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RESEARCH ARTICLE

CHEMICAL COMPOSITION OF THE *CEDRUS ATLANTICA* (ENDL.) MANETTI EX CARRIÈRE SEEDS ESSENTIAL OIL IN FUNCTION OF THEIR GERMINATION STAGES

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ABSTRACT

Forest seeds present a particular importance because they are the principal organs which condition the reforestation success. Furthemore, successful production of replanting seedlings for outplanting sites depends on many parameters, especially biological ones. Along this study, we were interested in the valorization of *Cedrus atlantica* seeds, by the chemical characterization of essential oils extracted by steam distillation, during different stages of their germination. The yield of the Atlas cedar seeds essential oils obtained varied between 0.7 and 1.45%, depending on the germination's stage. Also the results of chromatographic analysis by GC / FID and GC / MS revealed that the seeds are rich in monoterpene hydrocarbons (alcohols and esters). The chemical map of the essential oils depends on the seeds germination's stage of these organs which indicates that the biosynthesis of these compounds play an important role in the germination phases of Atlas cedar seeds.

INTRODUCTION

The cedar, at the present time, has gained a considerable importance in the forestry world of the Mediterranean's surrounding in view of its bio-ecological and socio-economic interest. The natural area of the *Cedrus* gender distribution is North Africa (*C. atlantica* in Morocco and in Algeria), the Eastern Mediterranean (*C. brevifolia* in Cyprus and *C. libani* in Turkey, Lebanon and Syria) and the Western Asia (*C. deodara* in India, in Afghanistan and the Himalaya North-East). In terms of area, the Morocco and the Turkey are in the foreground with more than 130, 000 ha in each of both countries (M'hirit *et al*., 2006). In Morocco, the Atlas cedar is an endemic species that occupies an average altitudinal range between 1600 and 2400 m. The main massifs are observed in the Rif, the central and Eastern Middle Atlas and the Oriental High Atlas. Its ecological amplitude is relatively wide. It is inserted in the wet and cold sub-humid bioclimates and displays an indifference vis à vis to the chemical nature of the substrates (Emberger, 1938; HCEFLCD, 2013). In fact, the cedar is a monoecious species, male and female organs are found on the same tree.

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The male organs are kittens whereas the female organs are inflorescences which are developing gradually in conelets then in cones. The seed is tender resinous, it involves at least one resinifere pocket on each side. This pocket's rupture definitely hinders the seed's germination. The free part of the wing is longer than the seed itself. The length and width's averages of the Atlas cedar seeds are of the respectively order of 12 mm and 5 mm (Toth, 1980). The seed's germination is the first stage of the plants life cycle to produce a new generation. It is defined as the radical's emergence (Bacchetta *et al*., 2006). The germination process is consisted of three phases. The first is called imbibitions phase, corresponding to the water's absorption by the seed ; the second phase is considered as the most important because it determines the seed's development, it is a stage of intense metabolic activity for the genes expression and the synthesis of enzymes which hydrolyze the nutrient reserves used for the seedling development, and finally the last stage is relative to the radical's emergence which foregoes the seedlings establishment (Bewley, 1997; Footitt *et al*., 2006 ; Finkelstein *et al*., 2008). However, the chemical composition of the Cedrus atlantica essential oils has done the object of several studies. The cedarwood essential oil derived from Morocco is in general largely dominated by the α-, β- and γ-himachalenes, respectively (5.72 - 15.8%; 14.62 - 39.7% and 4.8 - 9.5%). Other sesquiterpenes were also reported as (E) -α-atlantone (up to 30.8%), the deodarone (up to 7.7%), the himachalol (up to 7.1%), the (Z) -α- atlantone (up to 5.9%) and the (E) -γ- atlantone (up to 4.2%) (Aberchane *et al*., 2001; Aberchane *et* *al*., 2003; Aberchane *et al*., 2004; Satrani *et al*., 2006). Concerning the Cedrus atlantica essential oils needles (Morocco), they are dominated by α-pinene (14.85%), followed by himachalene (10.14%), β-himachalene (9.89%), γ-himachalene (7.62%), cis-α-atlantone (6.78%) himachalol (5,26%), α-himachalene (4,15%), germacrene D (3.52%), β-caryophyllene (3.14%), cadinene (3.02%), β-pinene (2.35%), humulene (2.30%) and copaene (2.26%) (Derwich *et al*., 2010).

In fact, a first study was conducted in Morocco on Atlas cedar seeds essential oils samples into dormancy coming from the Moroccan High Atlas has shown that the manool is the main constituent of these gasolines with a content of 49.22% followed by the α-pinene with 41.02% (Rhafouri *et al*., 2014). Thanks to these important results, we are interested in deepening our scientific researches to show the chemical variability of the *Cedrus atlantica* seeds essential oils, depending on the evolution of their germination stages and this is for a better valuation of the reproductive organ of the Moroccan Atlas cedar.

MATERIALS AND METHODS

Plant Material

The collecting cones of *Cedrus atlantica*, was conducted by the Regional Station of Azrou Forest Seed in October 2014 in the Eastern Middle Atlas. After a soaking for 48 hours into the water, they were manually disarticulated, then dried in the sun. The seed's extraction taken from cones was performed using an apparatus consisted of an extraction drum and a densimetric table. After that, the seeds are kept in tightly sealed plastic bags and stored in a refrigerator at 3°C temperature.

Methods

Germination procedure

A sample of 1.8 kg of Atlas cedar seeds was soaked during 48 hours in tap water, and then placed in a tray and settled in the cold room at 4°C during 28 days. These seeds are removed from the freezer and placed in blotting paper boxes. Three repetitions were realized at the rate of 600g per box. These boxes were deposed in a germoir type Rumed, set at 20 ± 1°C temperature with a relative humidity of 85% and during 16 hours under light. The seeds development in seedling comprises several physiological successive phases, starting by seed imbibing during 48 hours (Phase 1), followed by the micropyle opening step and the emergence of the radical which spread over a period of 15 days marking the end of the germination process (Phase 2), then by the development and the evolution of the epicotyl (stalk) in 20 days (Phase 3) and finally the stage of leaves deployment during 40 days (Phase 4). Test samples are taken according to the different stages of seed germination (Fig1).

Essential oils extraction

The essential oils obtaining of cedar seeds depending on the germination stage, was carried out by hydrodistillation in a

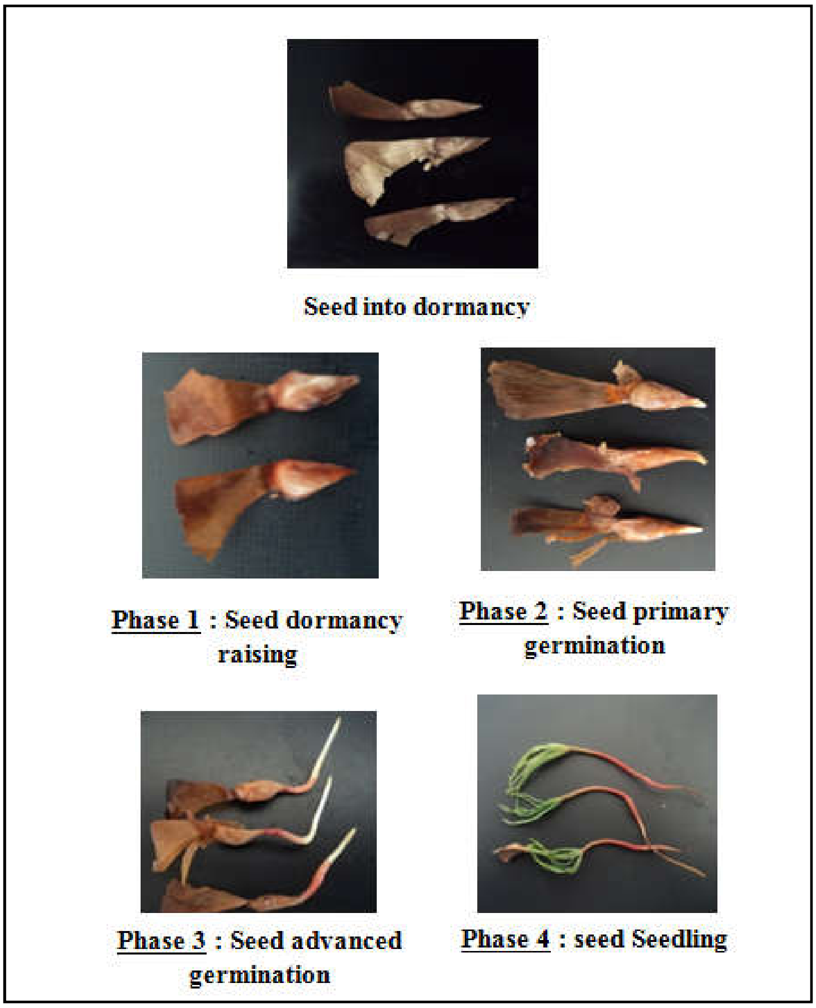

Fig. 1. Photos showing the *Cedrus atlantica* germination seed stages

Clevenger type apparatus (Clevenger, 1928). The applied method is the one described in the European Pharmacopoeia in 2008 (PE, 2008) and according to the recommendations of the 2008 French Agency for Sanitary Safety of Health Products (AFSSPS, 2008). Three distillations of 200 g of material plant were carried out during 4 hours. The essential oil was stored at 4°C in the obscurity with the presence of anhydrous sodium sulfate. Then, it is diluted in the methanol (1/20 v/v) before the chromatographic analysis operations by GC / FID and GC/MS according to the AFNOR standard (AFNOR, 2000).

Chromatographic Analysis

The Gas Chromatography (GC) analysis were performed using a Hewlett Packard Gas Chromatographer (HP 6890) with electronic pressure control, equipped with a HP-5MS capillary column (30 m x 0.25 mm, film thickness : 0.25 µm), a FID detector set at 250°C and using a H_2/Air mixture, and a *split-splitless* injector set at 250 °C. The injection mode was split (split ratio: 1/50, flow rate : 66 $ml.min^{-1}$) and the injected volume was about 1 µl. Nitrogen was used as carrier gas with a flow rate of 1.7 ml. min^{-1}. The column temperature was programmed from 50 to 200°C at a heating rate of 4°$C.min^{-1}$, during 5 min. The apparatus was controlled by a “Chemstation” computer system.

The Gas Chromatography/Mass Spectrometry (GC/MS) analysis were performed by a Hewlett-Packard gas chromatographer (HP 6890) coupled with a mass spectrometer (HP 5973). The fragmentation was performed by electron impact at 70 eV. The column used was HP-5MS (30 m x 0.25 mm, film thickness: 0.25 µm). The carrier gas is helium whose flow is fixed at 1.5 ml. min^{-1}. The injection mode was split (split ratio: 1/70, flow rate112 ml min^{-1}). The column temperature was programmed from 50 to 200 °C at a heating rate of 4 °$C.min^{-1}$, during 5 min. For the chromatographic analysis, essential oils were diluted in methanol (1/20 v/v). The identification of the components is based on the comparison of their mass spectra (GC/MS), respective with spectra of the library (NIST 98), of the bibliography (Adams, 2007) and on the basis of calculation of Kovàts indices (Kovàts, 1965).

RESULTS AND DISCUSSION

Yield and chemical composition

The yields of the cedar seeds essential oils of Eastern Middle Atlas, in function of seed germination stages (Seed into dormancy (SD), in dormancy raising (SDR), in primary germination (SPG), in advanced germination (SAG) and in

Table 1. Chemical composition of the *Cedrus atlantica* essential oils seeds in function of their germination stages

Kovats Indices (KI)	Compound	% SD*	% SDR*	%SPG*	% SAG*	% SS*
869	Isopentyl acetate	tr	tr	0,14	tr	tr
921	tricyclene	0,14	0,12	0,11	0,11	0,05
924	α-thujene	0,06	0,05	0,05	0,05	Tr
932	α-pinene	72,19	66,09	60,74	58,95	53,35
946	camphene	1,20	1,82	2,02	2,54	4,05
969	sabinene	0,67	tr	tr	tr	tr
974	β-pinene	6,23	5,70	5,83	5,94	6,35
988	myrcene	4,08	3,17	2,95	5,11	5,16
1002	α-phellandrene	0,10	tr	tr	0,64	tr
1014	α-terpinene	0,05	-	-	-	-
1020	p-cymene	0,05	0,11	0,10	0,12	0,15
1024	limonene	2,92	2,85	2,70	2,45	3,35
1054	γ-terpinene	0,07	-	-	-	-
1086	terpinolene	0,25	tr	tr	tr	tr
1089	p-cymenene	-	tr	tr	0,05	0,05
1095	linalool	0,30	0,40	0,47	0,42	0,44
1098	trans- sabinene hydrate	0,19	0,32	0,39	0,34	0,34
1099	α-pinene oxide	-	0,59	0,47	0,57	0,50
1102	perillene	-	tr	0,17	0,19	0,27
1111	6-comphenol	2,77	2,97	2,88	3,08	4,13
1122	α-compholenal	tr	0,06	0,06	0,07	0,26
1135	trans-pinocarveol	-	0,75	0,80	0,83	0,92
1139	cis-pinan-2-ol	tr	0,16	0,52	0,07	0,12
1141	camphre	tr	0,10	0,06	0,18	tr
1155	isoborneol	tr	0,27	tr	tr	tr
1160	pinocarvone	0,12	0,17	0,17	0,15	0,17
1165	borneol	tr	0,51	0,57	0,57	1,44
1174	terpinen-4-ol	0,43	0,58	0,30	0,35	0,64
1183	cryptone	0,31	0,14	0,13	0,15	0,15
1186	α-terpineol	0,18	0,47	0,14	0,45	0,46
1195	myrtenal	tr	tr	0,10	0,12	0,08
1204	verbenone	tr	0,08	0,73	0,46	0,44
1218	endo-fenchyl acetate	tr	0,05	0,40	tr	0,14
1235	Myrtenyl acetate	tr	tr	0,30	0,12	0,72
1239	carvone	-	tr	0,09	0,10	0,16
1249	piperitone	0,48	0,08	0,10	0,08	0,13
1287	bornyl acetate	3,46	5,24	5,34	5,94	7,02
1359	neryl acetate	0,17	0,18	0,21	0,20	0,19
1379	geranyl acetate	0,06	tr	tr	0,06	0,19
1454	trans beta-farnesene	1,05	1,21	1,47	1,52	1,62
2056	manool	1,90	2,02	2,65	2,79	2,88
	Total	99,64	96,48	93,28	94,88	95,99

*SD : Seed into dormancy ; *SDR : Seed dormancy raising ; *SPG : Seed primary germination ;
*SAG : Seed advanced germination ; *SS : Seed seedling ; tr : Trace (≤ 0,04%) ; - : absent

seedling (SS)), are respectively : 1.45 ; 0.9 ; 0.8 ; 0.7 and 0.28%. We observe that the essential oil content decreases in function of the germination seed advancement. This can be explained by the rapid increase in seed weight during the development stages, generated by their absorption of the necessary moisture in the germination triggering metabolisms. In fact, the essential oil rate for cedar dormancy seeds of the Eastern Middle Atlas (1.45%) is lower than that obtained for the origin of the High Atlas (Morocco) (2.6%) (Rhafouri *et al*., 2014) and also of that of Djurdjuran region in Algeria (1.7%) (Boudarene *et al*., 2004). The chromatographic analysis of the Cedrus atlantica seeds essential oils of different germination stages, have permitted to identify 36 compounds for the (SD) step and 39 compounds for other phases (SDR, SPG, SAG and SS), representing respectively 99.64; 96.48; 93.28; 94.88 and 95.99% of the total composition of these oils (Table 1).

It comes up from these chromatographic analyses that the essential oils of Atlas cedar seeds are mainly composed of the α-pinene (53.35 - 72.19%), accompanied with other components at varied rates: β pinene (5.70 - 6.35%), myrcene (2.95 - 5.16%), bornyl acetate (3.46 - 7.02%), limonene (2.45 - 3.35%), 6-comphenol (2.77 - 4.13%) and the manool (1.9 - 2.88%). It is worth noting the existence of other components in weak quantities. The study led by Boudarene et al. (Boudarene *et al*., 2004), has shown that the principal constituents of the Atlas cedar seeds essential oils from Algeria of both regions Oulad Yacoub and Tala Guilef are : α-pinene (37.1 - 5.5%), β-pinene (8.6 - 1.9%), myrcene (3.6 - 0.6%), limonene (2.5 - 0.6%), bornyl acetate (5.4 - 4.0%), (E) -β-farnesene (6.8 - 1.9%) and the manool (8.3 - 20.7%). The coming up of these counting, is obvious in the chemical composition of seeds into dormancy.

These latter, coming from Morocco, are richer in the α-pinene with a rate of 72.19% against only 37.1% for ones of Algeria (region Oulad Yacoub). It should be also noted that the seeds essence originating from Algeria is different from that of Morocco in quantity plan of certain components, such as : β-pinene (8.6 against 6.23%), bornyl acetate (5.4 against 3.46%), (E) -β-fernasene (6.8 against 1.05%) and the manool (8.3 against 1.9%). This observed difference between the chemical composition of the *Cedrus atlantica* seeds essential oils from Morocco and that of Algeria, could be explained by climatic conditions and the specific geographical factors to each region (Ghanmi *et al*., 2007; Mansouri *et al*., 2010). The high content of α-pinene (72.19%) of essential oils extracted from Atlas cedar seeds into dormancy is close to that of essential oil of *Juniperus phoenicea* ssp fruit. Lycia (79.08%) (Mansouri *et al*., 2011). Furthermore, we note the decrease in α-pinene during the evolution of seeds germination process passing from 72.19% for SD up to 53.35% for the SS. This remark may be explained by the involvement of α-pinene in the biosynthetic pathway of other molecules. In fact, studies (Shukla *et al*., 1968; Savithiry *et al*., 1998; Yoo *et al*., 2002) have shown that the α-pinene is a precursor of several training monoterpene compounds, such as: the pinocarveol, the limonene, the p-cymene, etc. The analysis of these results has also allowed noticing a variation in other compounds rates along the evolution of seed germination. In fact, the content of certain components has known an increase since the seed into dormancy phase until the seedling stage to be known: the bornyl acetate (from 3.46 to 7.02%), the camphene (from 1.20 to 4.05%), the 6-comphenol (from 2.77 to 4.13%), the myrcene (from 4.08 to 5.16%), the manool (from 1.9 to 2.88%) and the limonene (from 2.92 to 3.35%).

We also note that certain compounds which were absent or in the trace state, in dormancy stage, had appeared during the course of germination evolution till the seed seedling phase. We mention the borneol (1.44%), the trans-pinocarveol (0.92%), the oxide α-pinene (0.5%) and the perillene (0.27%). On the contrary, constituents present in dormancy stage such as the sabinene (0.67%), the terpinolene (0.25%), the γ-terpinene (0.07%) and the α-terpinene (0.05%), have become absent or in traces in the other germination phases. In general, the absence or the presence of some constituents can serve as indicators of each germination phase (Ghanmi *et al*., 2005). Concerning the chemical classes of the seeds essences of *Cedrus atlantica* (SD, SDR, SPG, SAG and SS), they are largely dominated by monoterpene hydrocarbons followed by alcohols and monoterpene esters (Table 2).

It resorts from these results that the rate of monoterpene hydrocarbons diminishes in the course of the evolution of the seeds germination from dormancy to seedling being respectively 88.00 and 72.51%. Consequently, the rates of other chemical classes of compounds have known a growing during these different stages, especially the monoterpene alcohols class (3.95 - 9.57%) and monoterpene esters (3.72 - 8.27%). The gasoline chemical composition of *Cedrus atlantica* seeds, varies in function of the evolution of the seed germination from the dormancy phase to the seedling one, which could play an important role in this germination. In fact, some terpenes will participate in particular to the seeds germination inhibition (Fischer *et al*., 1994; Tarayre *et al*., 1995).

Table 2. The chemical classes of identified compounds in the essential oils of Atlas cedar seeds

Compounds chemical class	Identified compounds number		Class chemical composition in function of the germination stages (in %)				
	Seed into dormancy (SD)	Seed of different stages after the dormancy raising	SD	SDR	SPG	SAG	SS
monoterpene hydrocarbons	13	12	88.00	79.98	74.59	75.98	72.51
Sesquiterpene hydrocarbons	1	1	1.05	1.21	1.47	1.52	1.62
monoterpene alcohols	8	9	3.95	6.59	6.91	7.03	9.57
diterpenic alcohol	1	1	1.9	1.85	1.83	1.91	1.88
monoterpenic esters	6	6	3.72	5.55	6.41	6.38	8.27
monoterpene cetones	5	6	0.96	0.59	1.27	1.11	1.02
monoterpene aldehydes	2	2	0.05	0.08	0.16	0.19	0.35
monoterpene oxides	0	2	0	0.62	0.64	0.76	0.77
Total	36	39	99.64	96.48	93.28	94.88	95.99

Some hormones which control seed dormancy, such as : the Abscisic acid (ABA) (Finch-Savage *et al*., 2006; Owen *et al*., 2005) or the triggering of the seeds germination process, such as : the gibberellins (Yamaguchi, 2008), are synthesized from different chemical reactions involving the terpenes (Chen *et al*., 2011 ; Pichersky *et al*., 2006). In addition, various studies of allelopathic power, have shown that the terpenes have an effect on the inhibition of seeds germination (Fischer *et al*., 1994; Tarayre *et al*., 1995; Tworkoski, 2002; Singh *et al*., 2006; Kaur *et al*., 2010; Hanana *et al*., 2014). However, studies conducted by several authors, show that the terpenes chemical composition nature in plants is modeled by some biotic and abiotic factors (Letchamo *et al*., 2001 ; Sudha *et al*., 2002 ; Naghdi-Badi *et al*., 2004 ; Penuelas *et al*., 2005).

Therefore, the biosynthesis of chemical compounds of gasoline seeds, make an essential part of direct and indirect defenses systems against the pathogens. In fact, the terpenes can act in the direct defense towards the bacteria, the fungi and the insects (Stoessl *et al*., 1976; Huber *et al*., 2004; Bohlmann *et al*., 2008). Furthermore, the essential oils seeds, dominated by the monoterpenes, can contribute for the defense against the free radicals coming up from the endogenous and exogenous stress. The results obtained by J.W. Chen et al. (Chen *et al*., 2009) show that the inhibition of the monoterpenes synthesis in *Hevea brasiliensis*, accelerates the oxidative stress in this species by increasing levels of hydrogen peroxide and that of malondialdehyde, products of oxidative degradation of polyunsaturated fatty acids. Moreover, other studies have shown that the monoterpenes may physically stabilize the thylakoid membranes, at high temperature (Sharkey *et al*., 2001) or detoxify reactive oxygen species, as the ozone, which can damage the membranes (Loreto *et al*., 2001).

Conclusion

The essential oils of *Cedrus atlantica* seeds, obtained by steam distillation of the seeds during the development stages (SD, SDR, SPG, SAG and SS), have provided variable yields: 1.45; 0.9; 0.8; 0.7 and 0.28%. Furthermore, the chemical composition, carried out by chromatographic analysis (GC/ FID and GC/ MS), of the essential oils of *Cedrus atlantica* seeds (SD) and (SDR, SPG, SAG, SS), have permitted to identify respectively 36 and 39 compounds. These essences are dominated by the α-pinene compound (72.19 - 53.35%). The evolution of the chemical composition of essential oils of seeds, is modulated by various endogenous and exogenous factors. The variation of the seeds essence is presupposed, to have specialized functions associated to the pathway participation of the biosynthesis hormone that inhibit or induce the germinal process on one hand and on the other hand, to the seed's preservation against the endogenous attacks, such as : the free radicals and / or exogenous, such as : the pathogenic germs.

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