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Liquid and vapour-phase bioactivity of *Hertia maroccana* (Batt.) Maire essential oil: an endemic Asteraceae from Morocco

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ABSTRACT

Analysis of essential oil (EO) obtained from *H. maroccana* revealed that germanicol (17.8 %), β- pinene (14.6 %), α- guaiene (5.83 %), germacrene D (5.55 %), α- pinene (5.3 %) and δ- cadinene (5 %) were found to be the major components of this essential oil. Antimicrobial potential of *H. maroccana* oil in liquid and vapor phase against different bacterial strains (*Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Salmonella abony* NCTC 6017) and fungal strains (*Alternaria* sp., *Pencillium expansum*, *Rhizopus stolonifer*, *Fusarium oxysporum* f. sp. *albedinis*and *Aspergillus brasiliensis* ATCC 16404) was determined by the disc volatilization method and agar dilution method. The EO is considered moderately active against gram-negative strains (*E. coli*, *S. abony*), and those of gram-positive (*S. aureus*, *B. subtilis*). *S. abony* was the most sensitive bacteria, providing the lowest growth with an MIC equal to 0.156 mg mL^{-1}. However, the mycelium growth was totally inhibited in the presence of the vapor generated by 0.5 µl mL^{-1} air for *Alternaria* sp.

INTRODUCTION

Hertia maroccana (Batt.) Maire, which belongs to the important Asteraceae's family, is a perennial spontaneous shrub with yellow flowers. It is endemic of Morocco very common in the High Atlas, Tafilalet region, outskirts of Midelt (El Oualidi *et al.*, 2012). *Hertia maroccana* which also known as *Othonnopsis maroccana* Batt is known by the Morocco vernacular names of "Talzazte". In the Moroccan traditional medicine, the plant is used as herbal medicines for the treatment of ophthalmic diseases, intestinal parasites and gastrointestinal disorders (El Rhaffari and Zaid, 2002; Bammou *et al.*, 2015). To the best of our knowledge, there is no published report on the composition and antimicrobial activity of *Hertia maroccana* essential oil harvested from the Tafilalet region in the Southeast of Morocco. The present work studies, both the chemical composition and antimicrobial activity by poison food (PF) technique and the volatile activity assay (VA) against five agricultural pathogenic fungi and four pathogenic bacteria.

MATERIAL AND METHODS

Plant material

The aerial part of *H. maroccana* was collected in the Tafilalet region (southeast of Morocco), during the flowering period (May-July, 2014). The voucher specimens have been deposited at the Biochemistry of Natural Products Laboratory, Department of Biology, Faculty of Sciences & Techniques, Errachidia, Morocco. The dried plant material is stored in the laboratory at room temperature (25 °C) and in the shade before the extraction.

Hydrodistillation apparatus and procedure

The extraction of essential oil of the aerial part of *H. maroccana* was conducted by steam distillation in a Clevenger apparatus (Clevenger, 1928). The obtained essential oil was dried over anhydrous sodium sulfate and after filtration, stored at + 4°C until tested and analyzed.

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Essential oil analysis

The essential oil was analyzed using gas chromatography (*TRACE™ GC Ultra*) coupled to the mass spectrometry GC / MS (Polaris Q ion trap MS), fitted with a *TRACE TR-5 GC* capillary column (60m x 0.32mm ID x 0.25 µm). The carrier gas was Helium; the program was 2 min isothermal at 40 °C, then the temperature increased by 5 °C/min to 280 °C. The injection port temperature was 220 °C and that of the detector was 280 °C.

Antibacterial activity

Bacterialstrains

The antibacterial activity was evaluated against four selected Gram-positive and Gram-negative species: *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Salmonella abony* NCTC 6017 and *Escherichia coli* ATCC 8739. Microorganisms were obtained from the culture collection of the Institute of Hygiene (Rabat).

Solid diffusion tests

The qualitative antimicrobial assay of the volatile fraction of *H. maroccana* was carried out by the disc diffusion method (NCCLS, 1999). It was performed using culture growth at 37°C for 18h and adjusted to approximately 10^8 CFU mL^{-1} of the microorganism under study. The culture medium used for the bacteria was Mueller Hinton Agar (MHA). 100 µL of the inoculums were spread over plates containing MHA and a Whatman paper disc (6 mm) impregnated with 5, 10 and 15µL of the volatile fraction was placed on the surface of the media. The plates were left 30 min at room temperature to allow the diffusion of the oil. They were incubated 24 h at 37°C. After an incubation period, the inhibition zone obtained around the disc was measured. Two controls were also included in the test, the first was involving the presence of microorganisms without test material and the second was standard antibiotic. The developing inhibition zones were compared with those of reference discs.

Vapor diffusion tests

Solidified medium was inoculated with 100 µL of bacteria suspension containing 10^8 CFU $mL^{-1}$of the microorganism under study. A sterile filter discs (diameter 6 mm) were laid in the center of the inside surface of the upper lid. Then, 5, 10 and 15 µL of pure essential oils were added to each disc (Lopez *et al.,* 2005). The plate inoculated with microorganisms were immediately inverted on top of the lid and sealed with parafilm to prevent leakage of essential oil vapor. Plates were incubated at 37°C for 24 h and the diameter of the resulting inhibition zone in the bacterial lawn was measured.

Dilution method

The minimal inhibitory concentration (MIC) of testing volatile fractions was determined using the Mueller Hinton broth (MHB) dilution method (Standards, 1999). All tests were performed in MHB supplemented with Tween 80 (1%) (Amezouar *et al.,* 2012). Bacterial strains were cultured overnight in MHB at 37°C. Tubes of MHB containing various concentrations of volatile fractions were inoculated with 10 µL of 10^8 CFU/ml of standardized microorganism's suspensions. Control tubes without testing samples were assayed simultaneously. The MIC was defined as the lowest concentration preventing visible growth (May *et al.,* 2000; Burt, 2004).

Antifungal activity

Fungal strains

Five agricultural pathogenic fungi were selected for their implication in the contamination and the deterioration of vegetables and fruits. The fungal species used in the experiments are *Alternaria sp*, *Pencillium expansum*, *Rhizopus stolonifer*, *Fusarium oxysporum* f. sp. *albedinis*and *Aspergillus brasiliensis* ATCC 16404. These fungi are obtained from the culture collection at Faculty of Sciences & Technology, Errachidia.

Poison food (PF) technique

The antifungal activity of essential oil of*H. maroccana* against mycelial growth of fungi was evaluated by modified poisoned food technique (PF) (Perrucci *et al.,* 1994). The essential oil was dispersed as an emulsion in sterile agar suspension (0.2%) (Remmal *et al.,* 1993) and added to PDA immediately before it was emptied into the glass Petri dishes (90×20 mm in diameter) at 45°C to achieve final concentrations of 0.125 to 2 µl mL^{-1}. The controls received the same quantity of sterile agar suspension (0.2%) mixed with a PDA. The tested fungi were inoculated with 6 mm mycelial plugs from 7-days-old cultures cut with a sterile cork and incubated for 3 days for *Rhizopus stolonifer* and 6 days for *Alternaria sp*, *Pencillium expansum*, *Fusarium oxysporum* f. sp. *albedinis*and *Aspergillus brasiliensis* at 25±2°C.

Volatile activity assay

The antifungal activity of essential oil of *H. maroccana* against mycelial growth was determined by following volatile activity assay (VA) (Soylu *et al.,* 2010).

The Petri dishes were filled with 20 mL of potato dextrose agar (PDA) medium and then seeded with a mycelial disc (6 mm diameter), cut from the periphery of 7-days-old mycelium culture of the tested fungi.

The Petri dishes (90×20 mm, which offer 80 mL air spaces after addition of 20 mL PDA), were inverted and sterile filter paper discs (9 mm in diameter) impregnated with different concentrations of essential oil (i.e. 0.125, 0.25, 0.5, 1 and 2 µl mL^{-1} air) are deposited on the inverted lid and incubated for 3 days for *Rhizopus stolonifer* and 6 days for *Alternaria sp*, *Pencillium expansum*, *Fusarium oxysporum* f. sp. *Albedinis* and *Aspergillus brasiliensis* at 25±2°C.

In both types of experiments, three replicate plates were inoculated for each treatment and the radial growth was recorded for each plate by calculating the average of two perpendicular diameters. Fungitoxicity of essential oil was expressed in terms of percentage of mycelial growth inhibition (I %) and calculated following the formula of Pandey *et al*. (1982).

Percentage of mycelial growth inhibition (IP) $= \left(1 - \left(\frac{Dc}{Dt}\right)\right) \times 100$

Where: D_c: Average diameter (in mm) of mycelial in control and D_t: Average diameter (in mm) of mycelial in treatment.

The fungistatic–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 fungistatic effect was where temporary inhibition of microbial growth occurred.

Statistical analysis

Results are presented as mean ± SD of three independent tests. All tests were carried out in an identical condition.

RESULTS AND DISCUSSION

Chemical Composition of the Essential Oil

The yellowish oils isolated by hydrodistillation from the areal part of *H. maroccana* were obtained in yield of 1.55%. The essential oil was analyzed by means of GC-MS. The components of the oil, the retention times (RT) the percentage constituent (%) are summarized in Table 1 and figure 1.

Table 1: Percentage composition of the essential oils of *H. maroccana*.

N°	Retention Time (min)	Chemical constituents	%Area
1	**12.85**	**α-Pinene**	**5.3**
2	13.88	Sabinene	2.1
3	13.15	Myrcene	2.4
4	**14.48**	**β-Pinene**	**14.6**
5	15.24	α-Phellandrene	0.79
6	15.58	Myrcene	0.76
7	**16**	δ-3-Carene	4.37
8	16.93	γ-Terpinen	0.8
9	17.88	p-Cymene	1.75
10	19.17	α- Campholenal	0.48
11	19.93	β-Thujone	3.4
12	20.85	α-Terpineol	1.45
13	21.43	Myrtenal	0.87
14	22.15	Benzene,1-methoxy-4-methyl-2-(1-methylethyl)	0.37
15	22.97	Pulegone	1.71
16	25.5	α-Cubebene	0.46
17	26.4	α-Copaene	3.74
18	**26.77**	**α-Guaiene**	**5.83**
19	27.71	Longifolene-(V4)	3.33
20	**29.48**	**Germanicol**	**17.8**
21	30.31	δ-Cadinene	**5**
22	31.85	Spathulenol	3.13
23	32.54	Aristolene epoxide	1.04
24	**33.59**	**Germacrene D**	**5.55**
25	34.33	Trans- Cycloisolongifol-5-ol	1.81
26	36.88	allo-Aromadendrene oxide	4.03
27	51.34	α-Amyrine	1.5
28	55.33	3-O-Acetyllupeol	2.34
		Total	**96.71**

The number of identified compounds was 28 representing 96.71% of the total composition. Germanicol (17.8 %), β- pinene (14.6 %), α- guaiene (5.83 %), germacrene D (5.55 %), α- pinene (5.3 %) and δ- cadinene (5 %) were found to be the major components of this essential oil. These results were in accordance with those previously reported in literature. Indeed, for *Hertia intermedia* growing wild in Iran, Akhgar *et al.*, (2012) reported five major constituents: β- pinene (32.8 %), α-pinene (22.7 %), α-thujene (10.9 %) and β- phellandrene (9.6 %) followed by germacrene D (4.6%).Similar results were found with *Hertia angustifolia*. The main constituents were β-pinene (51.5 %), β-phellandrene (16.5 %), α- pinene (13.9 %) and α-thujene (2.7 %) (Afsharypuor *et al.*, 2000).Whereas thymol (61%), 2.6-dimethoxy-phenol (12.83%), camphor (5.82 %) and terpinene-4-ol (5.48 %) were most prevalent in the *Hertia cheirifolia* collected in Tunisia (Attia *et al.*, 2012).

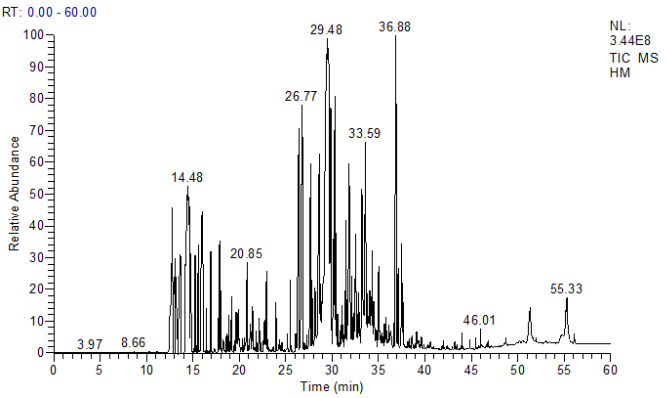

Fig. 1: Chromatogram of essential oil of aerial parts of *H. maroccana* from Morocco.

The essential oil's content showed some variations in the same genus of *Hertia*, harvested from different geographical origins, we noticed that there are some significant differences between the plants collected in Tunisia and the one collected in Iran. The differences recorded with regard to the chemical composition of the essential oil of *Hertia* might be due to several factors in particular soil composition, vegetative cycle phase, climate (Figueiredo *et al.*, 2008; Angioni *et al.*, 2006), physiological age of the plant in addition to the method of oil isolation (Díaz-Maroto *et al.*, 2005).

Antibacterial activity

The results of susceptibility of the strains tested are presented in table 2. Standard antibiotics also exhibited marked activity against Gram positive bacteria than Gram negative bacteria, with the exception of Penicillin which is inactive against *E. coli.*

The in vitro antibacterial activities of *H. maroccana* EO against the four bacteria species tested were assessed in liquid and in vapor phase by the presence or absence of inhibition zone, and by MIC values. As can be seen in Table 3, the EO showed higher activity in the liquid phase. Despite the fact that vapor has the advantage of being able to treat large areas and do not require direct contact with liquid oils, which can make them more suitable for use as disinfectants (Křůmal *et al.*, 2015).

S. abony was the only microorganism that was inhibited when in vapor contact, at 5 µl/disc the size of the inhibition zone equal to 24.00 ± 1.00 mm.

Table 2: Susceptibility of the strains tested.

	Antibiotics					
Microorganisms	**P_{10}**	**C_{30}**	**CN_{10}**	**S_{10}**	**AMC_{30}**	**CIP_5**
E. coli ATCC 8739	NA	26.33±0.57	21.67±0.57	12.00±0.00	17.00±1.00	12.33±0.57
S. aureus ATCC 6538	34.33±1.15	29.00±1.00	24.66±0.57	20.00±0.00	33.66±0.57	15.33±0.57
S. abony NCTC 6017	15.33±0.57	26.33±0.57	21.00±1.00	16.66±0.57	23.33±0.57	14.67±0.43
B. subtilis ATCC 6633	34.66±0.57	35.33±1.15	30.66±0.57	21.33±0.57	29.66±1.15	21.67±0.57

P_{10} : Penicillin ; C_{30} : Chloramphenicol (30µg/disc) ; CN_{10} : Gentamicin (10µg/disc) ; S_{10} : Streptomycin (10µg/disc) ; AMC_{30} : Amoxycillin/Calvulanic Acid (30µg/disc) ; CIP_5 : Ciprofloxacine (5µg/disc).NA : Not active.

Table 3: Antibacterial activity of EO of *H. maroccana* against the bacterial strains based on disc diffusion method.

	Liquid contact EO (µl/disc)			**Vapor contact EO (µl mL^{-1} air)**		
	Inhibition zone diameter [a]					
Microorganisms	5	10	15	5	10	15
Gram-negative bacteria						
E. c ATCC 8739	NA	15.33±0.58	21.00±1.00	NA	NA	NA
S. ab NCTC 6017	12.33±0.58	18.67±0.58	24.33±0.58	24.00±1.00	42.67±0.58	53.33±1.53
Gram+ positive bacteria						
S. a ATCC 6538	NA	10.67±0.58	13.67±0.58	NA	NA	NA
B. s ATCC 6633	NA	10.67±0.58	13.00±1.00	NA	NA	NA

E.c : *Escherichia coli* ; S.a : *Staphylococcus aureus* ; S.ab : *Salmonella abony* ; B.s :*Bacillus subtilis*
[a] Diameter of the zone of inhibition (mm) including disk diameter of 6 mm; NA: Not active.

Table 4: The MIC values of different extracts from *H. maroccana* against the bacterial strains.

Microorganisms	**MIC (mg mL^{-1})**
Gram-negative bacteria	
Escherichia coli ATCC 8739	0.312
Salmonella abony NCTC 6017	0.156
Gram+ positive bacteria	
Staphylococcus aureus ATCC 6538	1.248
Bacillus subtilis ATCC 6633	1.248

The essential oil is considered moderately active against gram-negative strains (*E. coli, S. abony*), and those of gram-positive (*S. aureus, B. subtilis*). The diameter of the inhibition zone varies from one bacterium to another. At 15 µl/disc the inhibition zone in the liquid phase generally increased in the following order: *B. subtilis* (13.00 ± 1.00 mm), *S. aureus* (13.67 ± 0.58 mm), followed by *E. coli* (21.00 ± 1.00 mm) and *S. abony* (24.33 ± 0.58 mm), the latter was the most susceptible bacterium.

The inhibition activity depends on plant taxonomy as well as on oil concentration and types of chemical radicals of the molecules in essential oils (Pinto *et al.*, 2006; Ouraïni *et al.*, 2005; Satrani *et al.*, 2011).

Essential oils from *Thymus saturejoides* L. were reported more effective than those from *Mentha pulegium* L. on human mycosis causing agents (Ouraïni *et al.*, 2005). *Artemisia herba alba* led to inhibition of some microorganisms like *Streptococcus agalactiae*, *Salmonella enteridis*. However, ithad no effect on *Pseudomonas aeruginosa* (Yashphe *et al.*, 1987). It was also reported that essential oils from *Origanum vulgare* and *Thymus zygis* were more effective on *E. coli* than those from *Rosmarinus officinalis*, *Lavandula* sp and *Thymus vulgaris*, whereas, *Origanum vulgare* was the only one effective on *Staphylococcus aureus* (Kaloustian *et al.*, 2008). Similarly, Soković *et al.*, (2009) observed the antibacterial activity of essential oils extracted from thyme and mint leaves against the *Staphylococcus aureus*, *Salmomella typhimurium* and *Vibrio parahaemolyticus*. Our results show a great variability in the bacteriostatic qualities of the oil towards the different strains. Only the strains Gram negati1ve *S. abony* and *E. coli* are more sensitive than the other bacterial strains tested, with a minimum inhibitory concentration, respectively of (0.165 and 0.312 mg mL^{-1}) (Table 4).

Antifungal activity

The essential oils isolated from the aerial parts of *H. maroccana* were tested for antifungal activity against five phytopathogenic strains. The results of antifungal activity assays for both studied methods showed that the oils significantly reduced the growth of all tested fungi in a dose-dependent manner (Figs. 2).

Using PF assay, the data (Fig.2 A) indicated that *Alternaria s*p. was the most sensitive strain producing a 100 % inhibition at 2 µl mL^{-1}. Furthermore, *F. oxysporumalbedinis, P. expansum,A. brasiliensis* and *R. stolonifer* were found to be susceptible to the EO of *H. maroccana* and were considerably inhibited in the presence of the liquid oil at 2 µl mL^{-1}. At same concentration, *F. oxysporumalbedinis* and *P. expansum* with the IP are 87.50 ± 1.14 %and 80.07 ± 1.7 %, respectively. Furthermore, the successful effects correlate with oil concentration (Ouraïni *et al.*, 2005) as well as with the type of fungal species. For example, with *M. pulegium*, 10 µL was necessary to inhibit growth of *Penicillium expansum* and *Alternaria alternate* (Satrani *et al.*, 2011). Whereas 20 µL was needed for *Penicillium sp* and 2 µg mL^{-1} for mycosis agents (Ouraïni *et al.*, 2005; Ouraïni *et al.*, 2007). Using VA assay, the results (Fig. 2 B) showed that the activity of the vapor of the *H. maroccana* essential oil was more pronounced for all strains tested.

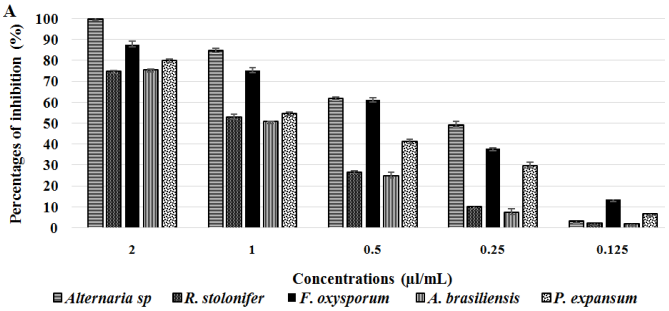

Fig. 2 A: Antifungal potential of *H. maroccana* oil liquid.

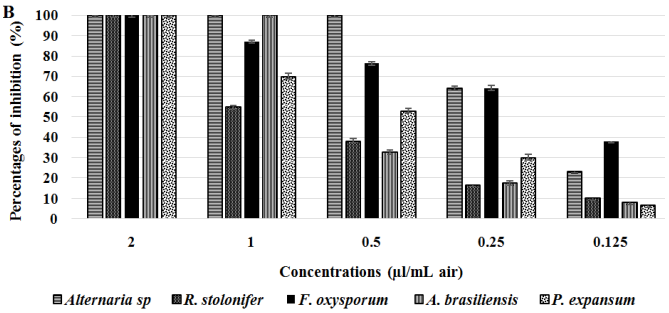

Fig. 2 B: Antifungal potential of *H. maroccana* oil vapor.

The mycelium growth was totally inhibited (100 ± 0.00 %) in the presence of the vapour generated by 2 µl mL^{-1} air for *F. oxysporumalbedinis, R. stolonifer* and *P. expansum.* Moreover, the mycelium growth of *A. brasiliensis* was totally inhibited at 1 µl mL^{-1} air. At same concentration, *F. oxysporumalbedinis, P. expansum* and *R. stolonifer* was only partially inhibited (87.12 ± 0.66 %, 69.63 ± 0.64 % and 54.81 ± 1.7 % respectively). Whereas, the concentration generated by 0.5 µl mL^{-1} air is sufficient to inhibit completely the mycelium growth of *Alternaria* sp.

It is interesting to know about the fungitoxic nature of this vapour oil against all fungal strains tested. To confirm this, we transfer the mycelial discs where growth inhibition was complete by *H. maraccana* vapor into the PDA medium not containing this oil.

From Table 5, it was clear that *H. maroccana* oil has shown its antifungal activity at a minimum inhibitory dose of 0.5 µl mL^{-1} air against *Alternaria* sp. All fungal species failed to restore growth even after six days incubation period without this oil, indicating a fungicidal activity of the oil at2 µl mL^{-1} air, except of *Alternaria* sp. where the MFC was 0.5 µl mL^{-1} air.

Table 5: Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) values in µl mL^{-1} air

Strains	MIC	MFC
Alternaria sp.	0.5	1
P. expansum	2	2
F. oxysporumalbedinis	2	2
A. brasiliensis (ATCC 16404)	1	2
R. stolonifer	2	2

The antifungal activity of *H. maroccana* is probably related to the high concentration of *α*- and *β*- pinene that were reported as effective antifungal agents (Hammer *et al.*, 2003). The work presented by Filipowicz *et al.*, (2003) reported that the antimicrobial activity of juniper oil is the result of the highest concentration of α-pinene, p-cymene and β-pinene. Thus, the activity of the oil probably results from the combination of all major compounds as well as from a synergic effect of the less dominant ones (Pinto *et al.*, 2006; Bouchra *et al.*, 2003).

It is evident that the vapor from *H. maroccana* oil showed higher antifungal activity against the pathogens tested compare to PF technique. The efficacy of essential oils in vapor state was probably attributable to the direct deposition of essential oils on lipophilic fungal mycelia together with an indirect effect via adsorption through the culture medium (Edris and Farrag, 2003).

Recently, there have been several studies confirms that vapor phases of EOs are more effective antifungal than their liquid phases, including *Foeniculum vulgare* (Sellam *et al.*, 2015), *Eucalyptus globules* (Tyagi and Malik, 2011), *Rosmarinus officinalis* (Survilienė *et al.*, 2009), *Citrus sinensis* (Sharma and Tripathi, 2006), and a range of others including thyme, fennel and lavender (Soylu *et al.*, 2006; Tullio *et al.*, 2007).

CONCLUSION

Following these results, we can say that *H. maroccana* EO, which is rich in Germanicol (17.8 %), β-pinene (14.6 %), α-guaiene (5.83 %), germacrene D (5.55 %), α-pinene (5.3 %) and δ-cadinene (5 %) possesses good antifungal activity against *Alternaria sp*, *Pencillium expansum*, *Rhizopus stolonifer*, *Fusarium oxysporum* f. sp. *albedinis*and *Aspergillus brasiliensis* ATCC 16404. In addition, this EO is highly effective in liquid phase and could potentially be used to combat bacterial pathogens (*Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Salmonella abony* NCTC 6017 and *Escherichia coli* ATCC 8739).

H. maroccana essential oil vapour may be considered as a potential agent for preventing phyto-pathogenic fungi. A further study in vivo condition is war ranted to confirm the antifungal activity of this plant, which may be used for preservation and/or extension the shelf life of raw and processed food.

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