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Coupling Ultrasound with Enzyme-Assisted Extraction of Essential Oil from Algerian *Artemisia herba-alba* Asso

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The composition of the essential oil (EO) of *Artemisia herba-alba* Asso, extracted by Hydro-Distillation (HD) and by coupling Ultrasound with Enzyme-Assisted Extraction (UE-AE) prior to HD from the plant's aerial parts were analyzed by GC-MS. Antibacterial, antifungal and antioxidant activities of the obtained EOs were evaluated. The yield of EO extraction after pretreatment of the desert wormwood leaves by coupling ultrasound with enzymes was in the range of 1.56%±0.07 compared to 1.01% ±0.08 in HD process; also, the total time necessary to complete EO extraction is 180min for HD and 120min for UE-AE. GC–MS profiling of the EOs showed changes in chemo type obtained by HD from camphor/1,8-cineole/α-thujone/chrysanthenone to a new chemotype in the case of UE-AE: camphor/α-thujone/1,8-cineole/filifolone; Then, an increasing of filifolone, α-thujone, 3-octyne and cis-limonene oxide characterize the UE-EO. The antifungal activity of the EO has slightly increased when extracted by UE-AE, however, both antibacterial and antioxidant activities were interestingly increased.

Keywords: *Artemisia herba-Alba* Asso, Essential Oil, Ultrasound & Enzymatic Assisted Extraction, Green Extractions

Introduction

Essential oils (EO) are complex mixture of volatile to semi-volatile organic compounds which contribute to the flavor and fragrance of the plant[1]. Due to their bioactivities effects, EO have been historically used as raw materials in many fields, such as pharmaceutical, cosmetic, perfume, agronomic, and food industries[2-7]. *Artemisia herba-alba* Asso, known as *desert wormwood* or *Shih* (Arabic name) in Northern Africa, had been studied for its characteristic bio molecules such as sesquiterpenes lactones and monoterpenes. Its EO is characterized by a high degree of polymorphism leading to the definition of several chemotypes[8-18]. Ultrasound-assisted extraction (UAE) increases the penetration of solvent into the plant cells via cavitation improving the EO extraction efficiency[19-20]. Recent studies shown that the enzymatic assisted extraction increases the yield of EO from *Amaryllidaceae, Apiaceae, Lamiaceae, Oleaceae* and *Zingiberaceae* without or with small chemical composition modifications[21-26], but only a few works on UAE and/or enzymatic pretreatment prior to EO extraction from *Asteracea* were studied. In this work, considering the large use of *Artemisia herba-alba* Asso, our work intended to enrich data about coupling ultrasound with enzymatic technologies prior to the EO extraction and the effect on its composition and bioactivities.

Experimental section

Plant material

The wild plant was collected in May 2014 from the steppe region of the western of Djelfa (Algeria) located at 300 km south of Algiers and the leaves were dried in open air. *Artemisia herba-alba* Asso plant used in this study was identified at the National High School of Agronomy (ENSA-Algiers). The dried leaves were thoroughly mixed and stored at ambient temperature, and protected from light in amber box before being subjected to the extraction experiments.

Chemicals and enzymes

All of the chemicals used in the experiments were analytical-grade and used without further purification.

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Mueller Hinton Agar, Mueller Hinton Broth and Sabouraud Dextrose Agar were obtained from CONDA, Madrid, Spain.2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich, USA. GENTAMICINE and FLUCONAZOLE were supplied from PFIZER Paris Cedex, France. Anhydrous sodium sulphate (Na_2SO_4) and Ethanol were purchased from FLUKA Chemicals (Buchs, Switzerland). Commercial enzymes used in this study consist of an enzymatic mixture from *Aspergillus aculeatus* containing cellulase (E.C. 3.2.1.4), β-glucanase (E.C. 3.2.1.6) and xylanase (E.C. 3.2.1.8) with a fungal β-Glucanase activity of 100 FBGU/g. Enzymes was procured from Novozym, Sigma–Aldrich (St. Louis. MO, USA). Distilled water was used in all extraction processes.

EO extraction methods

Hydro-distillation (HD) apparatus and procedure

Equivalence of 100 g of dried leaves of *Artemisia herba-alba* Asso were hydro-distillated with 1 L of distilled water using a Clevenger-type apparatus with heating mantle according to the European Pharmacopoeia until no more essential oil was obtained. The essential oil was collected and dried over anhydrous sodium sulfate and stored in amber vials at +4°C prior to analysis and bioactivity assessment. The yield of oil obtained was expressed as a percentage (w/w). The Artemisia EO obtained by HD was designated as the control EO.

Ultrasound/Enzymes-assisted extraction (UE-AE) procedure

Before coupling Ultrasound Assisted Extraction with Enzyme pretreatment of *Artemisia herba-alba* Asso leaves, preliminary experiments were carried out to optimize the suitable conditions of ultrasound pretreatment which was conducted in an ultrasonic bath (ELMA-SONIC H70; 37 kHz frequency; power range 100%). The UAE experiments were carried out with the same plant/solvent ratio (1:10) at the temperature of 20, 30 and 40°C, for 20, 30 and 40 min. then, the corresponding conditions allowing the maximum yield were combined with the second step consisting of enzymatic action. The enzymatic pretreatment was carried out by adding cellulolytic enzyme mixture equivalent to 2% to the flask after completion of the UAE experiments. The enzyme-aided hydrolysis reaction take place in citrate buffered water at pH adjusted to 4.5, 5.5 and 6.5 before UAE. The enzyme-plant contact periods of 60, 90, 120 min at the optimized temperature from UAE experiment were tested. After the Ultrasound-Enzyme pretreatment, the hydrolyzed *Artemisia* leaves were placed in a round bottom flask, connected to a Clevenger trap and distilled. The yield of oil obtained was expressed as a percentage (w/w).

Chemical composition by gas chromatography coupled to mass spectrometry (GC–MS)

GC/MS analyses were performed using a Hewlett-Packard 6890 plus series GC system (Agilent Technologies), equipped with an HP5-MS fused silica capillary column (30 m × 0.32 mm, 0.25 µm film thicknesses) coupled with a quadrupole mass spectrometer (model HP 5973).The oven temperature was initially held for 8 min at 60°C then increased by 2°C/min until 250°C and held for 10 min. Carrier gas was helium at a flow rate of 0.5 ml/min in split mode 1:50 with an injection volume of 1 µl. Injector and MS transfer line temperatures were set at 250°C and 280 °C, respectively. For MS detection, electron ionization at 70 eV was used in Full Scan mode, the temperature of the ion source was 175°C; Scan time and mass range were 1s and 50–550/29–550/ 34–450 m/z, respectively. The components of essential oils were identified by matching their recorded mass spectra with the data bank mass spectra (Wiley 7N and NIST 2002 libraries, (National Institute of Standards and Technology) using MSD Chemstation data analyses system.

Antimicrobial activities

Microbial strains and culture conditions

The obtained AEO were tested for their antibacterial activity against two Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis)* and two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa)* known to cause infections in humans. The antifungal activity of these EOs was also evaluated against one fungal strain (*Aspergillus niger)* and one yeast strain (*Saccharomyces cerevisiae).* As positive control, we have also included standard antimicrobial agents (GENTAMICINE as antibacterial agent and FLUCONAZOLE as antifungal agent) in the same assays.

Antimicrobial activities determination using disc diffusion method

For determination of antimicrobial activity of the two desert wormwood EOs (control EO obtained by HD and EO obtained by UAE-Enzymes), agar plates are inoculated by young culture from the broth medium (18–24 h for the bacteria and 48 h for fungal). Then, briefly, 0.1 ml from about 10^7-10^8

CFU/ml microbial suspensions was spread on the Mueller Hinton Agar (MHA) plates, and about 10^8 CFU/ml fungal broths on the SGA. A sterile disc of 6 mm in diameter was laid down on the surface of agar inoculated previously with 0.1 ml of the prepared microbial suspension. After this step, a drop of 50 µl of each desert wormwood EOs, previously dissolved four times in DMSO (25%) were added on the sterile disc and were left to disseminate for 30 min. Two plates were reserved for positive control experiment with GENTAMYCINE and FLUCONAZOLE. Also, pure DMSO was served as negative control in two other plates[27-29]. Inoculated Petri dishes were incubated at 37°C for 18–24 h for bacteria, and at 30°C for 24–72 h for fungus and yeasts. Reading is taken by measuring the diameter of the inhibition zone around each disc using a caliper. The results were expressed by the diameter of the inhibition zone measured in millimeters (IZ: mm) depending on the sensitivity of the strains against EO. All measurements were realized in triplicates.

Antioxidant activity by DPPH free radical scavenging

The evaluation of in vitro antioxidant activity of HD-EO and UE-EO was carried out by the DPPH free radical scavenging assay. In absence of antioxidants, the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical shows a maximum absorption at 517 nm (purple) but the color gradually turns to yellow, then absorbance decrease in presence of more amounts of antioxidants [30]. From each extraction methods, essential oils were diluted in methanol with the equivalent concentration of 4, 6, 8, 10, 15 and 20µl/ml. Then, 1 ml of each dilution was added to 2 ml of 0.2 mM methanolic DPPH solution. The mixtures were incubated under darkness at room temperature for 30 min. The absorbance was measured at 517 nm against the methanolic DPPH solution used as blank sample (negative control) using a UV-1800 type spectrometer SHIMADZU- UV SPECTROMETER. The scavenging of DPPH radical was calculated as radical scavenging capacity according to the following equation:

$$RSC\% = [(Ac - As)/Ac] \times 100 \ldots (1)$$

Where RSC% is the DPPH radical scavenging capacity; A_c and A_s are the absorbance values of the negative control and the sample, respectively. Ascorbic acid was used as positive control. Antioxidant activities of tested REOs were expressed as IC_{50}, defined as the required concentration scavenging 50% of the initial DPPH radicals, then, Antioxidant activities of tested REOs were expressed as IC_{50}, defined as the required concentration scavenging 50% of the initial DPPH radicals, then, lower IC_{50} values indicate higher free RSC. The IC_{50}(µl/ml) values were reported as mean ±SD, for this, each test was performed in triplicate.

Results and Discussion

Yield of essential oils extraction

Optimization of time and temperature of ultrasound pretreatment

Variation in the yield of *Artemisia herba-alba*Asso EO according to temperature and incubation time in the ultrasound bath (without enzyme treatment) is represented in Figure 1. It was observed that the optimum incubation period for a higher extraction yield was 40 min at 40°C.The extraction yield of EO increase with both increasing of temperature and ultrasound time, then, the yield passes from 1.05±0.08 % at 20°C for 20 min to 1.421% ±0.05 in case of ultrasound treatment for 40 min at 40°C. This yield is higher than the yield of control experiment without ultrasound treatment (1.01% ±0.08). Temperature is one of the main factors involved in cell wall destruction during ultrasound treatment. In general, an increase in this variable correlates with improvements in extraction yields due firstly to the increasing solvent diffusion and mass transfer throws the plant material, also, temperature starts the release of several compounds. However, temperature may increase mass transfer during extraction, but it may cause some degradation rates of the compounds obtained, especially when the UAE process reaches temperatures above 75°C[31].

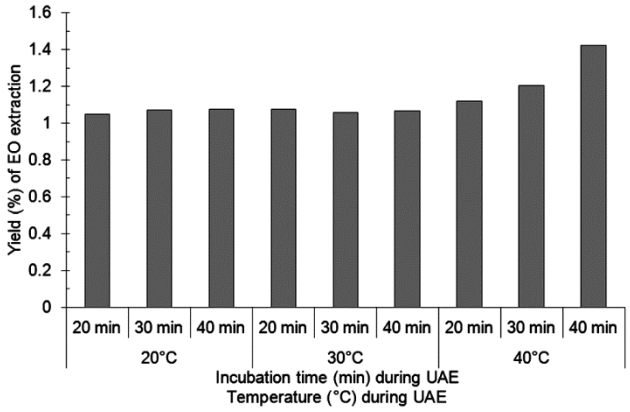

Fig. 1 — Time of exposition and temperature effect during ultrasound pretreatment before EO extraction from leaves of *Artemisia herba-alba*Asso

Optimization of pH and time of enzymatic pretreatment

The variation in EO yields according to enzyme activity at different pH levels and incubation time (at 40°C and enzyme concentration of 20 mg/l) is reported in Figure 2. The results demonstrated that the maximum enzyme activity was observed over a pH range of 4.5 and 5.5. It was also observed that among the tested time, the optimum duration of enzymatic action is 90 min. The highest yields of EO extraction was observed at pH5.5 for 60 and 90 min with 1.55% ±0.03 and 1.56% ±0.07 respectively, followed by extraction at pH4.5 for 60 and 90 min with 1.52% ±0.01 and 1.53% ±0.01 respectively, and then followed by the extraction after enzyme action for 120 min both at pH4.5 and 5.5 with 1.50%.The enzymatic action at pH6.5 gives the lowest EO yield for the three tested contact time with 1.44% ±0.04, 1.42% ±0.04 and 1.41% ±0.04 for 60, 90 and 120 min respectively. In concordance with our results, there are other works which reported that the enzyme assisted extraction of essential oil from several other medicinal plants like garlic, celery, fennel and cumin using individual enzymes (cellulase, pectinase, protease) or enzymes mixture resulted in a higher extraction yield at pH ranged between 4.5-5.5 for 60-90 min at 40-50°C [21-24]. This is because the enzymatic action on the plant material need proper contact time and suitable pH to breakdown the cell walls. In addition, to obtain the first essential oil droplet, 35 min for HD and just 20 min for UE-AE were needed. Then, the total time necessary to complete EO extraction is 180 min for HD and 110 to 120 min for UE-AE including the time of ultrasonic and enzymatic pretreatment. This result allowed us to confirm that the essential oil distillation process was facilitated by applying ultrasonic and enzymatic pretreatment. This could simply be attributed to cell wall degradation resulting in higher extraction efficiency.

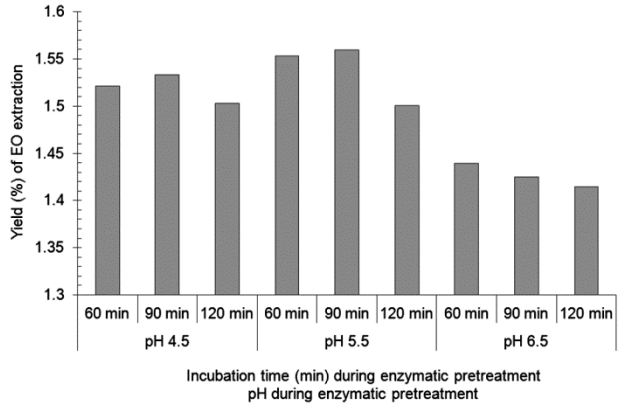

Fig. 2 — Effect of pH and time of enzymatic pretreatment before EO extraction from leaves of *Artemisia herba-alba* Asso

Chemical composition

A total of 26 compounds were identified by GC/MS in *A. herba-alba*Asso EO isolated by HD and UE-AE (Table 1).Chromatogram profile of the EO obtained by HD shows that the main components were camphor (22.44%); 1,8-cineole (11.98%); α-thujone (8.19%); chrysanthenone (7.26%); β-thujone

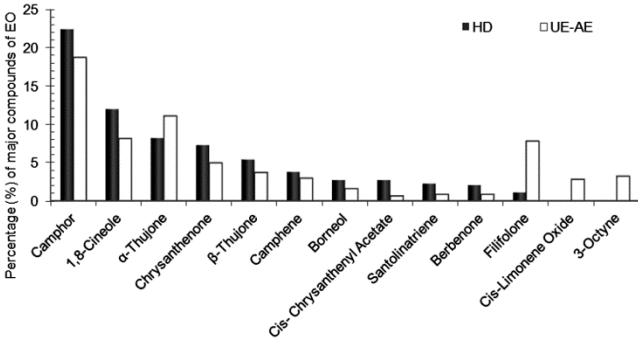

Fig. 3 — Change in percentage (%) of common and specific major compounds of EO fromleaves of *Artemisia herba-alba* Asso.obtained by HD and UE-AE

Table 1 — Chemical constituents of EO from leaves of *Artemisia herba-alba* Asso.obtained by HD and UE-AE

N°	Compound	RT (min)	Relative Area (%) HD	UE-AE
1	α-Pinene	10.80	0.79	1.21
2	Camphene	11.79	3.77	2.96
3	β-Myrcene	14.61	-	1.07
4	Artemisia Alcohol	21.78	0.82	-
5	1,8-Cineole	17.49	11.98	8.12
6	Filifolone	22.69	1.09	7.79
7	α-Thujone	23.16	8.19	11.09
8	Cis-Limonene Oxide	23.75	-	2.80
9	β-Thujone	23.97	5.37	3.70
10	Chrysanthenone	24.57	7.26	4.95
11	Terpenene-1-ol	24.756	-	1.04
12	Camphor	26.23	22.44	18.74
13	Cis-Verbenol	26.48	1.37	0.79
14	Pinocarvone	27.15	0.98	0.75
15	3-Octyne	27.75	-	3.19
16	Santolinatriene	27.89	2.22	0.83
17	Borneol	28.08	2.70	1.57
18	α-Phellandren-8-ol	28.29	-	0.72
19	Terpinene-4-ol	28.67	1.70	1.53
20	Cis- Chrysanthenyl Acetate.	34.032	2.69	0.63
21	Isopiperitenone	35.06	0.46	0.72
22	Cis-Jasmone	43.39	-	0.74
23	Berbenone	43.57	2.03	0.83
24	Germacrene D	48.40	1.48	0.16
25	Spathulenol	54.60	0.77	0.81
26	Davanone	54.85	1.21	-

(5.37%); camphene (3.77%); borneol (2.7%); cis-chrysanthenyl acetate (2.69%); santolinatriene (2.22%) and berbenone (2.03%). In this work, the EO of the Algerian *A. herba-alba*Asso from west Djelfa steppe region is characterized by the chemotype camphor/1,8-cineole/ α-thujone/chrysanthenone. Our results show high similarity with the chemotype reported by Boutekedjiret *et al*[16] (chrysanthenone, α-thujone, β-thujone, camphor, bornyl acetate and 1,8-cineole) and Vernin *et al*[13] (camphor/ 1,8-cineole/ chrysanthenone/α –thujone/β-thujone). Medium similarity is observed with the south Algerian chemotypes reported by Dahmani-Hamzaoui *et al*[17] (α-thujone/ camphor/ chrysanthenone/β-thujone/1,8-cineole/cis-jasmone/ davanone) but different from the chemotype described by Dob *et al*[18] who studied the composition of EO from a wild *A. herba-alba*Asso from M'sila-Algeria and reported that the EO contained camphor/ trans-pinocarveol/chrysanthenone/β-thujone as characteristic chemotype. In comparison with control EO obtained by HD, chromatogram profile of the EO obtained by UE-AE shows that the main components were camphor (18.74%); α-thujone (11.09%); 1,8-cineole (8.12%); filifolone (7.79%); chrysanthenone (4.95%); β-thujone (3.7%); 3-octyne (3.19%); camphene (2.96%); cis-limonene oxide (2.8%) and borneol (1.57%).Then, the initial obtained chemotype of the EO was changed into a new one: camphor/α-thujone/1,8-cineole/filifolone. However, it is interesting to note that the percentage composition of the main compounds has decreased except these of α-thujone that hase increased from 8.19% to 11.09%, also, apparition of new major compounds: filifolone, 3-octyne and cis-limonene oxide was noted, however, cis- chrysanthenyl acetate, santolinatriene and berbenone were present at trace rate (<1%). The difference in percentage composition of these components may be due to the effect of ultrasound wave which can elevate the temperature in the microenvironment during the bulbs implosion, then, Asfaw *et al*[32] reported that filifolone can be a thermal decomposition product of chrysanthenone. Moreover the presences of the enzyme mixture may also have played a major role in varying the percentage composition of individual components. Chandran *et al*[33] also observed the slight decrease of percentage recovery of mono-terpene hydrocarbons after enzyme pretreatment with respect of that of control of EO from black pepper and cardamom; this may be due to the loose of lower volatile mono terpene hydrocarbon compounds during the process. Our results are in well agreement with the findings of earlier researchers which showed that the enzyme assisted extraction lead to an increase in essential oil yield as well as a slight modification in the physicochemical properties of the volatile oil[21-23, 34].

Antimicrobial activity

The potential antimicrobial activity of both EO obtained by HD and coupling UAE-Enzymes was investigated through inhibition zones evaluation against two Gram-positive bacteria (*S. aureus* and *B. subtilis*), two Gram-negative (*E. coli* and *P. aeruginosa*), one mould (*A. niger*) and one yeast (*S. cerevisea*) as fungal strains. Results presented in Table 2 show that HD-EO and UAE-Enzymes-EO at 1:4 dilution in DMSO displayed a broad spectrum and variable degrees of antibacterial and antifungal activities against the tested strains. The essential oils from coupled ultrasound-enzymes treated samples were found as the most active against microbial strains, either Gram-positives or Gram-Negatives bacteria with a maximum inhibition against *S. aureaus* (33,1±1mm) witch get to close the positive control inhibition; the low inhibition activity was reported against *P. aeruginosa* (13±1mm) traited by standard HD-EO, then, coupling ultrasound-enzymes pretreatment procure more antibacterial proprieties of EO from *Artemisia herba-alba* Asso. Also, against fungal strains, EO obtained by coupling UAE to enzymes pretreatment demonstrates more effectiveness activity than the control HD-EO. The *chemotype* of control HD-EO with predominance of camphor, 1,8-cineole, alpha-thujone and chrysanthenome. Similar chemotypes were reported as effective

Table 2 — Diameter of inhibition zones (mm) and IC_{50} (µg/ml)of AEO

Bioactivities	HD	UE-AE	Positive Control
Staphylococcus aureus	28.3±1.5	33.1±1.0	35 [a]
Bacillus subtilis	22.0±2.0	26.3±1.5	35 [a]
Escherichia coli	16.5±1.5	24.5±0.5	27 [a]
Pseudomonas aeruginosa	13.0±1.0	18.7±1.5	28 [a]
Saccharomyces cerevisiae	15.0±1.0	17.7±0.5	25 [b]
Aspergillus niger	14.7±0.5	15.7±0.5	25 [b]
RSC-DPPH (IC_{50})*	12.5±0.5	8.2±0.75	5.5±0.75 [c]

Note- Negative control was carried out using DMSO and no inhibition zones were revealed for all the cases.
Positive controls were carried out using GENTAMICINE [(a)] against bacterial strains, FLUCONAZOLE[(b)] against fungal strains and ascorbic acid [(c)] for radical scavenging capacity.
* IC_{50} (µg/ml) of DPPH-RSC% of EO.

on microbial strains[20,35]. Then, the qualitative and quantitative change in terms of EO composition caused by the ultrasound and enzymatic pretreatment enhance antibacterial activity more than antifungal activity.

Antioxidant activity

The results of DPPH radical scavenging capacity of studied EO were presented as IC_{50} (µl/ml) in Table 2. *Artemisia herba-alba* AssoEO obtained by HD exerted an antiradical activity with an IC_{50} value of 12.5±0.5 µl/ml DPPH solution. This activity is relatively low comparatively to that of ascorbic acid with an IC_{50} value of 5.5±0.75 µg/ml DPPH solution. However the EO obtained by UAE coupled with enzymatic pretreatment showed an IC IC_{50} value of 8.27±0.75 µl/ml DPPH solution. The scavenging activity of *Artemisia herba-alba* Asso essential oil could be attributed to its high content of oxygenated monoterpenes such us camphor, 1,8-cineole, α-thujone, chrysanthenone and Cis- Chrysanthenyl Acetate, among others recognized for their potent antioxidant activity[36]. This scavenging activity was enhanced in the EO obtained by UE-AE method probably by the increasing of the content of α-thujone and the filifolone in comparison with the control EO obtained by conventional HD.

Conclusion

Coupling ultrasound with enzymatic pretreatment prior to EO extraction by hydro distillation from *Artemisia herba-alba* Asso leaves resulted in marked increase (54.45%) in the EO yield with decrease of the total time of extraction, which is significant from an economic viewpoint. *A. herba-alba*AssoEO exhibited weak *in vitro* antioxidant activity but interesting antimicrobial properties. Indeed, the agar diffusion method indicated a strong activity of *A. herba-alba*Asso essential oils against bacteria species more than fungi. However, the chemotype of EO obtained by HD was change from a profile marked by camphor/1,8-cineole/α-thujone/ chrysanthenone codominance to a modified profile marked by camphor/ α-thujone/1,8-cineole/filifolone codominance when EO was extracted by coupling ultrasound with enzymatic pretreatment. This profile modification has enhanced both antimicrobial and antioxidant activities of the desert wormwood EO. The results of our study indicated that coupling ultrasound with enzymatic pretreatment prior to hydro distillation can be adaptable by the herbal industries for greening and improving extraction process.

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