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Comparison of the Effect of Various Extraction Methods on the Phytochemical Composition and Antioxidant Activity of *Thymelaea hirsuta L.* aerial parts in Tunisia

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The present study aims to evaluate the effect of extraction methods namely soxhlet and cold maceration on the quality of *T. hirsuta* extracts, collected from different geographic regions within Tunisia. Hexane, ethyl acetate and methanol were used as solvent for both extraction processes. Yields varied considerably (from 0.61 to 8.11 %) according to sampling region, organic solvent and extraction method. The various chemical contents extracts were estimated by colorimetric methods, revealing important amounts of polyphenols (from 29.37 ± 1.34 to 259.63 ± 3.17 mg GAE/g) and flavonoids (from 26.22 ± 6.06 to 163.64 ± 3.32 mg QE/g). The antioxidant activity was measured using the DPPH-radical scavenging, the ABTS-radical scavenging and the ferric reducing antioxidant power (FRAP) assays. The antioxidant analysis showed that the methanol extract obtained by both processes exhibited the uppermost capacity to scavenge free radicals. However, the cold maceration technique leads to the richest extract in phenolic compounds compared to soxhlet method.

Keywords: *Thymelaea hirsuta L.*, phenolic compounds, DPPH, ABTS, FRAP.

Plants have the ability to synthesize compounds, through complex metabolic pathways[1]. These substances can be classified in two different groups such as primary and secondary metabolites. the latter play a key defensive role against the biotic and abiotic stresses[2]. However, plants contain a wide variety of phytochemicals (peptides, terpenes, phenolics, alkaloids, etc.) with very different physicochemical properties and original biological characteristics[3]. Despite the development of industrial compounds in recent years, people are more and more keen to rely on naturally-derived producrs. Consequently, a great number of industrial sectors (cosmetics, pharmaceuticals, agri-food) are increasingly incorporating these biomolecules in their formulations as an alternative to synthetic compounds. The valorization of these bioactive molecules represents an enormous economic potential. These compounds must be first separated from their plant matrix which requires several expensive and time consuming, such as extraction, analysis and identification. Among these steps, extraction is the crucial process involved in the discovery and quantification of bioactive molecule from natural and medicinal plants[4]. In this context, numerous papers have

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demonstrated the impact of extraction methods on chemical composition and biological activities of plant materials[5-7].

Thymelaea hirsuta L. is a flowering plant species which is widely distributed in the Canary Islands, Mediterranean region, north of Central Europe and Eastern Central Asia. It is an important medicinal plant belonging to the family of *Thymeleaceae*[8]. The aerial parts of this species are largely used as remedy to treat inflammation, hypertension and as an antiseptic[9-10]. Besides, this plant is particularly used as an ancient medicinal herb against diabetes [11-12]. In addition to that, the phytochemical screening of this species revealed the presence of high phenolic compound contents. Moreover, *T. hirsuta* extracts were reported to have antimicrobial[13], antitumor[9], antihypoglycemic[14], and antioxidant activities[15-16].

The main objective of this investigation was to evaluate the effects of extraction solvent and methods on the total phenolic content and *in vitro* antioxidant properties of various organic extracts obtained from *T. hirsuta* aerial parts.

MATERIAL AND METHODS

Chemicals and standards

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin, France).

Raw material

T. hirsuta aerial parts were harvested in September 2013 from different localities in Tunisia namely Tunis, Chebba, Kasserine and Fernana. The longitude and latitude of each area were listed in Table 1. The systematic identification of raw materials was confirmed by Mohamed Bousaid (Department of botany, National institute of applied sciences and technology, Tunis, Tunisia).The aerial parts were then dried and subsequently milled into powder with an electric grinder.

Extracts preparation

In order to optimize the bioactive compounds extraction, the aerial parts of *T. hirsuta* were extracted by two different methods namely Soxhlet extraction and cold maceration.

Soxhlet extraction

The plant materials were extracted successively using three different solvents with increasing polarity namely hexane, ethyl acetate and methanol. A total amount of 15 g of dried powdered were introduced into a cartridge and introduced inside a Soxhlet apparatus. Then, a volume of 150 ml of the extraction solvent was added to the solvent cup. Extraction was carried out with four cycles. Afterwards, the liquid extract was collected into a flask to remove the solvent.

Cold maceration

The powdered plant materials were successively extracted with solvents of increasing polarity namely hexane, ethyl acetate and methanol. In the first step, an amount of 5 g of dried powdered was placed into an Erlenmeyer flask contained 50 ml of hexane and macerated for 24h under continuous agitation. Once the extraction process is finished, the mixture was thoroughly filtered using a filter paper and the solvent was removed using a rotary evaporated.

After each extraction methods, the crude extracts were weighted and kept in a dark flask until further experiment. The extraction yield for each extract was calculated using the formula cited below:

$$\text{Yield}(\%) = \left(\frac{m}{M}\right) * 100$$

Where m: is the weight of residue in grams, M: is the weight of plant material in grams.

Estimation of total phenolics content

The estimation of phenolic content of each extract was done by the Folin-Ciocalteu method as outlined by Ghazouani *et al*[17]. To perform this assay, 100 µL of each extract were mixed with 500 µL Folin Ciocalteu reagent (0.2 N) and left at room temperature in the darkness for 5 min. Then, a volume of 400 µL of sodium carbonate solution (75 g/L, prepared in water) was added subsequently. The mixture also obtained was thoroughly shaken and incubated for 15 minutes in the darkness before reading the absorbance at 765 nm. The Gallic Acid was used as standard to plot a calibration curve. The amounts of phenolics content were calculated according to the calibration curve and expressed as milligram of Gallic Acid Equivalents (GAE) per g of dry weight (mg GAE/g dw).

Estimation of total flavonoids content

The total flavonoids content in extract was estimated by the colorimetric aluminum chloride methodology as described by Sifaoui *et al*[18]. In short, 250 µL of each extract were mixed with 1000 µL of water and 75 µL of a 15%

sodium nitrite solution ($NaNO_2$). The mixture was shaken and incubated for 6 minutes. 75 µL of aluminum chloride solution ($AlCl_3$, 10%) were added subsequently. After 6 minutes of incubation, 1000 µL of sodium hydroxide solution (NaOH, 4%) were added. The mixture was adjusted to 2500 µL by adding distilled water and allowed to stand for 15 minutes. Afterwards, the absorbance was read at 510 nm against the prepared blank using a UV visible spectrophotometer. The total flavonoids content was calculated from the calibration curve prepared by quercetinas reference. The results were expressed in terms of milligram of quercetin equivalents per g of dry weigh (mg QE/g dw).

Free radical scavenging activity

The free radical scavenging activity was tested using three different methods i.e. $DPPH^{\bullet}$, ABTS and FRAP.

DPPH· radical scavenging activity

The stable radical 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) was used to measure the free radical scavenging activity of *T. hirsuta* extracts by the procedure of Ghazouani *et al*[17].

In summary, triplicates of 100 µL of each extract was mixed with 900 µL of freshly prepared methanolic $DPPH^{\bullet}$ solution and the mixture was vigorously shaken. After 25 minutes of incubation in the darkness, the absorbance of the mixture was read at 524 nm against blank solution using a UV-vis spectrophotometer. The percentage of inhibition of the free radical scavenging activity of each extract was calculated came as:

$$\% \text{ inhibition} = (A \text{ (blank)} - A \text{ (sample)} / A \text{ (blank)}) * 100$$

Where A_{sample} is the absorbance of sample with $DPPH^{\bullet}$ solution and A_{blank} is the absorbance of $DPPH^{\bullet}$ solution without the extract. Results were expressed as IC_{50} (mg/L), defined as the concentration of the test material required to scavenge 50% of $DPPH^{\bullet}$ radicals which calculated from the inhibition percentage of radical scavenging activity. Ascorbic acid was used as a reference.

ABTS radical scavenging activity

The $ABTS^{\bullet+}$ (2,2'-azinobis-3-ethylbenzothiazoline- 6-sulphonate) radical cation scavenging assay was estimated based on the method outlined by Ghazouani *et al*.[17].

The $ABTS^{\bullet+}$ solution was prepared using: 1 mL of 7 mM ABTS (in distilled water) was mixed with 1 ml of 2.45 mM potassium persulfate solution ($K_2S_2O_8$, in distilled water). Prior to assay, the mixture was diluted in distilled water to give an absorbance ranging from 0.70 to 0.90. In this assay, about 100 µL of each extract at different concentration was taken in different test tubes and 900 µL of distilled water was added to each test tube. Afterwards, the mixture was vigorously shaken and allowed to stand for 6 min in the dark at ambient temperature. Then, Inhibition of $ABTS^{\bullet+}$ radical by the plant samples was measured at 734 nm using a UV-vis spectrophotometer. Results were expressed as IC_{50} (mg/L) and calculated with the same formula cited above in the $DPPH^{\bullet}$ scavenging assay. Ascorbic acid was used as standard. Triplicate analysis were carried out for each sample

Ferric Reducing Antioxidant Power (FRAP) assay

The ferric reducing ability of various *T. hirsuta* extracts was determined by the method outlined by Saoudi *et al*[19]. Briefly, the FRAP reagent solution was freshly prepared by mixing 1 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution (in hydrochloric acid (40 mM))), 1 mL of 20 mM Iron(III) chloride ($FeCl_3$) solution (in distilled water) and 10 mL of 300 mM acetate buffer solution (in distilled water, pH =3.6). Then, the mixture was shaken and warmed at 37 ºC. In order to perform this assay, 50 µL of each extract were added to 1500 µL of FRAP reagent solution and allowed to stand in the darkness for 30 minutes at 37 ºC. After that, ferric reducing ability of *T. hirsuta* extracts was measured at 595 nm. the results were expressed as $FeSO_4,7H_2O$ equivalent mM per gram of Dry Extract (mM /g DE) using a calibration curve. Triplicate analysis were carried out for each sample.

Statistical analysis

All results were expressed as means ± standard deviations and all measurements were done in triplicate. Statistical Analysis was carried out by one-way analysis of variance (ANOVA) and Duncan multiple range test using IBM SPSS Statistics 21. The PCA and SCA were performed using XLSTAT.

RESULTS AND DISCUSSION

Extraction yields

Extraction of *T. hirsuta* aerial part, collected from four different regions in Tunisia, was carried out by two different extraction methods such as successive soxhlet extraction and cold maceration with solvents of increasing polarity: hexane, ethyl acetate and methanol. The extraction yield was expressed as percent (%) and summarized in Table 2.

As shown in table 2, the yields of organic extracts prepared by cold maceration and soxhlet varied significantly (Á < 0.05) from 0.61 to 8.11 %, depending on the extraction method, origin geographic and solvent. The yield percent of *T. hirsuta* extracts through successive soxhlet and maceration techniques at different polarities of solvent shows that the highest extraction yield was recovered with solvent methanol (4.02 to 8.11 %), followed by ethyl acetate (1.41 to 2.35 %) either through maceration or soxhlet, whereas, the lowest yield was recovered by hexane (0.61 to 2.57 %). We should notice that the different polarities of solvent affect the extraction yield. The increase of the solvent polarities induces the enhancement of the extraction yield significantly. Such variation can be explained by the simple fact that the chemical composition varied considerably according to the solvent polarities, which could be related to the abundance of the polar compounds present in various *T. hirsuta* extracts. Despite the different solvent used, the soxhlet extraction method found to have higher yield over than cold maceration method. This result might be justified by the different conditions used in both extraction methods especially temperature and duration of extraction. In the soxhlet process, it is necessary to work at high temperature to boil the solvent. Whereas, the cold maceration process was carried out at ambient temperature. Raising the temperature leads the improvement the process efficiency. As regards the geographic variation, it clearly appears that the yield varies from one region to another. In maceration, the methanol extract yields percentage were found to be in the following order: Fernana > Tunis > Kasserine > Chebba, whereas, in soxhlet extraction it was in the order of: Chebba > Fernana > Kasserine > Tunis. these findings show that the extraction yield is highly influenced by factors such as extraction methods, geographic origin and solvent. To our knowledge, there are no previous studies investigating the extraction yield of *T. hirsuta*

Table 1. Geographical coordinates of various growing region of *T. hirsuta* where samples were collected

Regions	Latitude	Longitude	Altitude
Tunis	N 36°49'08"	E 10°09'56"	23
Chebba	N 35°14'13"	E 11°06'54"	3
Fernana	N 36°30'04"	E8°46'48"	143
Kasserine	N 35°10'03"	E 8°50'11"	674

Chemical composition of various *T. hirsuta* extracts

The chemical composition namely phenolic, flavonoids and tannins of *T. hirsuta* extracts obtained by the two extraction methods was estimated and summarized in Table 2 ~~It is appearing through the observation of the total phenol contents illustrated in table 2~~ which shows that the various extracts obtained from the aerial part of *T. hirsuta* are rich in phenolic compounds with amounts fluctuating between 29.37 ± 1.34 and 362.74 ± 3.49 mg EAG/g dw.

The content of polyphenols varies according to the polarities of solvent, extraction methods and geographical origin. In both extraction methods, the methanolic extract possess the highest

Table 2. Extraction yield of *T. hirsuta* aerial parts by two different methods

Regions	Samples	Extraction yield (%) Soxhlet	Cold maceration
Tunis	Hexane	2.57 ^{e}	0.61^{l}
	Ethyl acetate	2.35^{f}	1.83^{f}
	Methanol	5.17^{d}	4.88^{b}
Kasserine	Hexane	2.07^{h}	0.77^{k}
	Ethyl acetate	1.77^{k}	1.52^{h}
	Methanol	5.48^{c}	4.39^{c}
Chebba	Hexane	1.98^{i}	2.33^{e}
	Ethyl acetate	1.82^{j}	1.64^{g}
	Methanol	8.11^{a}	4.02^{d}
Fernana	Hexane	2.3^{g}	1.38^{j}
	Ethyl acetate	1.54^{b}	1.41^{i}
	Methanol	6.18^{b}	4.99^{a}

a-i: means within a column row with different letters were significantly different (Á<0.05)

total phenol contents (191.59 ± 2.09 to 362.74 ± 3.49 mg EAG/g dw), followed by the ethyl acetate (53.81 ± 2.19 to 165.47 ± 4.95 mg EAG /g dw). On the other hand, the hexane solvent weakly extracts the total phenolics content which proportions range from 29.37 ± 1.34 to 81.79 ± 0.43 mg EAG /g dw. Statistically the variation in the total phenolic content as a function of the type and the polarity of used solvents is significant ($p < 0.05$). Such variation might be attributed by the solvent extractive capacity. Therefore, it is known that the phenolic compound was better extracted by polar solvent[20]. Furthermore, table 2 showed also that the total phenolic content of *T. hirsuta* extract reveals that the cold maceration technique was more efficient than the soxhlet process, which yielded higher polyphenol content. The comparison between the areas tested shows that the total phenolic content differs from one region to another regardless the type of solvent used. This suggests that the phenolic compounds extraction was very affected by several factors which can be climatic conditions, altitude, latitude, longitude and soil.

About the total flavonoid content, we can notice that the amount ranged quantitatively from 26.22 ± 6.06 to 163.64 ± 3.32 mg QE/g dw and from 47.61 ± 2.29 to 162.61 ±2.60 mg QE/g dw for soxhlet and maceration techniques, respectively. These results show that the maceration method obtained higher amount of flavonoids in comparison with soxhlet method with the only exception of Tunis methanol extract and Fernana hexane extract. The amount of total flavonoids varies considerably according to the solvents used and geographical origin as well as extraction methods. Statistically, the difference between the flavonoid contents as a function of region is significant. This can be related to the chemical composition which changes from one region to another regardless the extraction method.

In maceration, we observed that the extracts corresponding to Chebba region are richer in flavonoids, followed by extracts relating to the Kasserine region, then the extracts from Fernana region and finally we found the extracts of Tunis which contain the lowest amount. In soxhlet, the highest amount of flavonoids was found in methanol extract of Tunis, followed by Kasserine methanol extract and then Chebba methanol extract. As for the Fernana methanol extract, it has a lower flavonoid content. Regarding the solvent used for both extraction methods, it seems like that

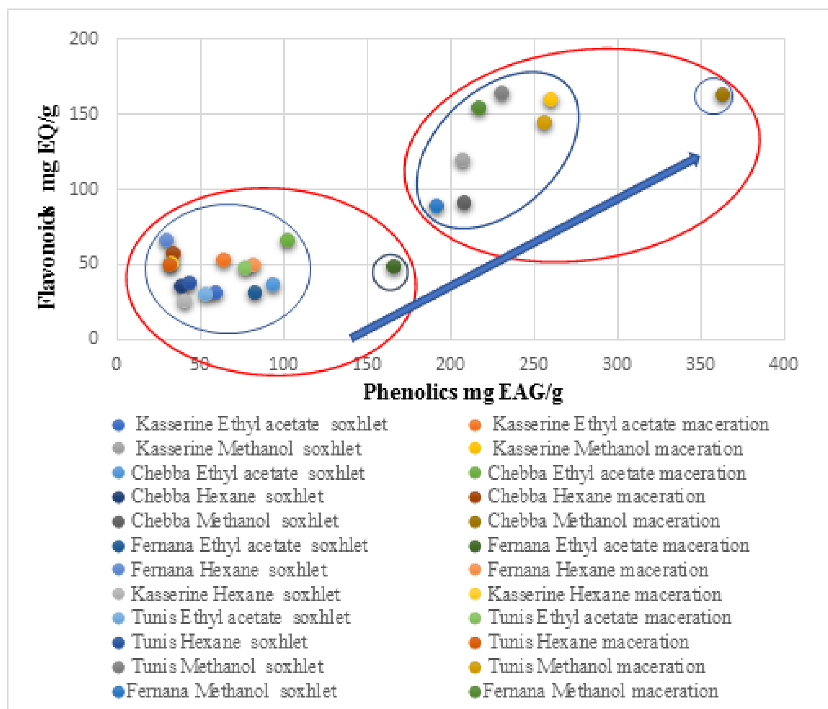

Fig. 1. Biplot obtained from scatter plot illustrating separation of groups

the methanol extracts gave the highest amount of flavonoids (from 88.76 ± 7.24 to 163.64 ± 3.32 mg QE/g dw (soxhlet) and from 144.32 ± 2.60 to 162.61 ±2.60 mg QE/g dw (maceration)), followed by ethyl acetate (from 30.45 ± 2.15 to 37.26 ± 0.51 mg QE/g dw (soxhlet) and from 47.61 ± 2.29 to 65.99 ±5.379 mg QE/g dw (maceration)), whereas, the lowest amount of flavonoids was obtained by hexane extract (from 26.22 ± 6.06 to 66.25 ± 5.17 mg QE/g dw (soxhlet) and from49.43 ± 1.55 to 57.62 ± 1.19 mg QE/g dw (maceration)). These results highlighting chemical differences between various extracts obtained in accordance with region, organic solvent and extraction method. This behavior is confirmed by scatter plot analysis (SCA) performed on the variability of polyphenol and flavonoids composition.

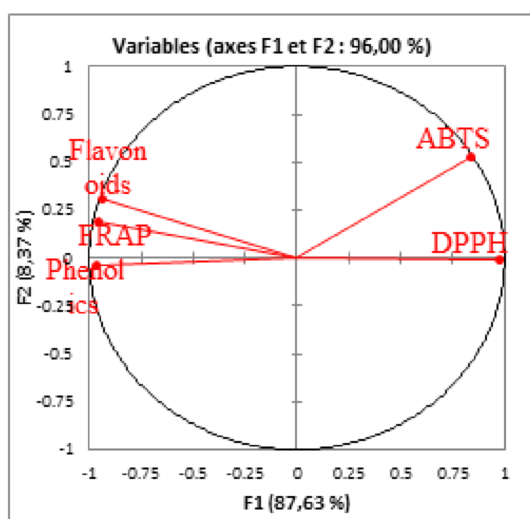

Fig. 2. Biplot obtained from PCA illustrating correlation between antioxidant activity and chemical composition mainly phenolic and flavonoid compounds

Figure 1 revealed a variability among the extraction solvent used in relation to phenolics and flavonoids composition by showing clear two separated groups. The first one groups the hexane and ethyl acetate extracts obtained by both extraction methods, indicating that these extracts possess almost similar phenolic and flavonoids composition, with the exception of macerated Fernana ethyl acetate extract that contain a higher phenolic compound compared to extracts from the same group. The second group contains only the methanol extracts, showing the richness of these extracts in phenolic compounds, with the exception of macerated methanol extract corresponding to Chebba region that is far to the methanol extracts of the same group, which shows a difference in the chemical composition (mainly phenolic compounds). This allows us to make inferences towards the possible impact of regional factor and extraction solvent on phenolic compounds.

This phytochemical study showed that the various macerated extracts were richer in phenolic

Table 3. Phytochemical composition from *T. hirsuta* extracts obtained by two different extraction methods

Regions	Samples	Chemical composition			
		Phenolics (GAE)*		Flavonoids (QE)*	
		Soxhlet	Cold maceration	Soxhlet	Cold maceration
Tunis	Hexane	43.40 ± 0.58$^{h, A}$	32.10 ± 0.43$^{h, B}$	37.95 ± 4.78$^{e, A}$	50.37 ± 1.19$^{f, g, A}$
	Ethyle acetate	53.81 ± 2.19$^{g, B}$	77.30 ± 1.16$^{f, A}$	30.45 ± 2.15$^{e, f, B}$	47.61 ± 2.29$^{g, A}$
	Methanol	230.19 ± 4.03$^{a, B}$	256.07 ± 4.03$^{b, A}$	163.64 ± 3.32$^{a, A}$	144.32±2.60$^{a, B}$
Kasserine	Hexane	40.51 ± 1.62$^{h, A}$	33.24 ± 1.09$^{h, A}$	26.22 ± 6.06$^{f, A}$	50.72 ± 2.73$^{f, g, A}$
	Ethyle acetate	59.14 ± 1.16$^{f, A}$	63.97 ± 2.09$^{g, A}$	31.83 ± 1.69$^{e, f, B}$	53.57 ± 1.79$^{e, f, A}$
	Methanol	206.70 ± 2.15$^{b, B}$	259.63 ± 3.17$^{b, A}$	119.47 ± 6.57$^{b, A}$	160.19 ±5.17$^{a, B}$
Chebba	Hexane	38.70 ± 2.12$^{h, A}$	33.75 ± 1.37$^{h, A}$	35.45 ± 0.99$^{e, B}$	57.62 ± 1.19$^{e, A}$
	Ethyle acetate	93.94 ± 1.0$^{d, A}$	102.29 ± 3.81$^{e, A}$	37.26 ± 0.51$^{e, B}$	65.99 ±5.379$^{d, A}$
	Methanol	208.35 ± 8.14$^{b, B}$	362.74 ± 3.49$^{a, A}$	91.52 ± 8.36$^{c, B}$	162.61 ±2.60$^{a, A}$
Fernana	Hexane	29.37 ± 1.34$^{i, B}$	81.79 ± 0.43$^{f, A}$	66.25 ± 5.17^{dA}	49.43 ± 1.55$^{f, g, A}$
	Ethyle acetate	82.89 ± 1.55$^{e, B}$	165.47 ± 4.95$^{d, A}$	31.05 ± 3.09$^{e, f, B}$	49.08 ± 2.15$^{f, g, A}$
	Methanol	191.59 ± 2.09$^{c, B}$	217.28 ± 4.59$^{c, A}$	88.76 ± 7.24$^{c, B}$	153.89 ± 2.60$^{b, A}$

*: mg/g dw; ±: Standard deviation; GAE: Gallic Acid Equivalent; QE: Quercetin Equivalent; CE: Catechin Equivalent.
a-i: means within a column row with different letters were significantly different ($p<0.05$).
A-B: means within a row with different letters were significantly different ($p<0.05$).

Table 4. Antioxidant activity (DPPH$^{\bullet}$, ABTS$^{\bullet +}$ and FRAP) of *T. hirsuta* extracts obtained by two different extraction methods

Regions	Samples	Antioxidant activities					
		DPPH$^{\bullet}$ (mg/L)		ABTS$^{\bullet +}$ (mg/L)		FRAP (mM/g)	
		Soxhlet	Cold maceration	Soxhlet	Cold maceration	Soxhlet	Cold maceration
Tunis	Hexane	$> 200^{d, A}$	$> 200^{f, A}$	$> 200^{h, A}$	$> 200^{c, d, A}$	$0.044 \pm 0.007^{g, A}$	$0.040 \pm 0.005^{e, A}$
	Ethyle acetate	$> 200^{c, A}$	$195.25 \pm 1.70^{c, B}$	$168 \pm 1^{g, A}$	$104 \pm 1.41^{b, c, B}$	$0.141 \pm 0.001^{f, B}$	$0.18 \pm 0.009^{c, d, A}$
	Methanol	$12.46 \pm 1.85^{a, A}$	$4.97 \pm 0.05^{a, A}$	$9.68 \pm 0.09^{a, A}$	$4.45 \pm 0.09^{a, B}$	$2.153 \pm 0.09^{a, A}$	$1.49 \pm 0.11^{a, B}$
Kasserine	Hexane	$> 200^{d, A}$	$> 200^{f, A}$	$> 200^{j, A}$	$> 200^{e, A}$	$0.038 \pm 0.002^{g, B}$	$0.066 \pm 0.003^{e, A}$
	Ethyle acetate	$> 200^{c, A}$	$> 200^{e, A}$	$124.25 \pm 3.30^{f, B}$	$168.5 \pm 2.12^{d, A}$	$0.136 \pm 0.009^{f, A}$	$0.177 \pm 0.004^{c, d, A}$
	Methanol	$17.82 \pm 1.25^{a, A}$	$4.75 \pm 0.07^{a, B}$	$9.53 \pm 0.11^{a, A}$	$4.11 \pm 0.10^{a, B}$	$0.88 \pm 0.059^{c, B}$	$1.59 \pm 0.072^{a, A}$
Chebba	Hexane	$> 200^{d, A}$	$> 200^{f, A}$	$> 200^{h, A}$	$> 200^{e, A}$	$0.031 \pm 0.007^{g, B}$	$0.08 \pm 0.005^{e, d, A}$
	Ethyle acetate	$> 200^{c, A}$	$198 \pm 1.73^{d, B}$	$74.65 \pm 1.52^{e, A}$	$49.25 \pm 1.76^{a, b, B}$	$0.175 \pm 0.0005^{e, f, B}$	$0.21 \pm 0.005^{c, A}$
	Methanol	$15.9 \pm 0.66^{a, A}$	$5.3 \pm 0.26^{a, B}$	$20.87 \pm 0.73^{d, A}$	$4.40 \pm 0.08^{a, B}$	$0.80 \pm 0.001^{d, A}$	$1.28 \pm 0.17^{b, A}$
Fernana	Hexane	$> 200^{e, A}$	$> 200^{f, A}$	$> 200^{i, A}$	$> 200^{e, A}$	$0.048 \pm 0.001^{g, A}$	$0.040 \pm 0.005^{e, A}$
	Ethyle acetate	$> 200^{b, A}$	$> 200^{d, A}$	$65.25 \pm 1.70^{d, A}$	$31.35 \pm 0.21^{a, B}$	$0.197 \pm 0.020^{e, A}$	$0.186 \pm 0.021^{c, d, A}$
	Methanol	$16.1 \pm 0.24^{a, A}$	$15.86 \pm 0.46^{b, A}$	$15.7 \pm 0.55^{b, A}$	$10.33 \pm 0.64^{a, B}$	$1.070 \pm 0.02^{b, A}$	$1.508 \pm 0.197^{a, A}$
Vitamin C	3,54 ± 0,08	2.94 ± 0.04		-			

±: Standard deviation; a-i: means within a column row with different letters were significantly different (Á<0.05).
A-B: means within a row with different letters were significantly different (Á<0.05).

compounds than the extracts obtained by soxhlet, indicating that extraction methods had a significant impact on the bioactive molecule extraction. These data allow us to suggest that maceration method was more efficient to extract aerial parts of *T. hirsuta*. Although the cold maceration technique is one of the most used methods to extract a group of fragile molecules but it is time consuming. However, the soxhlet extraction method needed a high temperature to extract the bioactive substances, which could be influences on the quality of extracts causing thermal degradation. Previous studies showed that some thermolabile compounds may decompose in the soxhlet method[21-22]. In fact, some phenolic compounds are thermo-sensitive mainly flavan-3-ol and derivatives as well as anthocyanin, which needed to be extracted under moderate temperature[23-24].

Only four studies in the literature reported the phenolic and flavonoids of *T. hirsuta extracts* prepared by cold maceration technique. Both Akrout *et al* (2011) and Trigui *et al* (2013) have found a greater phenolic content (345.2 mg GAE/g dry extract in 50:50 aqueous/ethanol extract and 147.6±1.8 to 6.1±1.5mg GAE/g dw, respectively) [9,13], whereas, Djeridane *et al*. (2006) and Amari *et al* (2014) have shown that the *T. hirsuta* aerial parts contain a small amount of total phenolic with a 6.81±0.4 mg GAE/g dw in 70:30 aqueous/ethanol extract and 147.6±1.8 to 6.1±1.5mg GAE/g dw in water extract [15-16]. About the flavonoid content a small amount were found by Akrout *et al* (2011) (36.6 mg RE/g extract in 50:50 aqueous/ethanol extract), Trigui *et al*. (39.3±2.4 to 15.3±0.2 mg QE/g dw), djeridane *et al*. (2006) (4.95±0.81 mg RE/g dw in 70:30 aqueous/ethanol extract) and Amari *et al*. (2014) (5.70±0.06 to 2.61±0.13 mg QE/g dw in water extract).

Antioxidant activity

To evaluate the antioxidant activities of the various extracts from *T. hirsuta* obtained by two different extraction methods, we used three different in vitro assays namely DPPH$^•$, ABTS$^{•+}$, and FRAP. In this study, the antioxidant activities were expressed as IC_{50} for DPPH$^•$ and ABTS$^{•+}$ while FRAP results were expressed in terms of mM $FeSO_4$/g dw. The results for DPPH$^•$, ABTS$^{•+}$, FRAP and standard antioxidant were summarized in Table 4.

As shown in Table 4, both IC_{50} values of DPPH$^•$ and ABTS$^{•+}$ had wide variability among areas, extraction methods and solvent. The DPPH$^•$ radical-scavenging activity of various methanolic extracts obtained by cold maceration varied from 4.75 ± 0.07 to 15.86 ± 0.46 mg/L. In fact, they have significantly higher antioxidant activity than methanolic extracts obtained by soxhlet extraction with IC_{50} values ranged from 12.46 ± 1.85 to 17.82 ± 1.25 mg/L. Such variation demonstrated the impact of extraction methods on the antioxidant compounds extraction. Despite the difference of polarities of solvent used, these data indicated that the cold maceration process always gives extracts rich in antioxidant molecule. This can be explained by the difference of experimental conditions carried out in both extraction methods especially temperature and duration of extraction. Indeed, the temperature can cause thermal degradation of antioxidants.

In terms of the impact of geographical sampling location, the DPPH$^•$ radical-scavenging ability of the methanolic extract can be arranged for maceration in the following order Kasserine >Tunis >Chebba >Fernana. Whereas, for the soxhlet methods it was in the order of Tunis >Chebba >Fernana >Kasserine. This allows to deduce that the chemical composition varies from localities.

This result was correlated with ABTS$^{•+}$ assay where ethyl acetate (31.35 ± 0.21 to 168.5 ± 2.12 mg/L) and methanolic (4.11± 0.10 to 10.33± 0.64 mg/L) extracts obtained by maceration methods were also found more active than ethyl acetate (65.25 ± 1.70 to 168 ±1 mg/L) and methanolic (9.53±0.11 to 20.87 ± 0.73 mg/L) extracts of soxhlet. Statistically, the variation of the antioxidant activity as a function of the extraction solvent used is highly significant ($p < 0.05$). Comparing the different solvents, the methanolic extract also exhibited a stronger antioxidant properties, followed by ethyl acetate that possess a moderate activity for DPPH$^•$ and ABTS$^{•+}$ assays. In contrast, the hexane extracts were not active for both extraction methods. The results obtained showed that the methanolic extracts present an antioxidant activity near to that found by standard antioxidant and especially in case of maceration extracts. All these results led us to note that the IC_{50} values obtained by both assays are in concordance where the methanolic extracts relative to the four

regions exhibit a notable antioxidant effect with respect to the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals. From data illustrated in Table 4, it can be clearly noticed that the IC_{50} values relating to $ABTS^{\bullet+}$ assay are lower than those found by $DPPH^{\bullet}$ assay. This variation can be related to the kinetics of reaction. In fact, reactions to the $ABTS^{\bullet+}$ test involve an electron transfer and occur at a faster rate, contrary to the $DPPH^{\bullet}$ radical where the discoloration degree is due to the ability to give hydrogen of the test compounds[25].

In the case of FRAP assay, the values obtained ranged from 0,04 ± 0,005 to 1.59 ± 0.072 mM/g DE(maceration) and from 0.031 ± 0.007 to 2.153 ± 0.09 mM/g DE (soxhlet) according to origin geographic origin and solvent. Regarding the polarities of solvent, it is important to notice that the methanol extract possessed a higher activity, followed by ethyl acetate extract, whereas, the hexane extract presents a lower activity regardless to extraction method and region. As presented in Table 4, it can be clearly observed that the results were similar to those found by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, where the maceration method (especially methanol extract)s was statistically more efficient for extraction antioxidant compounds except to the soxhlet methanol Tunis extract. The observed substantial antioxidant activity of the extracts could be explained by their richness in phenolic compounds.

According to the literature, these results are in agreement with data found by Amari *et al*. (2014), who revealed that the flower, leaf and steam extracts of *T. hirsuta* prepared by cold maceration have a greater antioxidant activity using three different methods $DPPH^{\bullet}$, $ABTS^{\bullet+}$and FRAP [16]. However, Akrout *et al* (2011) showed that the 50% aqueous ethanol extract obtained by cold maceration possessed a feeble DPPH activity (EC_{50}=3.025 mg/mL)[9]. Additionally, Djeridane *et al* (2006) revealed a moderate ABTS scavenging activity in 70% aqueous ethanol extract with a value of 17.62 mmol TEAC/ g dw[15]. Another study performed by Trigui *et al* (2013) indicated a good DPPH scavenging activity in the acetone (EC_{50}=39.2 µg/mL) followed by ethyl acetate (EC_{50}=88.69 µg/mL) and water extracts (EC_{50}=187.96 µg/mL)[13]. Our study is, to our knowledge, the first report on the antioxidant activity of *T. hirsuta* extracts obtained by soxhlet.

Correlation between phytochemical composition and antioxidant activity

The principal component analysis (PCA) was performed to evaluate the correlation between phytochemical composition and antioxidant activity. The results were presented in Figure 2.

Analysis of the results demonstrated a higher correlation between the $DPPH^{\bullet}$and $ABTS^{\bullet+}$. Conversely, no positive correlation was observed between phenolic contents and their antioxidant power measured by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. In fact, several parameters can be explained these results. The antioxidant radical scavenging capacity of various extracts could be justified by the presence of other bioactive molecules. On the other hand, the Frap results correlated well with phenolic and flavonoid compounds. Therefore, the highest Frap results were attributed by the presence of phenolic and flavonoid compounds.

CONCLUSION

The Present study describes the effect of extraction methods as well as the geographic sampling origin on the phytochemical content and antioxidant properties of extracts obtained. To the best of our knowledge, it was the first time that the *T. hirsuta* aerial part was extracted successively by soxhlet and cold maceration using hexane, ethyl acetate and methanol as solvents. Comparing the extraction methods, we have found that the cold maceration was more efficient than soxhlet to extract the phenolic compounds exhibiting a higher antioxidant property. Furthermore, it appears that the chemical composition differs quantitatively between geographical areas. In summary, our findings revealed that *T. hirsuta* aerial parts could be used as potential natural source of antioxidants. The aerial part of this plant show a potential as a therapeutic agent to treat or prevent against oxidative stress. Nevertheless, further studies are needed to identify bioactive substances, especially in macerated methanol extracts that possess a higher amount of phenolic compounds and a strong antioxidant activity.

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