Phytochemical screening and antioxidant activity of four Moroccan aromatic plant methanolic extracts and essential oils

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Abstract:

This work aimed to evaluate and to compare the antioxidant potential between essential oils (EOs) and methanolic extracts (MEs) of *Anthemis tenuisecta*, *Cladanthus arabicus* Cass., *Ismelia carinata* Schousb., and *Thymus broussonetii* Boiss subsp. broussonetii collected from Morocco. The EOs used in this study were isolated by hydrodistillation using a Clevenger-type apparatus. The MEs were obtained by maceration in methanol. The main compounds of EOs were identified using gas chromatography-mass spectrometry. The MEs of the four plant species were tested for total phenolics and total flavonoids. The *in vitro* antioxidant activity of EOs and MEs was carried out using diphenyl-1-picrylhydrazyl (DPPH) assay. The antioxidant activity of MEs showed high correlations with phenolic compounds. For both fractions, the best IC_{50} values were given by *T. broussonetii*. The MEs revealed significantly ($p \leq 0.05$) better antioxidant activity than EOs. The results suggest that *A. tenuisecta*, *C. arabicus*, *I. carinata*, and *T. broussonetii* may be potential sources of natural antioxidants.

Keywords: Phytochemical screening, antioxidant activity, methanolic extracts, essential oils, diphenyl-1-picrylhydrazyl (DPPH) assay.

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1. Introduction

Oxidative stress is recognized as a key contributor to many health problems and food deterioration (Pisoschi and Pop 2015; Prakash et al. 2015**)**. This occurs when antioxidant defense mechanisms are overwhelmed by free radicals due to environmental toxin exposure, chronic infections, or intrinsic dysfunction (Cadet et al. 2009; Čáp et al. 2012; Kalyanaraman 2013). Research has focused to reduce oxidative stress using antioxidant supplements but due to various negative effects related to synthetic antioxidants (Kahl and Kappus 1993; Sun and Fukuhara 1997), the investigation of natural antioxidants has drawn renewed interest.

Plant Secondary metabolites are several botanical compounds that are involved in plants adaptation to their environment and from which the plants are thought to get their remediating properties (Krishnaiah et al. 2011; Shahidi and Ambigaipalan 2015). They are produced by most vascular plant families in variable quantity and quality.

Asteraceae and Lamiaceae families are well known for their medicinal properties and their phytochemicals content that can replace synthetic antioxidants (Krishnaiah et al. 2011; Metrouh-Amir et al. 2015).

Anthemis tenuisecta, *Cladanthus arabicus* Cass., and *Ismelia carinata* Schousb. are Asteraceae species, growing as wild populations in Morocco (El Oualidi et al. 2012) and unexplored in pharmacological practices (Bellakhdar 1997).

Thymus broussonetii Boiss subsp. broussonetii is an endemic species of Morocco (El Oualidi et al. 2012). This Lamiaceae is widely used in the traditional medicine of Mediterranean areas due to its numerous virtues (Bellakhdar 1997). Several studies have been published on biological properties and chemical composition of *T. broussonetii* essential oil (Belaqziz et al. 2010; Elhabazi et al. 2006; El Ouariachi et al. 2014).

The study aimed the highlighting uncharacterized plant species that Moroccan diversity offers to reduce over-harvesting of those used locally in folk medicine and culinary practices. For that, the scavenging effects of three unexplored aromatic plants were evaluated and compared to *T. broussonetii*, considering two fractions: a volatile-nonpolar fraction (essential oils) and a polar one (methanolic extracts).

To assess the antioxidant potential of these plant phytochemicals, the major chemical groups of essential oils were identified. Also, the total phenols and flavonoids of methanolic extracts were screened.

2. Material and methods

2.1. Plant materials

The aerial parts of *A. tenuisecta*, *C. arabicus*, *I. carinata*, and *T. broussonetii* were collected at the flowering stage from different regions of Morocco and then they were dried and kept at room temperature. Plants were identified at the Scientific Institute of Rabat (Morocco). The botanic family, date, and site collection of plants are summarized in Table 1. Specimens were deposited in the herbarium of the laboratory of biotechnology at the National School of Applied Sciences, Ibn Zohr University; Agadir, Morocco.

Table 1: The scientific name, family, and date with a collection site of studied plants.

Scientific name	Family	Date of collection	Site of collection	Geographic coordinates
Anthemis tenuisecta	Asteraceae	April 2017	Agadir region at Tin Mansour	30°6'58"N, 9°33'6"W
Cladanthus arabicus Cass.	Asteraceae	March 2017	Agadir region	30°25'12"N, 9°35'53"W
Ismelia carinata Schousb.	Asteraceae	March 2017	Tiznit region at Tifnit	30°12'0"N, 9°37'48"W
Thymus broussonetii Boiss subsp. broussonetii	Lamiaceae	April 2017	Safi region at Sebt Gzoula	32°07"N, 9°05"W

2.2. Preparation of plant extracts

Essential oils (EOs) were obtained by hydrodistillation of the air-dried plants for 3 hours using a Clevenger type apparatus as recommended by European Pharmacopoeia (British Pharmacopoeia 1988).

For the extraction of the phenolic fraction, several studies have shown that methanol is the most suitable solvent for the extraction of phenolic compounds (De Abreu and Mazzafera 2005; Gálvez et al. 2005), especially flavonoids recognized by their antioxidant potential and which we aimed to evaluate in this research. Also, other works indicated that maceration presented the highest yield for the extraction of the phenolic fraction (Ćujić et al. 2016; Naima et al. 2015; Rocha et al. 2017). Hence the choice to use maceration in methanol in this study. Methanolic extracts (MEs) were prepared by maceration according to the Basma et al. (2011) method. Plant material (50 g) was extracted with 100 mL of methanol for ten days with frequent agitation at

room temperature in the dark. The mixture was then filtered and concentrated in the oven at 45 ℃. The extraction yield (w/w) of the crude extract and essential oil was determined for each plant. EOs were dried over anhydrous sodium and kept with the MEs in the dark at 4 °C.

2.3. Gas chromatography-mass spectrometry analysis

The chemical composition of essential oils was investigated by gas chromatography/mass spectrometry (GC/MS) using a Shimadzu system. A VB5 capillary column was used (30 m x 0.25 mm inner diameter, 0.25 µm film thickness), and Helium was the carrier gas at 1 mL/min. The GC oven temperature was conserved at 50 °C for 1 min and programmed to 280 °C at a rate of 10 °C/min, then kept constant at 280 °C for 2 min. The split flow was used; the injector temperature was 250 °C. The mass spectrometer was operating at ionization energy of 70 eV and 300 °C. The mass spectrometer range was from 20 to 350 m/z, and the interface was at 300 °C. A library search was composed using the "Wiley GC/MS Library" Nist147 database LIB (Adams 2007). The chemical composition of *T. broussonetii* EO used in this study was previously determined by our laboratory team (Chebli et al. 2019).

2.4. Phytochemical screening of MEs

2.4.1. Total phenolic contents (TP)

Total phenolics were determined according to the method described by Singleton and Rossi (1965) using the Folin-Ciocalteu reagent. The standard curve was evaluated using gallic acid concentrations ranging from 0 to 0.25 mg/mL (distilled water was used as a blank). In a test tube, 200 µL of plant extract (1 mg/mL) and 500 µL of 10% (v/v) Folin-Ciocalteu reagent were mixed. Then 500 µL of distilled water and 800 µL of 7.5% (w/v) Na_2CO_3 solution were successively added. The tubes were incubated in the dark at room temperature for 30 min. The absorbance was measured at 765 nm. TP were expressed in mg of gallic acid equivalent per gram of dry extract (mg GAE/g DW) and were calculated according to the following formula:

$$TP = GAE \times \frac{V}{W}$$

Where GAE is the gallic acid equivalent (mg/mL) determined from the calibration curve (y = 0.1457 x + 0.058; r = 0.83), V is the volume of extract (mL) and W is the weight of pure plant extract (g).

2.4.2. Total flavonoid contents (TF)

Aluminium chloride colorimetric method

The colorimetric method used is the one described by Basma et al. (2011). Briefly, 250 µL of extract (1 mg/mL) was mixed with 1 mL of distilled water, and then 75 µL of 5% (w/v) $NaNO_2$

solution was added. After 6 min of incubation, 75 µL of 10% $AlCl_3$ solution was added to the mixture. After the second 6 min incubation, 1 mL of 4% NaOH solution was added and the mixture was immediately made up to 2.5 mL with distilled water. After a third incubation of 15 min, the absorbance was measured at 510 nm against distilled water (the blank). Catechin concentrations from 0 to 60 µg/mL were used to establish the calibration curve of the standard. Results were expressed in mg of catechin equivalent per g dry extract (mg CE/g DW). Flavonoids in the dry extract were calculated using the following formula:

$$TF = CE \times \frac{V}{W}$$

Where CE is the catechin equivalent (µg/mL) established from the calibration curve (y = 0.0045x + 0.0429; r = 0.9981), V is the volume of extract (mL) and W is the weight (g) of the pure plant extract.

NEU's reagent method

TF were also estimated using NEU's reagent according to the method cited by El Hariri et al. (1991). In a test tube, 2 mL of extract (1 mg/mL) was mixed with 100 µL of methanol solution of 1% NEU reagent (2-aminoethyl diphenylboric). Quercetin (0.05 mg/mL) was treated with the same reagent and absorbance was measured at 409 nm. TF were expressed in mg of quercetin equivalent per g dry extract (mg EQ/g DW) and calculated according to the following formula:

$$TF = \frac{(Aext \times 0.05)}{(Aq \times Cext)} \times 100$$

Aext: Absorbance of the extract; Aq: Absorbance of quercetin; Cext: Concentration of the extract in mg/mL.

2.4. DPPH radical scavenging assay

The antioxidant activity was determined by the DPPH test. The potential of extracts to scavenge the DPPH radical was assessed according to Leitão et al. (2002) method using four concentrations (0.125-0.25-0.5-1 mg/mL). At equal volumes, each concentration of the extract and 0.004% DPPH methanol solution were mixed and then incubated for 30 min at room temperature in the dark. The absorbance was recorded against a blank sample (0.004% DPPH methanol solution) at 517 nm. BHT (butylated hydroxytoluene) and Covi-ox were used as positive controls. Each concentration and the blank sample were made in triplicate. The inhibition of DPPH free radical in percentage (I %) was calculated as follows:

$$I\% = \frac{\text{Absorbance of blank} - \text{Absorbance of concentration}}{\text{Absorbance of blank}} \times 100$$

The IC_{50} values were graphically calculated as the concentration of extracts neutralizing 50% of DPPH radicals, a lower IC_{50} concentration corresponds to a higher antioxidant activity.

2.5. Statistical analysis

All assays were carried out in triplicate. All data were statistically analyzed with one-way ANOVA using SPSS 16.0 software. The means comparisons were performed using Newman and Keuls tests. The results are presented as mean values ± standard deviation with different letters, which are statistically significant at 5 %. Correlations between phenolic compound contents in methanol extracts and their IC_{50} values were calculated using Pearson's test and evaluated according to Thumb's rule (Hinkle et al. 2003).

3.Results and discussion

Extraction Yield of EOs and MEs

The average yields of EOs and MEs based on the dry weight of the plants (w/w) are shown in Table 2. The yield in dry extract varies from 9.82 to 12.08 %. *A. tenuisecta* showed the highest dry extract yield when the one of *C. arabicus* was lower than that obtained by Aghraz et al. (2018).

The EO content varied from 0.1 to 1.5 %. *T. broussonetii* presented the highest EO yield, but it was lower than those reported in the literature (Belaqziz et al. 2010; El Ouariachi et al. 2014). The EO yield of *C. arabicus* was similar to that found in a previous study (Aghraz et al. 2017).

It has been reported that the extraction efficiency of EOs and methanolic extracts depends on different parameters as physical properties of the plant material and chemical composition (Dai and Mumper 2010).

Table 2: Extraction yield (%) of EOs and MEs.

Plant species	Extraction yield (%)	
	EOs	MEs
A. tenuisecta	0.4	12.08
C. arabicus	0.4	9.82
I. carinata	0.1	11.66
T. broussonetii	1.5	11.36

The chemical composition of EOs

The main compounds from the GC-MS analyses of the EOs used in this study are listed in Table 3. EO of *I. carinata* was dominated by oxygenated sesquiterpenes (tau-cadinol). *C. arabicus* EO contained a noticeable fraction of monoterpene hydrocarbons (β-pinene and α-pinene). Carvacrol (phenol) was the first main compound of EOs from *A. tenuisecta* (19.52 %) and *T. broussonetii* (39.51 %).

Table 3: Quantitative composition of major compounds identified in four essential oils by GC-MS.

EOs	**Fractions (%)**			
	Compound 1	Compound 2	Compound 3	Compound 4
A. tenuisecta	Carvacrol (19.52%)	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1R)- (16.76%)	5-Hepten-2-one, 6-methyl- (16.46%)	Camphene (13.18%)
C. arabicus	β-Pinene (23.58%)	tau-Cadinol (9.54%)	Diethyl phthalate (7.88 %)	α-Pinene (5.68%)
I. carinata	tau-Cadinol (65.93%)	-	-	-
T. broussonetii [a]	Carvacrol (39.51%)	Benzene, 1-methyl-2-(1-methylethyl)- (14.8%)	γ-Terpinene (10.32%)	α-Pinene (9.7%)

[a] (Chebli et al. 2019)

Phytochemical screening of MEs

The TP and TF of MEs varied significantly ($p \leq 0.05$) (Table 4). The total phenolics ranged from 65.43 to 74.51 mg GAE/g DW. The total flavonoids varied from 43.32 to 44.34 mg CE/g DW using a colorimetric method and from 0.95 to 3.12 mg QE/g DW based on the NEU's reagent assay. To sum up, the highest amounts of TP and TF were found in *T. broussonetii* whilst *I. carinata* contained the lowest ones.

C. arabicus extract has revealed phenolic contents higher than those found by Aghraz et al. (2018) using maceration in methanol, which were 7.97 mg GAE/g DW and 6.62 mg QE/g DW of TP and TF, respectively. Also in a preceding research, Sayout et al. 2015 obtained from *T. broussonetii* leaves extract 26.4 mg GAE/g DW and 5.33 mg QE/g DW of TP and TF, respectively. On the whole, the MEs of the four plant species showed potential phenolic contents compared to other Lamiaceae and Asteraceae species. For example, Roby et al. (2013) reported that the highest TP content found in ethanolic extracts of three Lamiaceae species was

8.10 mg GAE/g DW. The content of total phenolics and flavonoids of methanol extract of *Pulicaria mauritanica* and *Asteriscus imbricatus* (Senhaji et al. 2017), and *Anthemis praecox* (Belhaoues et al. 2020) were lower than those found in the current study. Also, the TP of *Artemisia herba-alba*, *Conyza canadensis*, *Pulicaria dysenterica*, *Senecio anteuphorbium*, *Anvillea radiata*, and *Inula viscosa* did not exceed 25.26 mg GAE/g DW (El Guiche et al. 2015).

Table 4: Total Phenolic compounds of MEs of *A. tenuisecta*, *C. arabicus*, *I. carinata* and *T. broussonetii* (TP: total phenolics, GAE: gallic acid equivalent, TF: total flavonoids, CE: catechin equivalent, QE: quercetin equivalent, DW: dry weight extract).

Plant species	**TP (mg GAE/g DW)**	**TF**	
		(mg CE/g DW)	**(mg QE /g DW)**
A. tenuisecta	70.43 ± 0.51 c*	43.76 ± 0.05 b	1.51 ± 0.02 c
C. arabicus	71.84 ± 0.53 b	43.46 ± 0.02 b	1.98 ± 0.03 b
I. carinata	65.43 ± 0.15 c	43.32 ± 0.01 c	0.95 ± 0.01 c
T. broussonetii	74.51 ± 1.18 a	44.34 ± 0.07 a	3.12 ± 0.01 a

Mean value ± Standard deviation.

*Letters indicate statistical difference to 5 % level probability according to Newman and Keuls test.

DPPH radical scavenging assay

The IC_{50} values of the antioxidant activity are summarized in Table 5. The IC_{50} values of EOs in the DPPH assay ranged from 0.73 to 1.93 mg/ml and those of the MEs ranged from 0.06 to 0.87 mg/mL (Table 5). Among the tested EOs, *T. broussonetii* exhibited the highest scavenging effect, even similar to that of the standards. The weakest antioxidant potential was recorded by *I. carinata* EO. However, all MEs revealed similar scavenging effects to that obtained by the standards BHT and Covi-ox because their statistical groups are nested ($p \leq 0.05$). The IC_{50} values of the MEs were all lower than those of the EOs.

Table 5: IC_{50} values (mg/mL) obtained of the antioxidant activity.

Samples	IC_{50} (mg/ml)	
	EOs	MEs
A. tenuisecta	1.84 ± 0.31 e*	0.78 ± 0.5 bc
C. arabicus	1.33 ± 0.08 d	0.23 ± 0.08 ab
I. carinata	1.93 ± 0.16 e	0.87 ± 0.42 c
T. broussonetii	0.73 ± 0.07 bc	0.06 ± 0.05 a
BHT	0.25 ± 0.05 ab	
Covi-ox	0.43 ± 0.05 abc	

Mean value ± Standard deviation.

*Letters indicate statistical difference to 5 % level probability according to Newman and Keuls test.

The antioxidant effect of *T. broussonetii* EO was probably related to the high percentage of phenols (39.51 %) reported being responsible for the potent antioxidant capacity of several EOs (Kumar and Rawat 2013; Tohidi et al. 2017). In contrast, *A. tenuisecta* EO showed low antioxidant activity despite its high carvacrol content (19.52 %). This result is probably related to its chemical profile, especially the highly volatile compounds and the effect of minor compounds.

Oxygenated sesquiterpenes have been reported to be potent antioxidants (Khan et al. 2008). For this purpose, the modest antioxidant activity of *I. carinata* EO was unexpected given its richness of these compounds. Similar results were observed by El-Gawad et al. (2019), who found a weak antioxidant of *Xanthium strumarium* EO which was also reported to be rich in oxygenated sesquiterpenes.

The antioxidant activity of *C. arabicus* EO was modest compared to its appreciable content of hydrocarbon compounds reported as notable antioxidants (Bouzenna et al. 2017; Song et al. 2001). In a previous study, Aghraz et al. (2017) presented a higher IC_{50} value of *C. arabicus* EO (55.4 mg/mL) than that found in our results.

By comparing the antioxidant capacity of EOs and that reported for their major compounds, we can deduce that the antioxidant properties of the main component of essential oil do not always reflect its antioxidant activity (Dawidowicz and Olszowy 2014). Because the chemical complexity of EOs can generate a synergism, antagonism, or additivity effect between the various compounds (Chouhan et al. 2017; Rahhal et al. 2019).

Previous studies on medicinal plant EOs showed high IC_{50s} to those of the EOs tested in the present study. From the Asteraceae family, IC_{50} values of 49, 9.9, 4.72, and 0.08 mg/mL were

reported to *Baccharis trinervis* (Sobrinho et al. 2016), *Artemisia campestris* (Boulanouar et al. 2013), *Artemisia herba-alba* (Aljaiyash et al. 2018), and *Brocchia cinerea* (Hamdouch et al. 2022), respectively. EOs from Lamiaceae plants also showed IC_{50s} of 34.29 and 60.1 mg/mL, respectively to *Calamintha glandulosa* (Ćavar et al. 2013) and *Origanum compactum* (Babili et al. 2011).

Except for *T. broussonetii* EO, it can be concluded that the antioxidant activity of the hydrodistilled EOs was modest. However, it was considerable compared to several medicinal plants.

Concerning the MEs, the comparison of our results was made to those of studies using the DPPH method and extraction in methanol or ethanol. Sayout et al. (2015) previously investigated the antioxidant activity of leaves and stem extracts of *T. broussonetii*, whose IC_{50} values were 6.484 and 0.132 mg/mL, respectively. The maceration extract of *C. arabicus* achieved 50% inhibition of DPPH at 0.033 mg/mL (Aghraz et al. 2018). Roby et al. (2013) observed IC_{50s} of 0.83, 0.77, and 1 mg/mL, respectively to the extracts of *Salvia officinalis*, *Origanum majorana*, and *Thymus vulgaris*. *Origanum compactum* extract showed an IC_{50} of 9.9 mg/mL (Babili et al. 2011). Also, Khaled-Khodja et al. (2014) tested four Lamiaceae; *Ajuga iva*, *Marrubium vulgare*, *Mentha pulegium*, and *Teucrium polium*, whose IC_{50s} were 1.168, 0.52, 0.05, and 0.1 mg/mL, respectively. From the Asteraceae family, Tlili et al. (2013) observed IC_{50} values of 0.73 and 0.33 mg/mL, respectively for *A. campestris* and *A. herba-alba*. In two previous studies, the IC_{50s} of *Anthemis arvensis* and *Anthemis praecox* extracts were 0.191 and 2.43 mg/mL, respectively (Belhaoues et al. 2020; Boulanouar et al. 2013). It can be suggested that the extracts of all four plants revealed significant antioxidant activity.

Also, the antioxidant activity of MEs of *A. tenuisecta*, *C. arabicus*, *I. carinata*, and *T. broussonetii* showed strong positive linear correlations with their total phenol and flavonoid contents (Table 6) which indicates that phenolic compounds largely contribute to the antioxidant capacity. Other researchers have also reported these correlations (Khaled-Khodja et al. 2014; Muddathir et al. 2017; Rahhal et al. 2020).

Table 6: Correlations between DPPH scavenging activity and the amounts of phenolics in methanolic extracts.

DPPH scavenging activity	**Correlation coefficient**		
	TP	TF (CE)	TF (QE)
	0.789	0.881	0.963

TP: total phenolic compounds, TF: total flavonoids, CE: catechin equivalent, QE: quercetin equivalent.

Regarding the comparison between EOs and MEs, other studies have also shown better antioxidant activity in favor of MEs; for example, *Artemisia campestris* (Boulanouar et al. 2013), *Origanum vulgare* (Teixeira et al. 2013), and *Origanum compactum* (Babili et al. 2011). Studies reported similar results linked them to low phenol contents in EOs compared to terpenes due to the thermal destruction of their structures after hydrodistillation (Guimarães et al. 2010; Teixeira et al. 2007). While it is the phenols that easily donate hydrogen atoms to quench the radicals formed in the DPPH and have been well associated with antioxidant activity (John et al. 2015; Rezaie et al. 2015).

Conclusion

The present study reports for the first time the comparison of scavenging DPPH effect between a polar and a volatile-nonpolar fraction of *A. tenuisecta*, *C. arabicus*, *I. carinata*, and *T. broussonetii*. The MEs possess a higher scavenging DPPH effect than EOs. The antioxidant activity of MEs was related to the phenolic contents. The results suppose that *A. tenuisecta*, *C. arabicus*, *I. carinata*, and *T. broussonetii* may be potential sources of natural antioxidants that suggest uses to avoid oxidative damages. Moreover, the relationship between phytochemical analysis and antioxidant activity could serve as models for the synthesis of natural antioxidant supplements although more studies in biological systems along with phytochemical analyses should be carried out to ensure effectiveness and safety.

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