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Effect of the harvest stage on the chemical composition and bioactivity of Moroccan *Artemisia herba alba* essential oils

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ABSTRACT

The richness and diversity of the Moroccan Saharan flora constitutes a veritable phytogenetic reservoir with a flora that includes more than 500 aromatic and medicinal species, many of which remain unexploited. Among these plants we find *Atremisia herba alba* in the region of Guelmim (Sahara of Morocco) which has a great potential in phytomass and which deserves to be better valued. The objective of this work is the comparative study of the yield and chemical composition of the essential oil derived from *Artemisia herba-alba* in Guelmim region (Sahara of Morocco) according to the date of harvest as well as the determination of its antibacterial and antifungal activity towards eleven microorganisms. Essential oils yields expressed in ml compared with 100 g of dry matter of *Artemisia herba-alba* harvested in March, June, September and December (2015) are respectively 1.62; 0.95; 0.64 and 1.85 %. Chromatographic analyses of essential oils revealed the predominance of camphor, cis-β-terpineol, camphene, trans-thujone and trans-verbenol whose rates vary according to the plant phenological stage. The essential oil of *Artemisia herba-alba* collected during March was the most active against all microorganisms tested.

1. Introduction

Since antiquity, plants have served as a natural drug library and pragmatic for humans. No one wanted to know why or how they acted, but it was an undisputed fact that seemed magical. Indeed, it is surprising that a leaf, a flower or a rot could cure or at least relieve a pathological state or organic disorders (Schauenberg & Ferdinand, 2006). Currently, the aromatic and medicinal plants (AMP) have created much interest in the therapeutic field. Knowledge of the natural substances extracted from these plants, in particular essential oils, have always occupied an important place in man daily life since prehistoric man used them already to perfume, to flavor food or even to heal (Robert, 2000).

The particular geographical Moroccan position as Northwest point of Africa continent with the coast bathed by Atlantic ocean and the Mediterranean sea, has a varied climate resulting from the combined effects of oceanic, Mediterranean and Saharan influences (Knippertz *et al.*, 2003; Weisrock *et al.*, 2006; Fennane & Ibn Tattou, 2008; Ghanmi *et al.*, 2014). This climatic and geographical variability is at the genesis origin of an exceptional biological diversity in ecosystems, species and ecotypes. Moreover, the vascular flora of Morocco comprises 155 families, divided into 5211 species and plant species of which 452 subspecies type antonymous, 872 additional subspecies and 918 genera. Of the 5211 existing species and subspecies, 951 (18 %) are endemic (Amvam Zollo *et al.*, 1998). Morocco is a traditional producer of AMP, it is an exclusive supplier of several essential oils, like the case of the *Artemisia herba-alba*, the wild chamomile the rosemary, the thyme, the annual tansy, etc. (Ghanmi *et al.*, 2014). The application importance manifested in the eighties of the last century by some local and foreign processing industries encouraged the intensification and the exploitation of spontaneous AMP; that makes Morocco the biggest producer of some aromatic and medicinal plants of essential oils, such as the *Artemisia* genus (Ghanmi *et al.*, 2011; Ghanmi *et al.*, 2014).

Artemisia herba-alba "Chih" belongs to the *Artemisia* genus, which contains more than 400 species (Mucciarelli & Maffei, 2002; Ghanmi *et al.*, 2011), distributed over the five continents. In Morocco, it is represented by thirteen species, among the most important ones, is *Artemisia herba-alba*. This seems of great economic interest to Morocco, which holds 90 % of the world market for the essential oil extracted from this plant (Ghanmi *et al.*, 2011). The *Artemisia herba alba* is found throughout the Mediterranean and develops frequently in clayey steppes, rocky and earthy pastures of dry region’s lowlands mountains and generally in areas with arid and semi-arid bioclimatic hot variants, tempered and fresh at the levels of thermomediterranean and mesomediterranean vegetation (Aafi *et al.*, 2002; Vernin *et al.*, 1995). This herbaceous plant 30-60 cm tall with numerous and tomentos stems, very leafy with a thick stump. In Morocco, it is encountered in the Oriental regions, Eastern

Rif, Middle Atlas, High Atlas and Saharan Anti-Atlas (Aafi *et al.*, 2002). Plant picking begins during the flowering period that depends on the region and extends from April to September.

Generally, it has been reported through scientific work that the *Artemisia* genus is rich with secondary metabolites, such as flavonoids, coumarins, essential oils, sterols and acetylene (Kundan & Anupam, 2010). Thus, the essential oils of *Artemisia herba alba* were the subject of several studies in Morocco (Benjilali *et al.*, 1982; Lawrence, 1993), Spain (Salido *et al.*, 2004; Akrout, 1999) and Algeria (Vernin & Merad, 1994; Vernin *et al.*, 1995). Indeed, the essential oil contained in the leaves of the *Artemisia* genus is known for its regulatory properties of the menstrual cycle and as a remedy for many diseases, such as diabetes, bronchitis, abscesses and diarrhea (Akrout *et al.*, 2001; Mirjalili *et al.*, 2007). The region of Guelmim (Sahara of Morocco) which has a great potential in phytomass of *Artemisia herba alba*. Furthermore, to our knowledge, no work has been done before on the chemical quality and bioactivity of *Artemisia herba alba* essential oils in Guelmim region (Sahara Morocco).

During this study, we were interested in monitoring the influence of harvest date on the yield and chemical composition of white wormwood's essential oils in Guelmim region (South of Morocco) as well as their antibacterial and antifungal activity had been estimated against four bacterial strains, three molds and four reference woods rot fungi.

2. Materials and methods

Plant material

The samples of white armoise (aerial part) were collected during the four seasons (March, June, September and December) during 2015 in the Guelmim region in South Morocco, according to AFNOR standard (AFNOR, 2000). The plants were taxonomically identified at the Forestry Research Center in Rabat (Laboratory of Botany, Vaucher Number: **Ar036/2015**).

Obtaining mode of essential oils

The essential oils production was carried out by hydrodistillation in a Clevenger-type apparatus (Clevenger, 1928). The method applied is which described in the European Pharmacopoeia (2008) and according to the 2008 recommendations of the French Agency for health products safety. Three replicates were carried out for each season for *Artemisia herba alba*. The yields (w/w) of essential oils were determined based on the dry weight of the plant materials in a well-ventilated area with a temperature not exceeding 35°C. Essential oils were dried over anhydrous sodium sulfate and stored under refrigeration (4 °C). It is then diluted in hexane (1/20 v/v) before analysis by GC and GC/MS according to the AFNOR standard (AFNOR, 2000).

Chromatographic analysis

Analysis of essential oils Gas chromatography (GC) analysis was 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 fed with a H_2/Air mixture, and a split splitless injector set at 250 °C. The injection mode was split (1:50) and the injected volume was 1µl. 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°$C.min^{-1}$. The apparatus was controlled by a Chemstation" computer system.

Gas chromatography/mass spectrometry (GC/MS) analysis was performed using a Hewlett-Packard Gas Chromatographer (HP 6890) coupled with a mass spectrometer (HP 5973). 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 injection mode was split (1:50). The column temperature was programmed from 50 to 200 °C at a heating rate of 4°$C.min^{-1}$.

The components of the essential oils were identified based on Kováts retention indices (Kovàts, 1965) and mass spectral database (NIST 98 library) (Jalali & Sereshti, 2007).

Microorganisms studied

The microorganisms studied were selected for their high frequency of contamination and pathogenicity.

Eleven microbial strains (below) were chosen for their pathogenicity and their frequent involvement in food contamination.

- Bacteria: *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus*.
- Fungi: *Aspergillus niger*, *Penicillium digitatum* and *Penicillium expansum*.
- Wood rot fungi: *Gloeophyllum trabeum, Poria placenta, Coniophora puteana* and *Coriolus versicolor.*

The bacterial strains are lots of "American Type Culture Collection" ATCC, they are deposited by transplanting on favorable nutrient agar for growth and the molds are grown on the nutrient medium PDA (Potato Dextrose Agar). The technical used, is the dispersion of EO in agar agar at 0.2 %.

The minimum inhibitory concentrations (MIC) of essential oil *Artemisia herba alba* are determined according to the reported method by Remmal *et al.*, (Remmal *et al.*, 1993) and Satrani *et al.*, (Satrani *et al.*, 2001).

3. Results and discussion

Yield and chemical composition of Artemisia herba alba essential oils

The average yields of essential oils from the samples of Guelmim *Artemisia herba alba* were expressed in ml compared to 100 g of aerial part dry plant.

The samples of the *Artemisia herba alba* harvested in December have provided the best rate in essential oil (1.85 ± 0.02 %) compared to those obtained during the March, June and September collects : respectively of 1.62 ± 0.03 %, 0.95 ± 0.02 % and 0.64 ± 0.02 %.

These seasonal variations in yields could be explained by the nature of the study area climate where there is significant rainfall during the December month. Indeed, the Guelmim region climate is said to be desert. The climate map of Köppen-Geiger classifies the climate as being of type hot desert climate (BWh). Over the year 2014, the average temperature is 18.9°C. The June month is the driest. However, the annual precipitation average reaches 119 mm and December has the highest precipitation rate with an average of 26 mm (M.I., 2015).

Moreover, this high level of essential oil obtained during the December month for the Guelmim *Artemisia herba alba* was also found for the Guerçif same species (eastern region) with 1.23 % (Ghanmi *et al.*, 2010). This essential oil important content supplies during the December month was also observed for the wormwood species: *Artemisia arborescens*, *Artemisia judaica* and *Artemisia sieberi* with respectively 0.3 %, 0.4 % and 1.7 % (ml/100g) (Abdelgaleil *et al.*, 2007; Ghasemi *et al.*, 2006). However, this essential oil level obtained for Guelmim *Artemisia herba alba* remains relatively low compared to the same species collected in Oued Asla (Laayoun East, Oriental-Morocco) which was 2.03 % (Ourid *et al.*, 2016a) and also for another *Artemisia* species (*Artemisia haussknechtii*) with 2.1 % yield (Jalali *et al.*, 2007).

Table1. Collection date effect on the armoise essential oil chemical composition in the Guelmim region

No	KI	component	M (%)	J (%)	S (%)	D (%)
1	921	tricyclene	-	-	-	1.12
2	924	α-thujene	0.12	0.27	0.05	0.06
3	928	α-pinene	0.16	0.98	0.53	0.07
4	**943**	**camphene**	**5.55**	**7.26**	**9.74**	**9.41**
5	951	thuja-2,4(10)-diene	-	0.07	-	-
6	966	sabinene	0.27	0.1	0.43	0.07
7	988	myrcene	1.16	2	0.4	4.92
8	1007	δ-3-carene	0.23	-	-	-
9	1015	α-terpinene	0.58	0.36	-	-
11	**1025**	**β-phellandrene**	**14.3**	**13.35**	**21.66**	**18.29**
12	1049	γ-terpinene	1.05	0.44	0.42	0.08
13	1060	Artemisia ketone	0.79	0.53	0.18	0.19
14	1073	cis-sabinene hydrate	0.2	1.5	0.20	0.12
15	1079	alcohol of Artemisia	0.18	-	-	-

16	1098	cis-thujone	0.25	0.28	1.23	0.52
17	**1108**	**trans-thujone**	**1.88**	**0.13**	**0.27**	**20.68**
18	**1144**	**cis-β-terpineol**	**28.55**	**18.5**	**4.07**	**0.18**
19	**1145**	**trans-verbenol**	**14.78**	**16.08**	**3.23**	**0.2**
20	**1145**	**camphor**	**6.85**	**25.93**	**46**	**35.39**
21	1149	trans-dehydro-α-terpineol	5.47	1.37	-	-
22	1157	cis-dehydro-β-terpineol	1.59	0.08	3.32	2.58
23	1162	cis-dehydro-α-terpineol	2.81	2.1	2.12	0.14
24	1166	δ-terpineol	0.62	-	-	-
25	1171	acetate of artemis	2.77	0.77	1.24	0.17
26	1175	terpin-4-ol	0.14	-	-	-
27	1182	thuj-3-en-10-al	0.44	0.03	0.03	1.09
28	1189	α-terpinèol	0.06	1.38	1.88	1.34
29	1201	γ-terpineol	0.29	0.23	1.01	0.12
31	1224	cis-hydrate sabinene acetate	0.19	0.28	-	-
32	1230	nor-Davanone	0.18	-	-	1.25
33	1244	trans acetate of chrysanthenyl	0.22	0.27	-	-
34	1255	trans hydrate sabinene acetate	0.32	0.28	0.32	0.29
35	1276	neoiso-3-acetate of thujanol	0.45	0.36	0.22	0.14
36	1479	γ-muurolene	2.15	0.33	-	-
37	1494	trans-muurola-4(14), 5-diene	1.22	0.34	0.29	0.07
38	1515	γ-cadinene	0.40	-	-	0.06
39	1557	davanone B	0.17	-	-	-
40	1577	caryophenic alcohol	0.35	-	-	-
41	1585	davanone	0.14	-	-	-
42	1605	trans-β-elemenone	0.75	0.20	-	-
		Total	**97.63**	**95.80**	**98.84**	**98.55**

KI: Kovats Index; M: March; J: June; S: September; D: December; -: absent; %: Area peak

The essential oils chromatographic analyzes interpretation of the Guelmim region *Artemisia herba alba* have revealed the presence of 40 compounds for the March collection against 32 ; 25 and 28 constituents for those harvested respectively in June, September and December (Table 1). These constituents represent about 97.63; 95.80; 98.84 and 98.55 % of all plant EO collected respectively during March, June, September and December.

Generally, the camphene, the β-phellandrene, the trans-thujone, the cis–β-terpineol, trans-verbenol and camphor are the main compounds of *Artemisia herba alba* in the Guelmim region (southern Morocco). The camphene and the β-Phlelandrene present in this species essential oils with percentages slightly stable for the four seasons (respectively 5.55 to 9.74 % and 13.35 to 21.66 %). However, we note that the Guelmim *Artemisia herba alba* essential oils obtained during the four collection periods show qualitative and quantitative differences. Indeed, each *Artemisia herba alba* collection season is distinguished by certain chemical constituents' dominance. Thus, March collection is characterized by cis-β-terpineol (28.55 %) and trans-verbenol (14.78 %), while in June, camphor is found (25.93%). In addition, to cis-β-terpineol and trans-verbenol (18.5 and 16.08 %, respectively). Whereas the September crop can be a source of camphor at 46 %, the December crop is rich in trans-thujone (20.68 %) and camphor (35.39 %).

It can be deduced from its results that one predominance or more of these four constituents: trans-thujone, cis-β-terpineol, trans-verbenol and camphor can serve as a specific chemical map to distinguish between the different phenological stages of The Guelmim white wormwood. In addition, this Guelmim wormwood is characterized by the chrysanrhenone complete absence, which is a constituent present in most *Artemisia herba-alba* essential oils (Ghanmi *et al.*, 2010; Ourid *et al.*, 2016b; Fleisher *et al.*, 2002).

In addition, the chemical composition of the Guelmim *Artemisia herba alba* essential oils (Southern Morocco) is different from that of Matmata (Tunisia), which consists mainly of α-thujone (43.85 %), trans sabinyl-acetate (17.46 %) and β-thujone (10.10 %) together with 1,8-cineole (3.30 %), chrysanthenone (2.32 %) and chrysanthenyl acetate (3.93 %) (Akrout, 1999). It is largely different from that of the M'sila region (Algeria) which is dominated by camphor (19.4 %), trans-pinocarveol (16.9 %), chrysanthenone (15.8 %) and B-thujone (15 %) (Charchari *et al.*, 1996). Similarly, the essence of the Jordan *Artemisia herba alba* has a different chemical profile than the Guelmim wormwood with α- and β-thujones (16.2 % and 8.5 % respectively), alcohol santolina (13.0 %), Artemisia ketone (12.4 %), trans-sabinyl acetate (5.4 %), D-germacrene (4.6 %), α-eudesmol (4.2 %) and caryophyllene acetate as major compounds (5.7 %) (Hudaib & Aburjai, 2006). As for the essential oils of the Guelmim *Artemisia herba alba* collected in September, they are similar to those of Algeria, Spain and Israel, which present camphor as the main compound with percentages between 15 and 68 % (Feuerstein *et al.*, 1988; Fleisher *et al.*, 2002; Vernin *et al.*, 1995). Previous studies done on *Artemisia herba alba* have revealed the other major compounds presence such as α-thujone acetate (25.6-40.9 %) (Boutekedjiret *et al.*, 1992; Fleisher *et al.*, 2002; Lawrence, 1995; Paolini *et al.*, 2010; Ourid *et al.*, 2016a), β-thujone (44 %) and davanone (18.1-51.2 %) (Benjilali *et al.*, 1982), chrysanthenone (54.5 %), camphor (22 %) (Boutekedjiret *et al.*, 1992; Paolini *et al.*, 2010; Ghanmi *et al.*, 2010; Ourid *et al.*, 2016a; Benjilali *et al.*, 1982), 1,8-cineole (3-50%), cis-chrysanthenol (24.5-30 %) (Feuerstein *et al.*, 1986) and cis-chrysanthenyl acetate (69 %) (Feuerstein *et al.*, 1988). We also mention that the davanone presence in samples from Spain (Salido *et al.*, 2001; Salido *et al.*, 2004). All this shows that the chemical composition of the essential oil of the *Artemisia herba alba* is too variable according to the plant phenological stage and the harvest origin.

Antimicrobial and antifungal activity of the *Artemisia herba alba*'s essential oil

In our investigations, the antimicrobial activity was studied by observing the inhibitory power of our samples *Artemisia herba alba* essential oils at different concentrations on the bacteria and fungi to be tested. The results are shown in Table 2.

Table 2. Antimicrobial activity of *Artemisia herba alba* essential oils according to the vegetative stage.

	1/100				1/250				1/500				1/1000				1/2000				1/3000				T
	M	**J**	**S**	**D**	**M**	**J**	**S**	**D**	**M**	**J**	**S**	**D**	**M**	**J**	**S**	**D**	**M**	**J**	**S**	**D**	**M**	**J**	**S**	**D**	
													Bacteria												
Ec	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Ml	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Sa	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Bs	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
													Molds												
An	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pex	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pd	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
													Wood rot fungi												
Cv	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Cp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Pp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+
Gt	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+

+ : Growth, - : Inhibition, M : March, J : June, S : September, D : December, Ec : *Escherichia coli,* Bs *:Bacillus subtilis,* Sa *: Staphylococcus aureus*, Ml *: Micrococcus luteus*, An *: Aspergillus niger,* Pd *: Penicillium digitatum,* Pex : *Penicillium expansum,* Gt : *Gloeophyllum trabeum,* Pp : *Poria placenta,* Cp *: Coniophora puteana,* Cv : *Coriolus versicolor.*

It is noted that the essential oils of Guelmim *Artemisia herba alba* have been active and have inhibited all microbial strains tested. With regard to the bacteria, the wormwood 's essential oil harvested during the period of March was the most effective and the growth of all the strains was stopped at the low concentration of 1/1000 v/v. Then comes in second, the wormwood's essential oil of June that inhibited all the bacteria to the concentration of 1/500 v/v.

With regard to the molds tested, only the essential oil of the March inhibited their growth at 1/500 v/v. As for the bioactivity of the June essential oil, *Aspergillus niger* was the most sensitive with an inhibition concentration of 1/500 v/v whereas *Penicillium expensum* and *P. digitatum* were inhibited at 1/250 v/v. At this essential oil's last concentration, all the molds were inhibited by the white wormwood's essences of September and December.

In the case of wood rot fungi, all the strains tested were sensitive to the *Artemisia herba alba*'s essential oil harvested in March even at the lowest concentration of 1/1000 v/v. The *Poria placenta* strain was the most vulnerable and its growth was inhibited at a very low rate of *Artemisia herba alba* essential oil (1/2000 v/v) in March and June. With regard to the *Artemisia herba alba* essential oils of September and December, the strains *Coriolus versicolor* and *Gloeophyllum trabeum* resisted to the concentration of 1/500 v/v whereas *Coniophora puteana* and *Poria placenta* were inhibited at 1/1000 v/v.

Following these results, it can be observed that the white wormwood's essential oil of March followed by that of June showed a great inhibitory power against all the microorganisms tested. This great anti-bacterial and antifungal activity of essential oils can be explained by their richness in terpene alcohols. Indeed, the white wormwood's essential oils of March and June contain 54.90 % and 40.10 % respectively, while those of September and December, only contain low respective rates of 15.80 % and 4.70 % (Table 3).

Table 3. Main chemical families of the white wormwood's essential oils in Guelmim according to the harvest period

Harvest Month	Terpenic alcohols (%)	Terpene Hydrocarbons (%)	Terpenic ketones (%)
March	54.92	27.58	11.01
June	40.10	27.28	27.07
September	15.81	33.90	47.88
December	4.70	33.34	58.03

This is in agreement with several works which have shown that the essential oils antimicrobial properties of several aromatic and medicinal plants are attributed to their chemical profile and especially to terpene alcohols (Satrani *et al.*, 2006a; Satrani *et al.*, 2006b; Satrani *et al.*, 2008; Hassane *et al.*, 2011). Indeed, the works done by Kordali et al. (Kordali *et al.*, 2005a; Kordali *et al.*, 2005b) demonstrated that the species of *Artemisia santonicum* and *A. spicigera* whose essential oils contain higher levels of monoterpene oxygenates have more antimicrobial efficacy than those of *Artemisia dracunculus* and *A. absinthium*.

This study concludes that the white wormwood's essential oil collected during March as well as its main constituents can represent new bio-resources for the development of different antimicrobial specific products for the control of various pathogens in the medical and agri-food sectors.

4. Conclusion

In this work, the best yield of essential oil of *Artemisia herba alba* was obtained in the month of December (1.85 ± 0.02 %). Chromatographic and spectrometric analyzes revealed the existence of six major compounds, camphor, cis-β-terpeneol, β-phellandrene, trans-thujone, trans-verbenol and camphene whose rates vary according to the plant phenological stage.

The essential oils obtained from the *Artemisia herba alba* samples of the four harvest periods were shown to be active against all the strains of bacteria and molds tested, but it was that of March which showed the greatest antimicrobial efficacy. This work could contribute to valorise better this plant of southern Morocco for a better use in different industrial sectors.

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