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Synergistic Antioxidant Activity and Chemical Composition of Essential Oils From *Thymus fontanesii, Artemisia herba-alba* and *Rosmarinus officinalis*

Fatima Benyoucef[1], Mohammed El Amine Dib[2*], Zoheir Arrar[1], Jean Costa[3], Alain Muselli[3]

[1]Faculté des Sciences, Département de Chimie, Université de Tlemcen, Laboratoire (COSNA), BP 119, 13000 Tlemcen, Algeria
[2]Laboratoire des Substances Naturelles et Bioactives (LASNABIO), Université de Tlemcen, BP 119, 13000, Algérie
[3]UMR CNRS 6134, Campus Grimaldi, Université de Corse, Laboratoire CPN, BP 52, 20250 Corte, France

Corresponding Author: Mohammed El Amine Dib, PhD, Professor, Laboratoire des Substances Naturelles et Bioactives (LASNABIO), Université de Tlemcen, BP 119, 13000, Algérie. Tel : +21-3554378192, Email : a_dibdz@yahoo.fr

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Abstract

Introduction: Oxidative stress is involved in many pathological mechanisms especially those due to aging, such as cancer, Parkinson's and Alzheimer's disease. Essential oils are known for their biological properties, especially as anti-nociceptive, anticancer, antiviral and antioxidative. The main objective of this study was to study the antioxidant activity of essential oils from *Thymus fontanesii*, *Artemisia herba-alba* and *Rosmarinus officinalis,* individually and in combinations.

Materials and Methods: Essential oils of plants aerial parts were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). The antioxidant properties were evaluated using two different methods, α, α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity and ferric reducing antioxidant power (FRAP).

Results: The essential oil of *T. fontanesii* was principally characterized by phenolic compounds represented by thymol (76.6%) and p-cymene (7.4%). The constituents identified from *A. herba-alba* essential oil were principally represented by camphor (32.3%) and chrysanthenone (25.6%). While, *R. officinalis* essential oil was characterized by 1,8-cineole (18.3%), camphene (15.4%) and α-pinene (12.8%). *T. fontanesii* essential oil indicated the significantly highest activity in quenching of DPPH radical, followed by *R. officinalis* and *A. herba-alba* essential oils with IC_{50} of 13.7, 24.5 and 79.4 mg/L, respectively. The combination of *T. fontanesii, A. herba-alba* and *R. officinalis* essential oils showed the greatest antioxidant activity with an IC_{50} of 2.6 mg/L almost equal to the synthetic antioxidant butylated hydroxytoluene (BHT).

Conclusions: The essential oils blend presented high antioxidant activity compared to individual oils. These findings provide a new source of antioxidant that can be used as a natural food preservative and alternative to chemical synthetic preservatives.

Keywords: Essential Oils, Antioxidant Activities, Synergistic Effects

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Introduction

In an attempt to preserve human health and avoid auto-oxidation affecting both the sensory and nutritional quality of foods, synthetic preservatives such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole were introduced in the food industry. However, with the current resurgence of interest in the application of safe organic materials instead of synthetic materials, which are suspected for their carcinogenicity, essential oils are increasingly sought as natural alternatives.[1,2] Essential oils represent a "green" alternative in the nutritional and pharmaceutical fields due to their incredible biological properties.[3,4] Essential oils are not only used in monotherapy but also have been used in combinations for many years.[5] They are used to act synergistically to further enhance their effects. The possible synergistic effect produced by the combination of plant essential oils was referred as an efficient strategy to inhibit or reduce the natural oxidation process of foods.[6] The combination of essential oils with antioxidant effects approach may lead to new naturel preservatives. A Few research have studied the synergistic effect of plant essential oils. Grosso et al suggested that the combination of thymol, carvacrol and thymoquinone in the volatile oil of *Satureja montana* may be responsible for the increase in antioxidant activity, using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging and rancimat methods.[7] *Thymus fontanesii* and *Rosmarinus officinalis* belong to the Lamiaceae family. While, *Artemisia herba-alba* belongs to the Asteraceae family. These species are especially known for their important biological properties such as antibacterial and antioxidant activities. To our knowledge, no study has investigated the antioxidant properties of *T. fontanesii*, *A. herba-alba* and *R. officinalis* essential oils blend. The purpose of the present study was (*i*)

to determine the chemical composition of the essential oil of these plants, (*ii*) to evaluate the antioxidant power of each essential oil by DPPH and FRAP assays and (*iii*) to investigate the possible synergistic impacts of the combination of three essential oils.

Materials and Methods

Plant Material and Extraction of the Oils

Aerial parts of *T. fontanesii*, *A. herba-alba* and *R. officinalis* were collected at the flowering stage on May to June, 2017. The plant materials were botanically identified by the Laboratory of Ecology and Ecosystem Management of the University of Tlemcen, Algeria. Voucher specimens were deposited with the Herbarium of the University of Tlemcen. *T. fontanesii* (T.on.04/2018), *A. herba-alba* (A.h.a.05.2018) and *R. officinalis* (R.of 04/2018).

Essential oils were obtained from fresh material (300-400 g) by hydrodistillation for 5 hours using a Clevenger-type apparatus with yields (w/w) of 3.7% for *T. fontanesii*, 0,7% for *A. herba-alba* and 0.4% for *R. officinalis*.

Gas Chromatography

Gas chromatography (GC) analyses were carried out using a Perkin Elmer Clarus 600 GC apparatus (Walhton, MA, USA) equipped with a single injector and two flame ionization detectors (FIDs). The apparatus was used for simultaneous sampling of 2 fused-silica capillary columns (60 m × 0.22 mm, film thickness 0.25 µm) with different stationary phases: Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). Temperature was programed 60 to 230°C at 2°C min^{-1} and then held isothermal 230°C (30 minutes). Carrier gas was hydrogen (0.7 mL min^{-1}). Injector and detector temperatures were held at 280°C. Split injection was conducted with a split ratio of 1:80. Injected volume was 0.1 µL.

Gas Chromatography-Mass Spectrometry (GC/MS)

The oils and the fractions obtained by GC were investigated using a Perkin Elmer TurboMass quadrupole analyzer, directly coupled with a Perkin Elmer Autosystem XL equipped with 2 fused-silica capillary columns (60 m × 0.22 mm, film thickness 0.25 µm), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). Other GC conditions were the same as described above. Ion source temperature was 150°C and energy ionization 70 eV; electron ionization mass spectra were acquired with a mass range of 35–350 Da and scan mass of 1 s. Oil injected volume was 0.1 µL and fraction injected volume was 0.2 µL.

Component Identification

The identification of each compound was carried out by comparison: (*i*) retention indices calculated respectively on polar and apolar columns with those of standard compounds of the laboratory library[8,9] or those reported in the literature; (*ii*) mass spectra (electronic impact) with those of standard compounds or those present in computerized banks.[10,11]

Determination of Antioxidant Activity of Essential Oils

DPPH Free Radical Scavenging Assay

The free radical-scavenging activity of essential oils and combinations were measured using DPPH, as described in the literature.[12] At first, 500 mg/L of each essential oil stock solution was prepared. A series of dilution with varying concentrations (0.1 to 100 mg/L) was prepared by dissolving various masses of essential oil in ethanol. In regards to essential oils blends, a ratio of 1: 1 by volume was mixed for each combination and a series of concentrations ranging from 0.1 to 50 mg/L were prepared. After on, 100 µL of each concentration was then mixed with 25 µL of 0.5 mM DPPH. After a 30 minutes incubation period at room temperature, the absorbance was measured at 517 nm using spectrophotometer. Ascorbic acid was used as standard and DPPH mixture without any sample served as blank. Inhibition of free radical DPPH in percent (I%) was calculated as follows:

$$I\% = \left[\frac{A_{blank} - A_{sample}}{A_{blank}}\right] x100\%$$

As presented above, A_{blank} is the absorbance of the control reaction (without oils), and A_{sample} was the absorbance in the presence of essential oils. From the obtained RSC values, the IC_{50} values, which represented the concentrations of the extracts caused 50% neutralization, were determined by linear regression analysis.

Ferric-Reducing Antioxidant Power Assay

The ferric reducing antioxidant power (FRAP) of the essential oils was tested as described earlier [13]. Different concentrations of the essential oils dissolved in ethanol (80%) were mixed with 2.5 mL of phosphate buffer (pH = 6.6) and 2.5 mL of potassium ferricyanide. Later, the mixture was incubated at 50°C for 20 minutes, and then trichloroacetic acid (10%, 1,25 mL) was added. After shaking the mixture vigorously, it was mixed with distilled water (5 mL) and ferric chloride (0.1%, 5 mL). After 30 minutes of incubation, absorbance was read at 700 nm against ethanol (80%) as blank. Analyses were performed in triplicate. Increased absorbance of the reaction meant increased reducing power and compared to the synthetic antioxidant BHT as reference.

Statistical Analysis

The data are presented as the means ± standard deviations from the three replicates. Calculations were performed using the SAS v. 9.1.3 program.

Results

Chemical Composition of Essential Oils

GC–FID and GC–MS analysis of *T. fontanesii, A. herba-alba and R. officinalis* oils accounted for 99.3%, 93.4% and 91.1% of oils, respectively and allowed the identification of 31, 20 and 19 components, respectively (Table 1). All components were identified by comparison of their mass spectrum and retention indices with those of our laboratory-produced "Arômes" library (Table 1). The essential oil of *T. fontanesii* obtained from the aerial parts was dominated principally by monoterpenoid phenol (84.7%). The main components were thymol (76.6%) and p-cymene (7.4%).

Table 1. Chemical Composition of the Essential Oils

No[a]	Compounds	lRI$_a$[b]	RI$_a$[c]	RI$_p$[d]	Essential oils			Identification[e]
					T. fontanesii	*A. herba-alba*	*R. officinalis*	
1	α-Thujene	932	924	1028	0.2	-	-	RI. MS
2	α-Pinene	936	931	1028	0.9	0.3	12.8	RI. MS
3	Camphene	950	945	1071	0.2	10.3	0.2	RI. MS
4	Oct-1-en-3-ol	962	962	1441	0.5	-	-	RI. MS
5	β-Pinene	978	972	1113	0.1	-	0.2	RI. MS
6	Myrcenes	987	982	1160	2.1	-	0.1	RI. MS
7	α-Phellandrene	1002	999	1161	0.2	-	0.2	RI. MS
8	3-Carene	1005	1006	1149	0.1	-	-	RI. MS
9	α-Terpinene	1008	1011	1270	1.7	-	-	RI. MS
10	p-Cymene	1015	1015	1270	7.4	-	1.5	RI. MS
11	1.8-Cineole	1024	1021	1211	-	8.4	18.3	RI. MS
12	(Z)-β-Ocimene	1029	1022	1234	0.6	-	-	RI. MS
13	Limonene	1039	1031	1967	-	-	4.2	RI. MS
14	γ-Terpinene	1051	1050	1245	2.3	0.2	3.8	RI. MS
15	trans-Sabinene hydrate	1051	1054	1445	0.1	0.1	-	RI. MS
16	Terpinolene	1082	1079	1281	0.2	-	-	RI. MS
17	Linalool	1083	1085	1538	1.7	0.5	-	RI. MS
18	Chrysanthenone	1110	1106	1504	-	25.6	-	RI. MS
19	Camphor	1123	1124	1506	0.1	32.3	-	RI. MS
20	α-pinocarvone	1135	1139	1632	-	-	1.4	RI. MS
21	Camphre	1144	1145	1532	-	-	15.4	RI. MS
22	Borneol	1148	1150	1688	0.3	4.5	12.7	RI. MS
23	Terpinen-4-ol	1164	1162	1591	1.1	3.2	1.2	RI. MS
24	α-Terpineol	1176	1176	1690	0.1	2.1	1.6	RI. MS
25	Verbenone	1183	1184	1723	-	-	12.7	RI. MS
26	Carvone	1214	1215	1278	-	3.5	-	RI. MS
27	Thymol	1266	1263	2181	76.6	-	-	RI. MS
28	Carvacrol	1278	1286	2193	0.6	-	-	RI. MS
29	Bornyl acetate	1285	1280	1600	-	-	2.6	RI. MS
30	Eugenol	1330	1329	2164	0.1	-	-	RI. MS
31	cis-Carvyl acetate	1343	1345	1858	0.1	-	-	RI. MS
32	(E)-β-Caryophyllene	1421	1416	1591	1.6	-	0.2	RI. MS
33	(E)-α-Bergamotene	1434	1435	1573	Trace	0.1	-	RI. MS
34	α-Humulene	1455	1448	1668	0.1	0.1	0.6	RI. MS
35	γ-Humulene	1483	1480	1702	Trace	0.1	-	RI. MS
36	β-Bisabolene	1503	1499	1721	0.1	0.2	-	RI. MS
37	δ-Cadinene	1520	1511	1760	Trace	0.5	-	RI. MS
38	(E)-α-Bisabolne	1530	1531	1755	0.1	0.3	-	RI. MS
39	Spathulenol	1572	1560	2120	-	0.5	-	RI. MS
40	Globulol	1589	1578	2067	-	0.6	-	RI. MS
41	Caryophyllene oxide	1578	1567	1969	0.1	-	1.4	RI. MS
Total identification %					**99.3**	**93.4**	**91.1**	
Monoterpene hydrocarbons %					8.6	10.8	21.5	
Sesquiterpene hydrocarbons %					1.9	1.3	0.8	
Oxygenated monoterpenes %					3.6	80.2	65.9	
Oxygenated sesquiterpenes %					0.1	1.1	1.4	
Non terpenic oxygenated compounds %					0.5	-	-	
Monoterpenoid phenol. %					84.7	-	1.5	

[a] Order of elution is given on apolar column (Rtx-1).
[b] Retention indices of literature on the apolar column (lRIa) reported from Konig.[9]
[c] Retention indices on the apolar Rtx-1 column (RIa).
[d] Retention indices on the polar Rtx-Wax column (RIp).
[e] Identification mode; RI. retention indices; MS. mass spectrometry in electron impact mode.

The constituents identified in the aerial parts of *R. officinalis* and *A. herba-alba* essential oils were principally oxygenated monoterpenes (80.2 and 65.9%, respectively), followed by monoterpene hydrocarbons (10.8 and 21.5%, respectively). The main components of *A. herba-alba* essential oil were camphor (32.3%), chrysanthenone (25.6%), 1,8-cineole (8.4%) and borneol (4.5%). While, *R. officinalis* essential oil was characterized by 1,8-cineole (18.3%), camphene (15.4%), α-pinene (12.8%), borneol (12.7%), verbenone (12.7%) and limonene (4.2%) (Table 1).

DPPH Free Radical Scavenging Assay

All essential oils were able to reduce the stable free radical DPPH to the yellow colored diphenylpicrylhydrazine. However, *T. fontanesii* presented the highest antioxidant effect based on DPPH test that had the lowest IC_{50} value of 13.7 mg/L. While, *A. herba-alba* and *R. officinalis* essential oils appeared to be the least active oils with IC_{50} values of 24.5 and 79.4 mg/L, respectively (Table 2).

Essential oils blends were very effective compared to the individual essential oils tested. The combination of *T. fontanesii*, *A. herba-alba* and *R. officinalis* essential oils showed a significant increase in antioxidant activity with an IC_{50} of 2.6 mg/L, almost equal to the synthetic antioxidant used as reference (IC_{50} = 2,3 mg/L) (Table 3). The combination of essential oils of *T. fontanesii* and *R. officinalis* showed also good antioxidant activity with an IC_{50} of 7.2 mg/L. However, the essential oils blends of *T. fontanesii* and *A. herba-alba*, and *A. herba-alba and R. officinalis* had promising antioxidant activities with IC_{50}s of 23.9 and 39.2 mg/L, but were still lower than the synthetic antioxidant (BHT) (Table 3).

Ferric Reducing Antioxidant Power Assay

FRAP assay is a widely used method that uses antioxidants as reductants in a redox-linked colorimetric reaction, wherein Fe^{3+} is reduced to Fe^{2+}. Figure 1 depicts the reducing power of individual and blend essential oils. All oils showed the presence the reductive effects, which increased with an increase in concentration. However, the combination of *T. fontanesii, A. herba-alba* and *R. officinalis* essential oils has been as effective on reducing power, compared to the synthetic antioxidant BHT. The individual and blends essential oils displayed chelating effects on ferrous ions, suggesting that it can sequestrate Fe-ions or reduce the concentration of metal.

Discussion

As a result, *T. fontanesii* presented the highest antioxidant effect, probably related to its chemical profile, especially the relatively high percentage of phenolic compounds. Thymol and p-cymene are the most frequently occurring constituents of essential oils obtained from thyme species, with many biological activities. They also act as antioxidants (free radical scavenger, anti-lipid peroxidative agent, etc).[14-16] The moderated antioxidant activity obtained from *R. officinalis* essential oil could be the consequence of appreciable content of camphene, borneol, verbenone, 1,8-cineole and α-pinene, that represented more than 71% of the total oil. The lowest antioxidant activity was recorded for *A. herba-alba* oil where chrysanthenone, camphor, camphene and 1,8-cineole represented more than 66.3% of the total oil. The antioxidant activity obtained from *A. herba-alba* and *R. officinalis* essential oils could be the consequence of appreciable contents of oxygenated monoterpenes (80.2 and 65.9%, respectively)

Table 2. 2.2-Diphenyl-1-picrylhydrazil Radical Scavenging Activities (%)

Samples		Antioxidant Activity						IC_{50}
Thymus fontanesii	Concentration (mg/L)	0.2	1	5	10	15	20	13.7±1.1
	DPPH radical scavenging activity (%)	3.1	5.8	19.8	37.2	54.6	72.1	
Rosmarinus officinalis	Concentration (mg/L)	5	10	15	20	25	30	24.5±2.1
	DPPH radical scavenging activity (%)	5.9	17.2	28.5	39.8	51.1	62.4	
Artemisia herba-alba	Concentration (mg/L)	10	20	30	50	70	90	79.4±2.8
	DPPH radical scavenging activity (%)	10.1	15.8	21.6	38.8	44.6	50.4	
BHT	Concentration (mg/L)	0.2	0.5	1.0	2.0	3.0	4.0	2.3±0.8
	DPPH radical scavenging activity (%)	28.4	35.6	47.9	71.9	85.1	86.6	

Table 3. Antioxidant Activity of Essential Oils Blends

Samples		Antioxidant Activity						IC_{50}
Thymus fontanesii + *Rosmarinus officinalis*	Concentration (mg/L)	0.5	1.0	3.0	5.0	7.0	10	7.2±1.8
	DPPH radical scavenging activity (%)	4.1	7.8	29.8	38.1	52.5	62.1	
Thymus fontanesii + *Artemisia herba-alba*	Concentration (mg/L)	5.0	10	15	20	25	30	23.9±2.3
	DPPH radical scavenging activity (%)	6.8	18.3	29.5	40.4	53.2	63.4	
Artemisia herba-alba+*Rosmarinus officinalis*	Concentration (mg/L)	5.0	10	15	20	30	40	39.2±3.1
	DPPH radical scavenging activity (%)	12.1	17.5	24.6	40.8	46.2	50.5	
Thymus fontanesii+*Rosmarinus officinalis*+*Artemisia herba-alba*	Concentration (mg/L)	0.5	1.0	3.0	5.0	7.0	10	2.6±0.4
	DPPH radical scavenging activity (%)	20.5	34.5	59.9	65.3	80.7	93.7	
BHT	Concentration (mg/L)	0.2	0.5	1.0	2.0	3.0	4.0	2.3±0.6
	DPPH radical scavenging activity (%)	28.40	35.6	47.9	71.9	85.1	86.3	

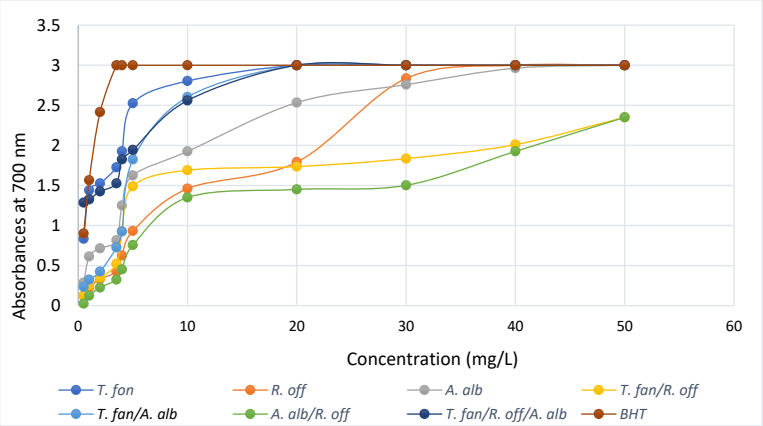

Figure 1. Ferric-Reducing Antioxidant Power Assay of Individual and Blend Essential Oils. Abbreviations: *T. fan, Thymus fontanesii*; *R. off, Rosmarinus officinalis*; *A. alb, Artemisia herba-alba*; BHT, butylated hydroxytoluene.

such as 1,8 cineole, camphor, chrysanthenone, verbenone and borneol. Indeed, Dawidowicz and Olszowy[17] showed that camphor, one of the main components in essential oil of *Salvia hispanica*, has lower antioxidant activity. This is while, 1,8 cineole and borneol were naturally found in many aromatic plants and showed weak antioxidant activity.[18,19] However, no information was found in the available literature about the biological activities of chrysanthenone and verbenone. Furthermore, monoterpene hydrocarbons are known to have noticeable antioxidant activities.[20] The obtained findings showed that essential oil combinations applied a promising synergistic antioxidant effect by decreasing the half maximal inhibitory concentration. The strong synergistic effect was found by combining *T. fontanesii, A. herba-alba* and *R. officinalis* essential oils (Table 3). It seems that the association of thymol and p-cymene with 1,8-cineole, chrysanthenone, camphor, borneol and verbenone increases the antioxidant activity. *T. fontanesii* and *R. officinalis* essential oils blend also showed a good antioxidant capacity when compared to the value of the synthetic antioxidant. It is evident that this synergistic effect was found when thymol and p-cymene was paired with 1,8-cineole, borneol and verbenone. However, the blends of *T. fontanesii* and *A. herba-alba, A. herba-alba* and *R. officinalis* essential oils showed the lowest antioxidant activities. It seems that the combination of thymol and p-cymene with chrysanthenone, camphor and 1,8-cineole produce low antioxidant power. While, the absence of thymol and p-cymene of blend significantly decreases this power.

In conclusion, the results showed that essential oil of *T. fontanesii* is a good source of monoterpenoid phenols as thymol and p-cymene. The essential oil had the best antioxidant activity using DPPH and FRAP methods. While, *R. officinalis* and *A. herba-alba* essential oils rich with oxygenated monoterpenes showed the lowest antioxidant activities by means of highest IC_{50} values. On the other hand, blends of essential oils showed an increase of antioxidant capacity. It seems that the monoterpenoid phenols (i.e. thymol and p-cymene) associated with oxygenated monoterpenes such as 1.8-cineole, chrysanthenone, camphor, borneol and verbenone play a pivotal role in this activity and produce stronger synergistic effect. These results, therefore, represent a basis for further studies that could lead to the development of a new treatment based on the combination of these essential oils as natural antioxidant agents, both in food and pharmaceuticals fields.

Authors' Contributions

FB prepared the samples and carried out the experiments; MAD wrote the manuscript; AZ conceived the original idea; AM performed the analyzes; JC supervised the project.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

Ethical Approval

Not applicable.

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