007	1,7
Camphene	950	952	1046	1,4
Sabinene	973	975	1123	1,3
β-Pinene	978	981	1088	8,2
Myrcene	987	992	1132	2,9
α-Terpinene	1013	1017	1180	0,4
p-Cymene	1015	1021	1236	0,6
Limonene	1025	1030	1201	0,3
1,8-Cineole	1024	1030	1207	18,4
Z–β-Ocimene	1029	1034	1221	0,5
γ-Terpinene	1051	1056	1231	0,8
Terpinolene	1082	1085	1253	0,2
α-Thujone	1089	1098	1409	22,2
β-Thujone	1103	1107	1425	8,4
Camphor	1123	1128	1490	
trans-Pinocarveol	1126	1128	1620	9,4
transPinocamphone	1139	1143	1490	1,1
cis Thujol	1149	1155	1532	1,4
Terpinen-4-ol	1164	1167	1575	0,8
Myrtenal	1172	1174	1590	0,2
α-Terpineol	1176	1178	1671	0,3
Myrtenol	1178	1184	1754	0,4
trans-Caryophyllene	1421	1416	1561	2,6
α-Humulene	1455	1453	1628	3,7
γ-Muurolene	1474	1474	1649	0,1
Caryophyllene oxyde	1578	1567	1926	0,4
Globulol	1589	1581	2036	5,9
epoxyde d'Humulene 2	1602	1590	1980	0,4
E-Biformene	2017	2033	2601	1,3
			TOTAL	96,3

IR/apol = indice retention on the apolar column (Rtx-1)
IR/ pol = indices retention on the polar column (Rtx-Wax)
% apol= relative percentages of components.

Table 2: Percentages of inhibition of mycelial growth of B.Cinera , P. expansum and R. stolonifer at various concentrations of *Salvia Officinalis* essential oil.

Strain	*B.cinerea*	*P. expansum*	*R. stolonifer*
EO concentration (µL/mL)		Incubation times	
	11 days 25 ± 2 °C	7 days 25 ± 2°C	60 hours 25 ± 2 °C
2.00	64.40±5.64^{Aa}	100 ±0.00^{Aa}	38.42±4.9^{Aa}
1.00	38.76±2.71^{Ab}	73.70±6.47^{Ab}	27.37±1.88^{Ab}
0.50	25.49±2.95^{Ac}	68.94±3.0^{Ac}	14.15±3.59^{Ac}
0.25	10.77±3.08^{Ad}	30.04±7.60^{Ad}	8.97±2.07^{Ad}

Mean values (±standard deviation) followed by different numbers and letters in each row (line and column) indicate significant differences (p<0.05) by least significant difference test (LSD)

These results indicate that the percentage inhibition of mycelial growth increased with increasing concentrations of *Salvia Officinalis* oil for all strains tested. This result suggests that the essential oil has a significant activity ($p < 0.05$) and inhibited the mycelial growth of all strains in a dosedependent manner. It was clear that P. expansum showed high sensitivity to S.Officinalis essential oil at a concentration of 2 µL/mL. The inhibition rate reached 30.04 % for P. expansum at 0.25 µL/mL, and 100% at 2 µL/mL,indicating that this latter concentration was the minimal inhibitory concentration (MIC) of *S.Officinalis* oil against P.expansum, whereas it displayed moderate high antifungal activity against B.cinerea and R. stolonifer, the percentage inhibition increases moderately with the concentration reaching respectively the maximum value of 64.4% and 38.42% to 2µL/mL , demonstrating that its MIC are more than 2 µL/mL.This might be attributable to the mode of resistant of the fungi against various substances present in the essential oil.We also noted that B.Cinera and R. stolonifer had the same degree of

sensitivity to different concentrations of the oil (sigficant difference, $p < 0.05$). Therefore, the plant pathogens studied can be classified according to their sensitivity to the oil in the following order: P. expansum>B.Cinera> R. stolonifer.Moreover, it is important to know the fungitoxic nature of the essential oil at 2 µL/mL of P. expansum Indeed, the transfer of a mycelial disk of the plates containing a PDA medium and 2 µL/mL of the essential oil on fresh PDA (without oil) showed that the mycelia of P. expansum has not grown after incubation for seven days, indicating a fungicide effect of this oil on this strain (no fungistatic activity) at 2 µL/mL (Table 3).

Table 3: Evolution of mycelial growth (in cm) of P. expansum after transfer of discs where inhibition was complete (2 µL/mL) during 7 days of incubation. Values are means (n = 3) ± standard deviations.

Strain	*P. expansum*						
EO quantity (µL/mL)	Incubation time (days)						
1	2	3	4	5	**6**	7	
2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

It must also be noted that the inhibitory effect of the vaporphase of *Salvia Officinalis* essential oil on mycelial growth (Table 4)wasgreater than the inhibitory effect exerted during PF technique(Table 2).

Table 4: Percentage of inhibition of mycelial growth of B.cinerea , P. expansum and R. stolonifer according the amount of *S.Officinalis* essential oil.

Strain	*B.cinerea*	*P. expansum*	*R. stolonifer*
EO concentration (µL/Disc)	Incubation times		
	10days 25 ± 2 °C	6days 25 ± 2°C	48hours 25 ± 2 °C
160	76.41 ±10.40^{Aa}	100 ±0.00^{Aa}	100 ±0.00^{Aa}
80	48.30±3.54^{Ab}	100 ±0.00^{Aa}	100 ±0.00^{Aa}
40	44.92 ±3.71 ^{Ac}	68.07 ±2.83^{Ab}	90.76±5.01^{Ab}
20	35.26 ±2.58 ^{Ad}	65.69 ±10.07 ^{Ac}	88.23 ±1.02 ^{Ac}
10	15.32±1.87^{Ae}	44.08±3.71^{Ab}	79.46±0.33 ^{Ad}

After growth inhibition had been established with *S.Officinalis* oil, the mycelial discs were transferred onto PDA medium withoutthe essential oil. The results for both strains of P. expansum and R. stolonifer are presented in Table 5.

Table 5: Evolution of mycelial growth (in cm) after transfer of discs where inhibition was completes after 6 days of incubation for P.expansum and 48 h for R. stolonifer. Values are means (n=3)±standard deviations.

		Strain *P. expansum*				
EO quantity (µL/disc)	Incubation time (days)					
1	**2**	**3**	**4**	**5**	**6**	
80	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
160	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Strain (µL/disc)	EO quantity	Incubation time (hours)				
		12	24	36	48	
R. stolonifer						
80		0.00±0.00	1.33±0.00	2.34±0.00	5.36±0.00	
160		0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	

From these results, there is no growth of fungi P.expansum for six days at, concentrations of 80 and 160 µL / disc (MIQ = 80 µL/disc), on the other side

R. stolonifer, there is a mycelial growth from the first day at a concentration of 80 µL/disc, then no mycelial growth was observed after treatment with oil to a concentration of 160 µL/disc. So *S.Officinalis* showed fungicidal activity against R. stolonifer at 160 µL/disc (MIQ = 160 µL/disc) and fungistatic 80 µL/disc.

DISCUSSION

According to the GC/MS analysis in this study, thirty one compounds, accounting for 96.30% of the total oil, were identified.Recently, interests have been generated in the development of essential oils to control phytopathogens in food andagriculture. Most of the essential oils have been reported to inhibit postharvest fungi in vitro conditions [26-31].Several researchers have reported that the mono- and sesquiterpenes, as the major components of various essential oils, have enormous potential for strongly inhibiting the growth of microbial pathogens. The antifungal activity of these compounds may be attributed to their interference with certain enzymatic reactions evolved in the cell wall synthesis. Indeed, the chemical composition and antifungal activity relationships deduced from our study demonstrates that the antifungal activity of *Salvia Officinalis* is probably related to the nevertheless, high content content of both α-Thujone (22.2%) and 1,8-Cineole (18.4%).The importance of this compound was demonstrated by M.Marino et al who reported that 1,8-cineole could be made to the antifungal activity [32].Moreover, According to the results obtained in this study, VA method in disc volatilization has better antifungal activity against the pathogens tested than that in liquid phase observed in the PF assay. This result indicates that the substances in the well diffusion method were less efficient than that in the disc volatilization method. The efficacy of essential oils in vapour state was probably attributable to the direct deposition of essential oils on lipophilicfungal mycelia together with an indirect effect via adsorption through the agar medium [33]. These results are in agreement with those reported in the literature [33, 34].The essential oils are complex mixtures of many differentaromatic components with various degrees of lipophilicity and relative hydrophilicity given by the presence ofconstituents with polar functional groups [35]. When added to a medium, the essential oils distributes more or lessinto the aqueous phase depending on its relative hydrophilicity. Hence, an essential oils with constituents with lowwater solubility should dissolve little in aqueous medium, and consequently should show a weak activity. Theseessential oils, however, showed very good activity when assayed by VA and this might related to their high volatility [33]. These observations suggest that the physical and chemical properties (solubility and volatility) canhave considerable effect on the in vitro antimicrobial activity [36].As shown in table 2 and table 3, the percentage of spore production inhibition of P.expansum, was visibly higher than that of other tested fungi, this can be attributed to greater sensitive of P.expansum than the other fungi to the essential oil of *Salvia Officinalis*. It has been observed that this essential oil vapour used in this study is more effective on spore production than mycelial growth. This result might reflect the effects of volatile components produced by oil on surface mycelial development and/or perception/transduction of signals involved in the switch from vegetative to reproductive development [30]. In addition, the partial inhibition of spore production could be attributed to mycelial destruction or inhibition of fungal growth [37].

CONCLUSION

In conclusion, these results demonstrate that the essential Oil of *Salvia officinalis* is an effective antifungal agent against B.cinera and R. stolonifer, but especially for P.expansum. This essential oil may beconsidered a potential alternative to synthetic fungicides for theprotection of apples from phytopathogenic fungi and may alsoprevent the spoilage of other food commodities during storage,because these types of essential oils have low mammalian toxicity,are biodegradable, and do not persist in the environment. Evaluationof the antifungal activity of this essential oil against thesephytopathogenic fungi in vivo is required before it can be marketed.

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