INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-4, Issue-1, Jan-Mar-2013 Coden : IJABPT Copyrights@2013

ISSN : 0976-4550

Received: 25th Nov-2011 *Revised: 02nd Dec-2011* *Accepted: 14th Dec-2012*

Research Article

CHEMICAL COMPOSITION AND ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS ISOLATED FROM *JUNIPERUS OXYCEDRUS* L.

Ismail Amri[1,2], Lamia Hamrouni[2], Samia Gargouri[3], Mohsen Hanana[4], Bassem Jamoussi[5]

[1]Faculté des Sciences de Bizerte. Bizerte, 7021, Tunisie.
[2]Laboratoire d'Ecologie Forestière, Institut National de Recherches en Génie Rural, Eaux et Forêts. BP 10, 2080 Ariana, Tunisie
[3]Laboratoire de Protection des Végétaux, Institut National de la Recherche Agronomique de Tunisie, Rue Hédi Karray 2049, Tunisie.
[4]Laboratoire de Physiologie Moléculaire des Plantes, Centre de Biotechnologie de Borj-Cédria, BP 901, Hammam-lif 2050, Tunisie.
[5]Institut Supérieur d'Education et de Formation Continue. Tunis, Tunisie.

ABSTRACT: The chemical composition of essential oils isolated from the leaves of *Juniperus oxycedrus* by hydrodistillation was analyzed by GC-MS. 42 compounds, representing 96.73% of total oil, were identified. *J. oxycedrus* oil was found to be rich in α-pinene (39.63%), manoyl oxide (12.34) and z-caryophyllene (4.1%) and characterized by relatively high amounts of monoterpenes hydrocarbons and sesquiterpenes. Results of the antifungal testing by in vitro contact assay showed that the oil significantly inhibit the growth of nine plant pathogenic fungi.

Keywords: *Juniperus* oxycedrus, α-pinene, manoyl oxide, Z-caryophyllene, antifungal activity.

INTRODUCTION

Pests are the largest competitor of agricultural crops and severely reduce the crop production in the range of 25–50% (Pimentel, et al., 1991; Oerke, 2006). To protect agricultural crops enormous amount of synthetic pesticides are used. As per Agrow (2007) report, the total value of world's agrochemical market was between US$31–35 billion and among the products, fungicides accounted for (22%). However, the excessive use of synthetic pesticides in the crop lands, urban environment, and water bodies to get rid of noxious pests has resulted in an increased risk of pesticideresistance, enhanced pest resurgence, toxicological implications to human health and increased environmental pollution. In fact, combating of environmental pollution and its ill-effects on the life is one of the most serious challenges before the present day world. Efforts are thus being made to replace these synthetic chemicals with biological products, which are safer and do not cause any toxicological effects on the environment. The natural pest and disease control either directly or indirectly using natural plant products including essential oils, holds a good promise (Isman, 2006; Bakkali, et al., 2008). *Juniperus oxycedrus* (Cupressaceae) is a shrub or small tree growing wild in stony places of the Mediterranean and Near East countries. That is one of the most appreciate plants for its essential oil richness and its plethora of biologically active compounds extensively used in folk medicine. *J. oxycedrus* was used for the treatment of various diseases, such as hyperglycemia, obesity, tuberculosis, bronchitis and pneumonia (Sanchez de Medina, et al., 1994). There are many reports on the chemical composition of the oils from *Juniperus* species (Altarejos, et al, 1999; Milaos and Radonic, 2000; Salido et al, 2002. Loizzo, et al., 2007), most of these reports indicate that α-pinene, manoyl oxide and Z-caryophyllene are the main constituents of these oils. The chemical composition of Tunisian *J. oxycedrus* have been reported (Medini, et al., 2010), however to the best of our knowledge, no report on the antifungal activity of *J. oxycedrus* essential oils.

The aims of this study were to determine the chemical composition of essential oils extracted from the aerial parts of *J. oxycedrus* L. and to determine their antifungal activity against nine plant pathogenic fungi.

Coden : IJABPT Copyrights@2013 ISSN : 0976-4550

MATERIALS AND METHODS

Plant material

The leaves of *J. oxycedrus* were collected from the INRGREF arboretum (Tunisia) in December 2009. Identification was performed in the Laboratory of Genetic of INGREF. A voucher specimen is deposited in the Herbarium of this laboratory.

Isolation of the essential oils

The essential oils were extracted by hydrodistillation of dried plant material (100 g of leaves in 500 mL of distilled water) using a Clevenger-type apparatus for 5 h. The oils were dried over anhydrous sodium sulphate and stored in sealed glass vials at 4°C prior to analysis. Yield based on dried weight of the sample was calculated.

Analysis of the essential oils

Gas chromatography analysis/mass spectrometry analysis conditions

Gas chromatography analysis

The essential oils were analysed using a Hewlett Packard 5890 II GC equipped with Flame Ionization Detector (FID) and HP-5 MS capillary column (5% phenyl/95% dimethylpolysiloxane: 30 m×0.25 mm id, film thickness 0.25 µm). Injector and detector temperature were set at 250 °C and 280 °C, respectively. Oven temperature was kept at 50 °C for 1 min then gradually raised to 250 °C at 5 °C/min and subsequently, held isothermal for 4 min. Nitrogen was the carrier gas at a flow rate of 1.2 ml/min. Diluted samples (1/100 in hexane, v/v) of 1.0 µl were injected manually and in the splitless mode. Quantitative data were obtained electronically from FID area percent data without the use of correction factors.

Gas chromatography analysis/mass spectrometry analysis

Analysis of the oils was performed using a Hewlett Packard 5890 II GC, equipped with a HP 5972 mass selective detector and a HP-5 MS capillary column (30 m×0.25 mm id, film thickness 0.25 µm). For GC/ MS detection, an electron ionization system, with ionization energy of 70 eV, a scan time of 1.5 s and mass range 40–300 amu, was used. Helium was the carrier gas at a flow rate of 1.2 ml/min. Injector and transfer line temperatures were set at 250 and 280 °C, respectively. Oven program temperature was the same with GC analysis. Diluted samples (1/100 in hexane, v/v) of 1.0 µl were injected manually and in the splitless mode. The identification of the compounds was based on mass spectra (compared with Wiley 275.L, 6th edition mass spectral library) or with authentic compounds and confirmed by comparison of their retention indices either with those of authentic compounds or with data published in the literature as described by Adams (2001). Further confirmation was done from Kovats Retention Index data generated from a series of n-alkanes retention indices (relative to C9–C28 on the HP-5 MS capillary column). (Davies, 1990).

Antifungal activity assays

Nine plants pathogenic fungi were obtained from the culture collection (INRAT). Cultures of each of the fungi were maintained on potato dextrose agar (PDA) and were stored at 4 °C. The fungal species used in this study were: *F. equisiti, F. culmorum, F. oxysporum, F. solani, F. verticillioides, F. nygamai, Botritys cinerea, Microdochium nivale var nivale, Alternaria sp.*

Antifungal activity was studied by using a contact assay (*in vitro*), which produces hyphal growth inhibition (Cakir, et al., 2004). Briefly, potato dextrose agar (PDA) plates were prepared using 9 cm diameter glass Petri dishes. The essential oil was dissolved in tween water solution (1%, v/v) and required amounts of the solutions were added to each of the PDA plates containing 20 ml of agar at 50 °C.

A disc (5mm diameter) of the fungal species was cut from 1-week-old cultures on PDA plates and then the mycelia surface of the disc was placed upside down on the centre of a dish with fungal species in contact with growth medium on the dish. Then, the plates were incubated in the dark at 25 °C. The extension diameter (mm) of hyphae from centers to the sides of the dishes was measured after 6 days. Mean of growth measurements were calculated from three replicates of each of the fungal species. PDA plates containing tween–water solution (1%, v/v), without essential oil were used as negative control. The percentage of growth inhibition by treatment was calculated using the following equation:

% Inhibition = (C-T)/Cx100.

Where *C* is the mean of four replicates of hyphal extension (mm) of controls and *T* is the mean of four replicates of hyphal extension (mm) of plates treated with essential oil and the compound solutions.

Coden : IJABPT Copyrights@2013 ISSN : 0976-4550

Statistical analysis

Data of antifungal activity assays were subjected to one-way analysis of variance (ANOVA), using the SPSS 13.0 software package. Differences between means were tested through Student-Newman-Keuls (SNK) and values of $p \leq 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

Chemical composition

The oil yield obtained from hydrodistillation of the *Junipers oxycedrus* leaves was 0.18 % (v/w), considerable differences were observed in the essential oil yield obtained in Croatia (0.05%) (Milaos and Radonic, 2000) and Spain (0.27%) (Salido, et al., 2002). Table 1 show the constituents identified in the essential oil with their area percentage and retention indices. 42 compounds, representing 96.73% of the essential oil were identified, the chemical composition of our *J. oxycedrus* oil was dominated bymonoterpene hydrocarbons (51.73%), oxygenated sesquiterpenes (21.55 %) and sesquiterpene hydrocarbons (16.05%). while the monoterpene hydrocarbons and diterpenes were present by low percentage, respectively, 1.5 and 5.9%. Like the essential oil from other *Juniperus* species, the major constituents of the oil were α-pinene (39.63%), manoyl oxide (12.34%), Z-caryophyllene (4.1%), δ-3-carene (3.9%), geranyl acetone (3.69%) and caryophyllene oxide (2.67%). The essential oil of *J. oxycedrus* was previously investigated in other countries, and in agreement with our result, generally it was shown that α-pinene and manoyl oxide were the major components in the oil but with different levels (Altarejos, et al., 1999; Milaos and Radonic, 2000; Salido, et al., 2002; Loizzo, et al., 2007, Medini, et al., 2010). These differences in the oil composition and yield may be due to several factors such as time of collection, geographic and climatic conditions.

Antifungal activity of essential oils

The essential oils isolated from the leaves of the *J. oxycedrus* were tested for their antifungal activity against nine important agriculturally fungal species. These results showed that the oil significantly reduced the growth of the fungal species over a very broad spectrum (table 2). The obtained results confirm the antifungal activity of conifer essential oils reported by others reports (Lis- Balchin, et al., 1998). As seen table 2, and according the statistical analysis, the oils exhibited different degrees of inhibition on the growth of tested fungi; *Microdochium nivale*, *F. culmorum* and *F.equisiti* were the most sensitive to the action of the oil, while *Botrytis cinerea* was the most resistant. Generally, *Juniperus* species are known to possess an antifungal activity (Parajuli, et al., 2005; Pepeljnjak, et al., 2005). In our study, *J. oxycedrus* oil was considered rich monoterpenes and oxygenated sesquiterpenes, in addition, there was a correlation between the antifungal activity and percentage of some components, Table 1 indicated that *J. oxycedrus* essential oils were characterized by the relatively high content of α-pinene, manoyl oxide, Z-caryophyllene and geranyl acetone, which are known to possess an important antifungal activity (Sokovic and Griensven, 2006; Hui-Ting, et al., 2008). Indeed, many authors have attributed the antifungal capacity of essential oils from different *Juniperus, Calocedrus, Pistacia* and *Cupressus* species to the presence of α-pinene, Z-caryophyllene and other sesquiterpenes (Duru, et al., 2003; Pepeljnjak, et al., 2005; Hui-Ting, et al., 2008; Mazari, et al., 2010).

Several studies have demonstrated the antifungal proprieties of these compounds; for example, Sokovic and Griensven (2006) showed that α-pinene and limonene posses an important antifungal activity against *Verticillium fungicola* and *Trichoderma harzianum* (MIC 4.0–9.0 µl/ml). Other studies have tested the antifungal activity of some sesquiterpenes and they showed that oxygenated sesquiterpene were more effective than sesquiterpene hydrocarbons which are more effective than hydrocarbon monoterpenes components of the oil (Hui-Ting, et al., 2008), for these reasons the antifungal activity of our oil was attributed to the presence of both sesquiterpenes and monoterpenes and the synergism between components does play an important role. But the exact mechanism of action of essential oils and its components on fungi remains unclear however, a number of effects and hypothesis have been reported by many authors. In general, the majority of reports agree that essential oils result in significant morphological changes to the hyphae, most noticeably a reduction in hyphae wall thickness, possibly related to interference by essential oil components in the enzymatic reactions of cell wall synthesis leading to incorrect assembly of wall components, such as chitin, glucans and glycoproteines (Helal, et al., 2006; Sharma and Triphati, 2006).

Coden : IJABPT Copyrights@2013 ISSN : 0976-4550

Table 1: Chemical composition of *Juniperus oxycedrus* L. Essential oil.

Peaks	RI[a]	Compounds	Area %	Identification
1	926	Tricyclene	0.15	MS RI
2	931	α-thujene	0.03	MS RI
3	939	α-pinene	**39.63**	MS RI
4	953	camphene	0.33	MS RI
5	953	α-fenchene	0.12	MS RI
6	956	thuja-2,4(10)-diene	0.01	MS RI
7	976	sabinene	0.41	MS RI
8	980	β-pinene	0.55	MS RI
9	991	myrcene	2.37	MS RI
10	1005	α-phellandrene	0.22	MS RI
11	1011	δ-3--carene	3.9	MS RI
12	1018	α-terpinene	1.21	MS RI
13	1026	p-cymene	0.22	MS RI
14	1031	limonene	1.65	MS RI
15	1062	δ-terpinene	0.83	MS RI
16	1088	α-terpinolene	0.1	MS RI
17	1125	α-compholenal	0.19	MS RI
18	1143	camphor	0.1	MS RI
19	1177	terpinen-4-ol	1.1	MS RI
20	1189	α-terpineol	0.11	MS RI
21	1372	α-ylangene	0.31	MS RI
22	1384	β-bourbonene	0.19	MS RI
23	1390	β-cubebene	1.3	MS RI
24	1391	β-elemene	0.9	MS RI
25	1402	langiofolene	1.87	MS RI
26	1418	Z-caryophyllene	**4.1**	MS RI
27	1428	β-copaene	0.73	MS RI
28	1454	α-humulene	1.35	MS RI
29	1455	geranyl acetone	**3.69**	MS RI
30	1460	bornyl iso-butanoate	0.49	MS RI
31	1461	α-muurolene	1.18	MS RI
32	1480	Germacrene D	2.99	MS RI
33	1508	β-bisabolene	0.1	MS RI
34	1524	δ-cadinene	0.21	MS RI
35	1538	α-cadinene	0.21	MS RI
36	1542	α-calacorene	0.61	MS RI
37	1565	Nerolidol	0.26	MS RI
38	1581	Caryophyllene oxide	2.67	MS RI
39	1684	Eudesma-4(15),7-diene-1-β-ol	2.1	MS RI
40	1990	Manoyl oxyde	**12.34**	MS RI
41	2054	Abietariene	3.38	MS RI
42	2080	Abietadiene	2.52	MS RI
Total Identified Compounds (%):			96,73	
Monoterpene hydrocarbons (%):			51,73	
Oxygenated monoterpenes (%):			5.19	
Sesquiterpene hydrocarbons (%):			16.05	
Oxygenated Sesquiterpenes (%):			17.86	
Diterpenes (%):			5.9	

Coden : IJABPT Copyrights@2013 ISSN : 0976-4550

Table 2 : Antifungal activity of *J. oxycedrus* essential oil.

Fungi	Control Growth (mm)	Essential oil (4µL/mL)	
		Growth (mm)	Inhibition%
F. nygamai	62±1.52	24.66±0.33	60.18±0.73b
Alternaria sp	60.66±0.66	26.66±0.88	56.00±1.89ab
F. solani	64±0.66	24±2.08	62.39±3.65b
Microdochium nivale	68.66 ±1.76	19±0.57	72.30±0.89c
F. culmorum	71.66±0.88	20±0.57	72.07±0.96c
Botrytis cinerea	84±0.33	42±1.52	50.40±1.68a
F.equisiti	72±1.15	19.66±0.33	72.67±0.64c
F.oxysporum	71.33±0.88	31.33±0.88	56.09±0.68ab
F.verticilloides	73±0.57	30.66±1.2	57.99±1.60b

Means in the same column by the same letter are not significantly different of the test Student-Newman-Keuls ($p \leq 0.05$).

Plasma membrane disruption , mitochondrial structure disorganization, decreases in both lipid and saturated fatty acid content , increases in insatured fatty acids and Mg^{2+}, Ca^{2+} and K^{+} leakage from exposed cells have been reported(Zamboneli, et al., 1996; Helal, et al., 2006; Sharma and Triphati, 2006) . Other reports suggested that the components of the essential oils cross the cell membrane, interacting with the enzymes and proteins of the membrane such as the H+-ATPase pumping membrane, so producing a flux of protons towards the cell exterior which induces changes in the cells and, ultimately, their death. Besides, (Lucini et al., 2006; Cristani, et al., 2007; Viuda-Martos, et al., 2008; Tatsadjieu, et al., 2009) reported that the antimicrobial activity is related to ability of terpenes to affect not only permeability but also other functions of cell membranes, these compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites. Other reports, showed that the essential oils would act on the hyphae of the mycelium, provoking exit of components from the cytoplasm, the loss of rigidity and integrity of the hypha cell wall, are resulting in its collapse and death of the mycelium (Sharma and Tripathi, 2008).

These findings need to be explored as a viable, alternative source to commercially available agrochemicals for plant pathogenic fungi. Further studies are under way to isolate and characterize the major active principles of the oil and test the compounds on different microorganisms and against various plant diseases, where in the information procured would further serve as a strong evidence for the plant as potent antimicrobial agent.

REFERENCES

A. Cakir, S. Kordali, H. Zengin, S. Izumi and T. Hirata (2004). Composition and antifungal activity of essential oils isolated from *Hypericum hyssopifolium* and *Hypericum heterophyllum*, Flavour. Frag. J. *Vol.* 19, 62–68.

Agrow (2007). agrow's Top 20: 2007 Edition—DS258. Informa Health Care, London, UK.

A. Zambonelli, A.A. Zechini, A. Bianchi and A. Albasini (1996). Effects of essential oils on phytopathogenic fungi, Phytopathology. Vol. 144, 491-494.

C. Hui-Ting, C. Ying-Hung, W. Chi-Lin, C. Shang-Tzen, Tun-Tschu and Y.S. Chang, (2008). Antifungal activity of essential oil and its constituents from Calocedrus macrolepis var. formosana Florin leaf against plant pathogenic fungi, Bioresource Technol. Vol. 99, 6266–6270.

C. Regnault-Roger (1997). The potential of botanical essential oils for insect pest control. Integrated Pest Manage Vol. 2, 25–34.

D. Pimentel, L. Mclaughlin, A. Zepp, B. Lakitan, T. Kraus, P. Kleinman, F. Vancini, W. Roach, E. Graap, W. Keeton, and G. Selig (1991). Environmental and economic effects of reducing pesticide use. BioScience: Vol. 41, 402–409.

E.I. Lucini, M.P. Zunino, M.L. Lopez and J.A. Zygadlo (2006). Effect of monoterpenes on lipid composition and sclerotial development of Sclerotium cepivorum Berk. Journal of *Pathology:* Vol. 154, 441-446.

F. Bakkali, S. averbeck, D. averbeck, and M. Idaomar (2008). Biological effects of essential oils a review. *Food* and Chemical *Toxicology:* Vol. 46, 446–475. E.C. Oerke (2006). Crop losses to pests. J. agr. Sci. 144, 31–43.

F. Sanchez de Medina, M. J. Gamez, I. Jimenez, J. Jimenez, J.I. Osuna and A. Zarzuelo (1994). Hypoglycemic activity of juniper berries, Planta Med. Vol. 60, 197–200.

G.A. Helal, M.M. Sarhan, A.N.K. Abu Shagla and E.K. Abou El-khiar (2006). Effects of Cymbopogon citrates L. essential oil on the growth, lipid content and morphogenesis of Aspergillus niger ML2-strainn, J. basic microb. Vol. 46, 456-469.

H. Medini, A. Elaissi, M.L. Khouja, I. Chraief, F. Farhat, M. Hammami , R. Chemli and F. Harzallah-Skhir (2010). Leaf essential oil of Juniperus oxycedrus L. (Cupressaceae) harvested in northern Tunisia: Composition and Intra-Specific Variability, Chem Biodivers. Vol. 7, 1254-1266.

J. Altarejos, C. Fernandez, and A. Camacho (1999). The Leaf Essential Oils and Taxonomy of Juniperus oxycedrus L. subsp. oxycedrus, subsp. badia (H. Gay) Debeaux, and subsp. macrocarpa (Sibth. & Sm.) Bali, J. Essent. Oil Res. Vol. 11, 167-172.

K. Mazari, N. Bendimerad, C. Bekhechi1 and X. Fernandez (2010).Chemical composition and antimicrobial activity of essential oils isolated from Algerian Juniperus phoenicea L. and Cupressus sempervirens L., J. Med. Plants Res. Vol. 4, 959-964.

M. Milos and A. Radonic (2000). Gas chromatography mass spectral analysis of free and glycosidically bound volatile compounds from Juniperus oxycedrus L. growing wild in Croatia, Food Chem. Vol. 68, 333-338.

M.B. Isman (2006). Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annual Review of Entomology Vol. 51, 45–66.

M. Lis-Balchin, S.G. Deans and E. Eaglesham (1998). Relationship between bioactivity and chemical composition of commercial essential oils, Flavour Fragr. J. Vol. 13, 98-104.

M.R. Loizzo, R. Tundis, F. Conforti, A.M. Saab, G. Statti and F. Menichini (2007). Comparative chemical composition, antioxidant and hypoglycaemic activities of Juniperus oxycedrus ssp. oxycedrus L. berry and wood oils from Lebanon, Food Chem. Vol. 105, 572–578.

M.E. Duru, A. Cakir, S. Kordali, H. Zengin, M. Harmandar, S. Izumi, and T. Hirata (2003). Chemical composition and antifungal properties of essential oils of three Pistacia species, Fitoterapia: Vol. 74, 170-176.

M. Viuda-Martos, Y. Ruiz-Navajas, J. Fernandez-Lopez, and J. Perez-Alvarez (2008). Antifungal activity of lemon (Citrus lemon L.), mandarin (Citrus reticulata L.), grapefruit (Citrus paradisi L.) and orange (Citrus sinensis L.) essential oils. Food Control: Vol. 19, 1130-1138.

M. Sokovic and L.J.L.D. Griensven (2006). Antimicrobial activity of essential oils and their components against the three major pathogens of cultivated button mushroom Agaricus bisporus, Eur. J. Plant Pathol. Vol. 116, 211–224.

M. Arslan and S. Dervis (2010). Antifungal activity of essential oils against three vegetative-compatibility groups of Verticillium dahliae. World Journal of Microbiology and Biotechnology : Vol. 26, 1813-1824.

Coden : IJABPT Copyrights@2013 ISSN : 0976-4550

M. Cristani, M. Arrigo, G. Mandalari, F. Castelli, M.G. Sarpietro, and D. Micieli (2007). Interaction of four monoterpenes contained in essential oils with model membranes: Implications for their antibacterial activity. Journal of Agricultural and Food Chemistry: Vol. *55*, 6300-6308.

N.W. Davies (1990). Gas chromatographic retention indexes of monoterpenes and sesquiterpenes on methyl silicone and carbowax 20M phases. Journal of Chromatogr A, Vol. 503, 1-24.

N.L. Tatsadjieu, P. Jazet, M.B. Ngassoum, F.X. Etoa, and C.M.F. Mbofung (2009). Investigations on the essential oil of Lippia rugosa from Cameroon for its potential use as antifungal agent against Aspergillus flavus Link ex. Fries. Food Control: Vol. 20, 161-166.

N. Sharma and A. Triphati (2006). Fungiotoxicity of the essential oil of Citrus sinesis on post-harvest patogenes, World. J. microb. biot. Vol. 22, 587-593.

R.P. Adams (2001). Identification of Essential Oil Components by Gas Chromatographye Quadrupole Mass Spectrometry. Allured, Carol Stream,IL, USA.

R.R. Parajuli, R.d. Tiwari, R.P. Chaudhary and V.N. Gupta (2005). fungitoxicity of the essential oils of some aromatic plants of manang against alternaria brassicicola, Scientific World. Vol. 3, 39-43.

S. Pepeljnjak, I. Kosalec, Z. Kaloera and N. Blazevic (2005). Antimicrobial activity of juniper berry essential oil (Juniperus communis L., Cupressaceae), Acta Pharm. Vol. 55, 417–422.