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Effect of solvent polarity on the content of biomolecules and antioxidant activity of *Thapsia garganica* (Apiaceae)

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Abstract: For the first time, we have evaluated the antioxidant properties of principal secondary metabolites of methanol, ethyl acetate, hexane and water extracts of *Thapsia garganica* leaves. In this study, phenolic compounds were extracted and isolated l from *T. garganica* leaves. Phytochemicals and antioxidant capacity of the used extracts were firstly investigated. The ethyl acetate extract exhibited the highest total phenol content (11.72 ± 1.8 mg AGE/g DW), in addition to the flavonoids concentration (3.45 ± 0.06 mg CE/g DW) and the anthocyanins (33.56 ± 4.25 mg/l). In the other hand, the highest level of tannins content was measured in the polar aprotic solvent ethyl acetate extract (4.73 ± 0.22 mg CE/g DW). The different extracts of *T. garganica* were evaluated for their radical scavenging activities by means of the DPPH assay. The strongest scavenging activity was observed in ethyl acetate fraction scavenged radicals effectively with IC_{50} values of 0.16 ± 0.02 mg/ml of extract. Similarly, the total reducing power of ethyl acetate extract was found higher than other extracts in both potassium ferricyanide reduction (FRAP) and $ABTS^{\bullet+}$. The present study found that ethyl acetate extracts of *T. garganica* have effective antioxidant and radical scavenging activities as compared to other extracts.

Keywords: Antioxidant activity, *Thapsia garganica* leaves, extracting solvents, phenolic compounds.

I. Introduction

Since the prehistoric time, many medicinal plants were used in folk medicine [1, 2, 3, 4, 5]. They have been used all over the world for thousands of years as natural medicines possessing therapeutic and other pharmacologic effect. Today, according to the World Health Organization (WHO), as many as 80% of the world's people depend on traditional medicine for their primary health-care needs. The preliminary results of a study on behalf of WHO have shown that the number of individuals using medicinal plants is large and in increase, even among young people [6]. Medicinal plants or parts of these plants (leaves, rhizomes, roots, seeds, flowers) can be utilized in different forms such as fresh crude form and preparations as teas, decoctions, powdered plant material, or extracted forms of medicinal agents (juices, water or alcohol extracts, tinctures, essential oils, resins, balsams). Medicinal plants are generally known and popular for a number of health benefits such as blood pressure decreasing, prevention of cardiovascular diseases, or reducing the risk of cancer also due to their antioxidant activity [7, 8, 9, 10, 11, 12]. The preservative effect of many plant spices and herbs suggests the presence of antioxidative and antimicrobial constituents in their tissues [13]. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity [14]. Apiaceae is a big plant family, regrouping up to 3000 species. This family contains many plants with medicinal properties and used in traditional medicine [15]. The characteristic of this family is the presence, in all organs, of bioactive secondary metabolites such as

essential oils, polyphenols: flavonoids, phenolic acids; coumarins (furano- and pyranocoumarins), saponins, alkaloids and polyacetylenes [16, 17, 18]. *T. garganica* L. (Apiaceae) is an umbelliferous plant growing in the Mediterranean area. Advantage of the skin irritating effects of the plant has been taken in traditional Arabian medicine for millennia [19], and the resin of the root was last included in the 1937 edition of the French Pharmacopoeia. Also the toxic effects of parts of the plant in fodder have been known for centuries [19]. In spite of the ancient knowledge of the effects of the plant, the chemistry and the pharmacology was not understood until the early 1980's. Moreover, a number of new either biosynthetically or pharmacological very interesting compounds have been isolated from plants belonging to the genus *Thapsia* [20]. An ancient remedy, *Resina thapsiae*, used against pulmonary diseases, catarrhs and rheumatic pains, was obtained from the roots of the Mediterranean plant *T. garganica* L. [21, 20] The resin, which is located in schizogenous secretory canals in the root bark, provokes a vigorous contact dermatitis expressed as erythema, itching and small vesiculae. As recently as the nineteenth century, the resin was used in medicine, especially by the Arabs of north Africa, who named the resin *Bou néfa* (father of health), and the plant, *Dérias* [22]. The resin of *T. garganica* and a medical plaster has been included in the 1937 edition of the French pharmacopoeia. However, there is rare study on the effect of solvents with various polarities on extracting active compounds from *T. garganica* related to their antioxidant activities. Because, the determination of the best solvent extract for *T. garganica* with measurement of total phenolic content and various antioxidant activity assays was one important factor to increase extraction process efficacy. The aim of this study was to examine the effect of different extracting solvents with different polarity on phenolic compounds and antioxidant activity (Ferric reducing antioxidant power [FRAP], 2,2-diphenyl-1-picrylhydrazyl [DPPH] and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid [ABTS]) of *Thapsia garganica*.

II. Experimental Section

II.1 Plant materials

Fresh leaves from *T. garganica* were collected in Mai 2014 in Djebel Bou Ramli (34°31'8" N and 8°32'48" E), Gafsa (South Tunisian) (**Fig. 1**). The plant was identified by Pr. Mohamed Chaieb, botanist at the University of Science (Sfax, Tunisia).

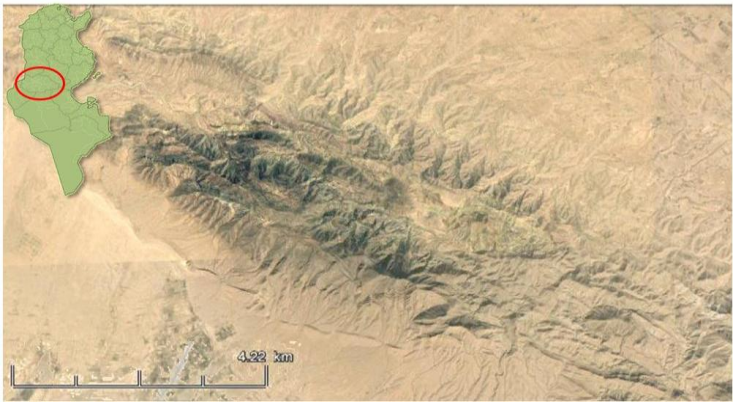

Fig. 1. *Location map of the study area (Google earth).*

II.2 Extraction procedure of biomolecule

Methanolic fraction: The leaves of *T. garganica* (1 g) were broken into small pieces and macerated in methanol (20 ml) at room temperature for 48 h. The extract was then separated from the residue by filtration through Whatman 0.45 µm filter paper. The solvent was evaporated under vacuum at 60 °C. The residue was weighed and dissolved in methanol (3 ml) for further analysis. Eventually, the solutions were stored at -20 °C. The remaining aqueous solution was fractionated successively with hexane and ethyl acetate (**Fig. 2**).

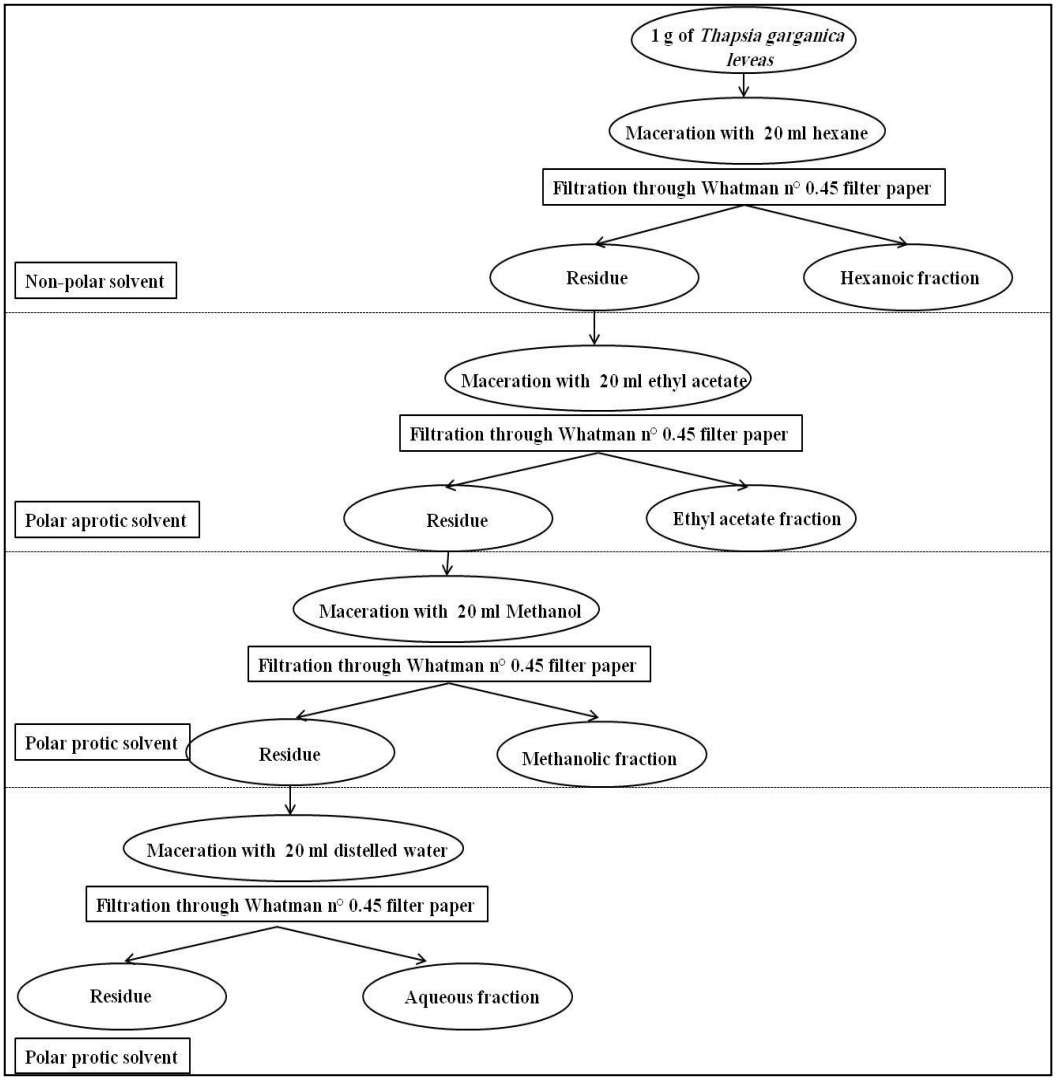

Fig.2. *Extraction procedure of biomolecules.*

II.3. Chemicals

Butylated Hydroxytoluene (BHT), 2,20-diphenyl-1-picryl-hydrazyl (DPPH), catechin, 2,4,6-tripyridyl-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid (trolox) and 2,2-azinobis (3-ethyl benzothiazoline- 6-sulfonic acid) diammonium salt (ABTS) (**Fig. 3**)

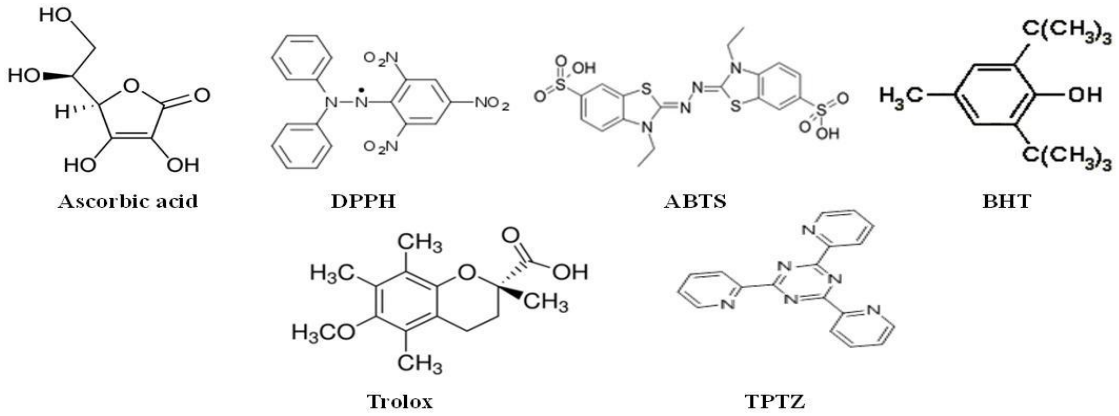

Fig.3*. Chemical structures*

II.4. Determination of total phenolic content

Total phenolics content of *T. garganica* was measured by Folin–Ciocalteu's phenol reagent [23, 24]. First, 200 ml of appropriately diluted sample or gallic acid standard were added to 2.6 ml of distilled deionized water. Then, 200 ml of Folin–Ciocalteu's phenol reagent was added at time zero and mixed. After 6 min, 2 ml of 7% (w/v) Na_2CO_3 solution was added and mixed. After incubation for 90 min at room temperature, absorbance was measured at 750 nm versus a prepared blank. The blank consisted of 200 mL 50% (v/v) methanol instead of sample. Gallic acid in 50% (v/v) methanol solution in concentrations of 0.1, 0.3, 0.5, and 0.7 mg/ml was used as a standard and a calibration curve was drawn for each day of analysis. The content of total phenolics was expressed as mg gallic acid equivalent (GAE)/ g of dry weight. All samples were analyzed in triplicate.

II.5. Total flavonoids content

Total flavonoid content was measured according to the method of [25]. Sample extract was added with 0.3 ml of 5% sodium nitrite and well mixed. After 5 min of incubation, 0.3 ml of 10% aluminum chloride solution was added. Then, after 6 min, 2 ml of 1 M sodium hydroxide was added to the mixture and made up the volume to 10 ml with water. The absorbance was measured at 510 nm with UV–visible spectrophotometer. Total flavonoids were measured from catechin (0–0.3 mg) standard curve and expressed as mg catechin equivalents/g of dry sample.

II.6. Determination of anthocyanins content

Monomeric anthocyanin content of *T. garganica* was measured using a spectrophotometrically pH differential protocol according to [26]. Extracts were mixed thoroughly with 0.025 M potassium chloride pH 1 buffer and similarly with sodium acetate buffer pH 4.5 in 1:10 ratio of extract to buffer. The absorbance of these solutions was measured at 510 and 700 nm. The anthocyanin content was calculated as follows:

Total monomeric anthocyanins (mg/l) = Abs x MW x 1000/ (Ɛ x C)

Where Abs is absorbance = (A515 - A700) pH 1.0 - (A515 - A700) pH 4.5; MW is molecular weight for cyanidin 3-glucoside = 449.2; Ɛ is the molar absorptivity of cyanidin 3-glucoside = 26900; and C is the concentration of the buffer in milligrams per milliliter. Anthocyanin content was expressed as milligrams of cyanidin 3-glucoside equivalents 1l of the triplicate extracts.

II.7. Determination of condensed tannins

Condensed tannins content was determined by the vanillin method as described by [27]. 3 ml of vanillin (4% in methanol) were added to 0.5 mL of the different extract. 1.5 ml of highly concentrated HCl was then added. The mixture was then kept in the dark for 15 min at 20°C. The absorbance was read at 500 nm. A calibration curve was prepared with a solution of catechin. The results were obtained in mg of catechin equivalent per g of dry weight (mg CE/g DW).

II.8. Determination of the antioxidant activity

The antioxidant potential of *T. garganica* organic extracts was determined by radical scavenging assays: the 2, 2 diphenylpicrylhydrazyl radical ($DPPH^{\bullet}$), FRAP, the 2,2′-azinobis-(3 ethylbenzothiazoline-6 sulfonic acid radical ($ABTS^{\bullet+}$) and reducing power.

II.8.1. Free radical scavenging activity on 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$)

The free radical scavenging capability of each extract solution on DPPH radicals was determined as described previously [28]. Briefly, 4ml of methanol solution of DPPH (0.1mM) was mixed with 1 mL of

each of extract (methanol, hexane, water and ethyl acetate) solution at different concentrations (0-0.4 mg/ml). The reaction mixture was incubated in a dark room for 30 minutes and the free radical scavenging ability was estimated by measuring the absorbance at 515 nm with the spectrophotometer. The reaction was carried out in capped glass test tubes that were tightly wrapped with aluminum foil. The DPPH radical stock solution was freshly prepared every day for the reaction, and precautionary measures were taken to reduce the loss of free radical activity during the experiment. The inhibition percentage of DPPH radicals was calculated as:

Inhibition (%) of DPPH radicals = (Ac – As) × 100/ Ac

Where Ac is absorbance of the control reaction (all reagents except plant extract) and As is absorbance of the sample (plant extract).

II.8.2. Ferric reducing antioxidant power (FRAP) assay

The procedure described by Benzie and Strain was followed [29]. The principle of this method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, colored form in the presence of antioxidants. Briefly, the FRAP reagent contained 2.5 ml of a 10 mmol/L TPTZ (2,4,6- tripyridy-*s*-triazine, Sigma) solution in 40 mmol/l HCl plus 2.5 ml of 20 mmol/L $FeCl_3$ and 25 ml of 0.3 mol/l acetate buffer, pH 3.6 and was prepared freshly and warmed at 37°C. Aliquots of 40 µl sample supernatant were mixed with 0.2 ml distilled water and 1.8 ml FRAP reagent and the absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37°C for 10 min. Value of FRAP was expressed as Trolox equivalents per gram of dry weight (TE/g DW).

II.8.3. Free radical-scavenging ability by the use of ABTS radical cation (ABTS assay)

Antioxidant activities of *T. garganica* were also analyzed by investigating their ability to scavenge the $ABTS^{\bullet+}$ free radical using a modified methodology previously reported by [30]. When combined with an oxidant (2.45 mM potassium persulfate), ABTS (7 mM in 20 mM sodium acetate buffer, pH 4.5) reacts to create a stable, dark blue-green radical solution following 12–16 h of incubation in the dark (4 °C). The solution was then diluted to an absorbance of 0.7 ± 0.01 at 734 nm to form the test reagent. Reaction mixtures containing 20 µl of sample and 3 ml of reagent were incubated in a water bath at 30 °C for 30 min. As unpaired electrons are sequestered by antioxidants in the sample the test solution turns colorless and the absorbance at 734 nm is reduced. The final result was expressed as mM of Trolox equivalents (TE) per g of dry weight (DW).

II.8.4 Reducing power

The ferric reducing antioxidant power assay was used to assess the reducing capacities of extracts of *Thapsia garganica*. Different dilutions (0.05-0.5 mg/ml) of each plant extract (ethyl acetate, methanol, hexane and water). Reducing power of both extracts of *T. garganica* were measured by method of [31] with a slight modification [32]. According to this method, the reduction of Fe^{3+} to Fe^{2+} was determined by measuring absorbance of the Perl's Prussian blue complex. This method is based on the reduction of (Fe^{3+}) ferricyanide in stoichiometric excess (0.1, 0.2, and 0.4 mg/ml) of water, methanol, ethyl acetate and hexane extracts of sumac *T. garganica* in 0.75 ml of distilled water were mixed with 1 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1 ml (1%) of potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50 °C for 20 min. After 20 min of incubation, the reaction mixture was acidified with 1 ml of trichloroacetic acid (10%). Finally, 0.25 ml of $FeCl_3$ (0.1%) was added to this solution. Distilled water was used as blank and for control. Absorbance of this mixture was measured at 700 nm using a UV spectrophotometer. Decreased absorbance indicates ferric reducing power capability of sample. Ascorbic acid and Butylated hydroxytoluene (BHT) were used for comparison.

II.9 Statistical analysis of data

Data were presented as means ± SD of at least triplicate experiments. Analysis of variance was performed on the data obtained. Significance of differences between means was determined by least

significant differences (LSD) at P ≤ 0.05. Pearson's rank-correlation was performed using XL-Stat software to determine the correlations between the phenolic compounds and the antioxidant activity in the different extracts of *T. garganica*.

III. Results and Discussion

III.1. Phytochemical screening

Previous studies indicated that different solvents could lead to different extraction efficiencies of bioactive compounds [33, 34 , 35] Therefore, this study determined the impact of four different extraction solvents with various polarity indexes to identify the most effective solvent for further optimization using response surface methodology. According to our knowledge, the chemical analysis of phenolic content, flavonoids, anthocyanins and condensed tannin of *T. garganica* from a Tunisian arid area is not yet investigated. The concentrations of total phenolics (TPC), total flavonoids (TFC), anthocyanins content and condensed tannins in extracts are presented in **Table 1**.

III.1.1. Total phenolic content in different extracts (TPC)

Polyphenols have attracted considerable attention because of their various biological activities including: antioxidant, antimutagenic, antitumor, anti-inflammatory, neuro-protective and cardio-protective effects [36]. Phenolic compounds are health benefactors because they act as antioxidative agents [28]. The total phenolic contents (TPC) in *T. garganica* leaves have been evaluated. The quantitative determination of TPC is expressed as milligram gallic acid equivalents per gram dry weight of sample. The ethyl acetate extract of the leaves exhibited the highest content (11.72 ± 1.8 mg EAG/g DW) followed by the methanolic fraction (10.44 ± 1.3 mg EAG/g DW). [37] indicated that total phenolic content of ethanol/water extract of *T. garganica* leaves is about 7.63 ± 0.61 mg GAE/g DW, which seems to be less important than the results obtained in the present study. According to [38] the total phenolic content of three species of Apiaceae family (*Centella asiatica, Hydrocotyle sibthorpioides and Hydrocotyle bonariensis*) ranged from 0.28 to 0.77 mg GAE/ g DW, with *Centella asiatica* showing the highest value of 0.77 mg GAE/g DW, followed by *Hydrocotyle sibthorpioides* and *Hydrocotyle bonariensis* with TPC values of 0.58 and 0.28 mg GAE/g DW, respectively. In addition, several studies revealed that phenolic compounds content differed with solvents polarities [39, 40]. [41] reported that solvent with different polarity had significant effect on phenolics compound and antioxidant activity in higher content in more polar solvents [42, 43]. This finding could be the result of non specific reactions of Folin–Ciocalteu reagent with other components of the water extract which could overestimate the phenolic content in these extracts [44]. In addition, it is difficult to compare our results with historical data. Indeed, the extraction of phenolic compounds from their natural matrix is complicated by their diversity and their susceptibility to oxidation and hydrolysis [45]. Concerning, the hexanoic and water fractions are less enriched in total phenolic compounds (8.66 ± 0.58 mg EAG/g DW and 3.15 ± 0.009 mg EAG/g DW respectively). Methanol, hexane, ethyl acetate and aqueous extracts of *T. garganica* leaves showed significant difference (***p < 0.001) in total phenolic content. We can conclude that ethyl acetate and methanol extracts had the highest total phenolic contents. In fact, the use of solvents with different polarities leads to differences in phenolic content and antioxidant capacity [46, 47]. In this regard, several authors have remarked the importance of the extraction method and the solvent used [47, 48, 49]. In addition, in plant preparations (extracts, decoctions), the content and composition of antioxidants depend also on extraction technique, its conditions (extraction time and temperature), and solvents [33].

III.1.2. Determination of total flavonoids content

Flavonoids (phenolic plant compounds) possess a wide range of pharmacological properties. Its importance to human health lies in its capacity to scavenge free radicals [50]. In the present investigation, the aluminum chloride colorimetric method was used for detection of flavonoids content, which increased from the hexanoic to ethyl acetate fractions. In this study the ethyl acetate extract presents the highest flavonoids content (3.45 ± 0.06 mg CE/g DW) followed by the methanolic fraction (2.05 ± 0.26 mg CE/g DW). Water and hexanoic extracts showed the lowest flavonoids content (1.34 ± 0.1 and 1.14 ± 0.03 mg CE/g DW, respectively). Methanol, hexane, ethyl acetate and aqueous extracts of *T. garganica* showed significant difference (***p < 0.001) in flavonoids content. According

to [37, 51], the total flavonoids content of *T. garganica* leaves extracted with aqueous methanol (80%) ranged from 4.04 ± 0.42 to 1.25 mg RE/g DW. Moreover, [52]. indicated that the flavonoids content of the aqueous extract of *T. garganica* is about 2.83 ± 0.03 mg RE/g DW. In comparison to the present study, the findings of the previous studies dealing with *T. garganica* are less important. In fact, the increase or decrease of flavonoids content was depended on the extraction solvent polarity [53, 40].

III.1.3 Determination of anthocyanins content

Anthocyanidin, an important plant pigment, is responsible for most of the purple, red and blue colors in plants. It is a flavonoid compound synthesized from phenylalanine and malonylcoa through a series of enzymatic reactions [54, 55, 56]. It has been known that anthocyanins possess antitumor, antiulcer and anti-inflammatory properties [57, 58, 59, 60]. In the present study, the anthocyanins content of methanol, hexane, ethyl acetate and water extracts of *T. garganica* leaves was determined by the pH differential method and results are shown in **Table 1**. The ethyl acetate extract of *T. garganica* presented the highest anthocyanins content (33.56 ± 4.25 mg/ kg DW) followed by the methanolic fraction (26.45 ± 2.67 mg/ kg DW). The least concentration of anthocyanins was observed in hexanoic fraction (11.85 ± 1.84 mg/kg DW) followed by aqueous fraction (17.54 ± 3.22 mg/kg DW). Methanol, hexane, ethyl acetate and aqueous extracts of *T. garganica* showed significant difference (***p<0.001) in of anthocyanins content. In fact, anthocyanins are polar molecules which are normally extracted from raw plant tissues by conventional solvent extraction (CSE) methodologies, using polar solvents [61]. However, the type of matrix and solvent used plays an important role for the extraction of anthocyanins [62].

III.1.4. Determination of condensed tannins

Many tannin-rich medicinal and food plants have been appreciated for their beneficial effects without being troubled by any obvious toxicity [63]. Research on the tannins in traditional medicinal plants, presented here, started when the chemical, biological and pharmacological properties of tannins in most medicinal plants were not yet subjected to modern analysis [64, 65, 66, 67, 68]. In the present study, the tannins content of methanol, hexane, ethyl acetate and aqueous extracts of *T. garganica* were determined by the vanillin method and results are presented in Table 1. The highest level of tannins content was measured in the ethyl acetate extract (4.73 ± 0.22 mg CE/g DW) followed by the water extract (3.74 ± 0.25 mg CE/g DW). Methanol extract of *T. garganica* and hexanoic fraction showed low tannins content compared to ethyl acetate extract (2.24 ± 0.17 and 1.98 ± 0.21 mg CE/g DW, respectively). The different extracts of *T. garganica* showed a significant difference (***p < 0.001) in condensed tannins amounts. In comparison with other materials, these extracts contained higher condensed tannin than those obtained from *Cytisis purgans* (1.2 mg CE/g DW), *Cytisis scoparius* (0.8 mg CE/g DW) and *Galendramus occidentalis* (0.7 mg CE/g DW) [69, 70]. Generally, the extraction efficiency of bioactive compounds is largely depending on the solvent used [71, 72, 73]. The present study shows that among all the solvent; ethyl acetate and water were better solvents for effective extraction of tannins as compared to other solvents like hexane and methanol. According to [74], the polar-aprotic solvent that cannot provide OH-ions, whereas the polar protic solvent that can provide OH-ions, making it easier to interact with polar functional groups on the tannins. Therefore, the polar-aprotic solvents are less compatible for tannins extraction than polar-protic solvent (eg. ethanol). In the end, the biomolecules composition of methanol, hexane, ethyl acetate and aqueous extracts of *T. garganica* leaves were resumed in **Fig. 4**.

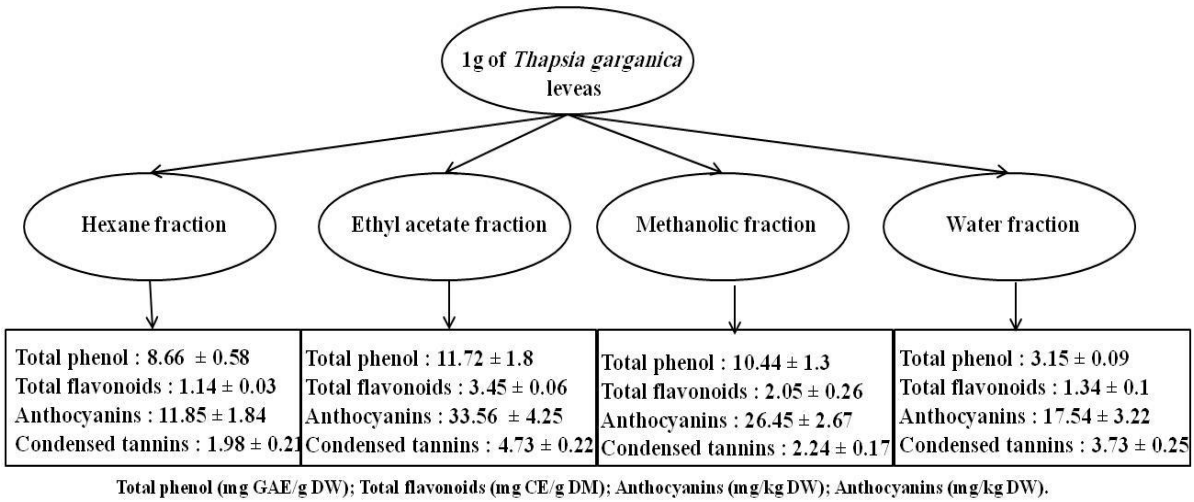

Fig. 4. *Total phenolic contents (TPC in mg GAE/g DW), total flavonoid contents (TFC in mg CE/g DW) and total condensed tannins (TCT in mg CE/g DW) of T. garganica leveas.*

Table 1: Phytochemical composition of T. garganica (L) leaves.

Extracts	Total phenol (mg GAE/g DW)	Phenolic compounds		
		Total flavonoids (mg CE/g DM)	Anthocyanins (mg/kg DW)	Condensed tannins (mg CE/g DW)
Ethyl acetate fraction	11.72 ± 1.8	3.45 ± 0.06	33.56 ± 4.25	4.73 ± 0.22
Methanolic fraction	10.44 ± 1.3	2.05 ± 0.26	26.45 ± 2.67	2.24 ± 0.17
Hexanoic fraction	8.66 ± 0.58	1.14 ± 0.03	11.85 ± 1.84	1.98 ± 0.21
Water fraction	3.15 ± 0.09	1.34 ± 0.1	17.54 ± 3.22	3.73 ± 0.25
F values	22.25***	34.14***	43.42***	24.8***

Data are presented as mean ± SD of three individual determinations. GAE = gallic acid equivalents; CE = Catechin equivalents; DW = Dry weight; ***Correlation is significant at the 0.001.

IV. Antioxidant activity of *T. garganica* organic extracts

Extraction of active compound in natural plants is potent to protect biological system against damaging effect of natural oxidation process in organism. In this study, the antioxidant capacity of *T. garganica* using different extracting solvent was evaluate by 4 assays; DPPH, FRAP, $ABTS^{+\bullet}$ and Reducing power. Each antioxidant assay possesses its own unique mechanism to evaluate the antioxidant activity in sample.

IV.1 DPPH radical scavenging activity

The antioxidant potential of methanol, hexane, and aqueous extracts of *T. garganica* was evaluated on the basis of their ability to scavenge stable free DPPH radicals and results are shown in **Fig. 5**. This test is based on change in color of DPPH solution from purple to yellow, due to scavenging of stable free DPPH radicals [75]. A stronger yellow color indicates a greater ability of the extract to scavenge free DPPH radicals and stronger antioxidant potential. The strongest scavenging activity was observed in ethyl acetate fraction followed by methanol extract, with IC_{50} values of 0.16 ± 0.02 and 0.24 ± 0.04 mg/ml of extract respectively. The hexane and water extracts of *T. garganica* leaves have the lowest $DPPH^{\bullet}$ radical scavenging ability over other extracts, with IC_{50} values of 0.27 ± 0.06 to 0.36 ± 0.01 mg/ml of extract respectively. ANOVA test shows a significant difference between the used extracts (***$p < 0.001$) in DPPH scavenging assay. The highest antioxidant activity in ethyl acetate fraction could be related to the high phenolic compounds content showed by this extract. [51]

reported that DPPH scavenging ability of *T. garganica* leaves extracted in aqueous methanol (80%) is about 17.06 ± 0.02 mg/l of extract, suggesting that our results are more important. In the DPPH$^{\bullet}$ assay, the free radical scavenging activities decreased from ethyl acetate extract to methanol, hexanoic and aqueous extracts for *T. garganica* leaves evidencing a clear secondary metabolite content due to the solvent–solvent partitioning processes [76]. An increase in DPPH scavenging ability was observed with increase in concentration of extracts. In comparison with other genus belonging to the Apiaceae family, [77] reported that the DPPH IC_{50} of *Prangos ferulacea, Chaerophyllum macropodum* and *Heracleum persicum* extracted with methanol was 0.242, 0.623 and 0.438 mg/l of extract respectively, which is close to the results obtained by the present study. The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants [78]. We can conclude that the ethyl acetate extract was the most effective in this respect.

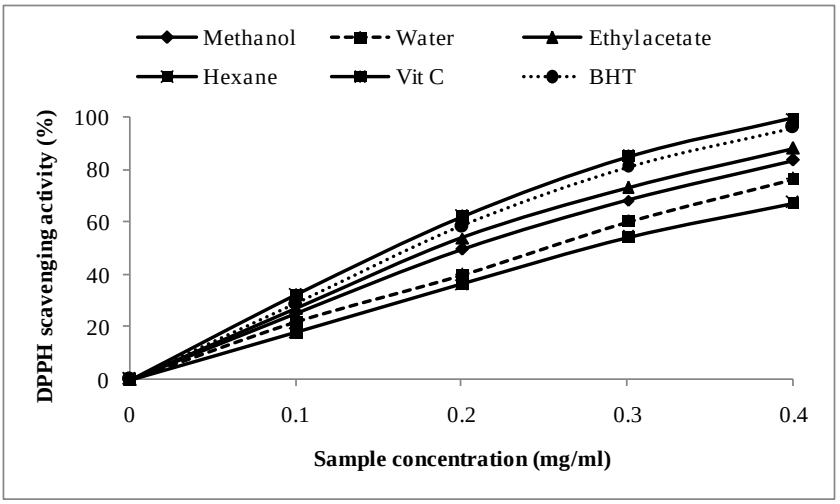

Fig. 5. *DPPH radical scavenging activities (%) of ethyl acetate, hexanoic, aqueous and methanolic fractions from T. garganica leaves. Ascorbic acid and BHT were used as positive control.*

IV.2. FRAP analysis

FRAP method was used to present rather quick and simple method measuring antioxidant presents in *T. garganica* leaves. The FRAP assay is based on the ability of phenolics to reduce yellow ferric tripyridyltriazine complex (Fe(III)-TPTZ) to blue ferrous complex (FE(II)-TPTZ) by the action of electron donating antioxidants [79]. In the present study, antioxidant activities evaluated by the FRAP scavenging capacity of methanol, ethyl acetate, hexanoic and water extracts of the samples are presented in Table 2. The leaves of *T. garganica* showed higher antioxidant activity in ethyl acetate extract (1.21 ± 0.01 mg TE/g DW) followed by methanolic fraction extract (1.08 ± 0. 21 mg TE/g DW). [37]. obtained in aqueous methanol extracts from *T. garganica* 0.06 mg TE/g DW, which is much lower than the results obtained by the present study. The least amount of FRAP scavenging was observed in water extract (0.48 ± 0.02 mg TE/g DW) followed by hexanoic fraction (0.74 ± 0.01 mg TE/g DW). In FRAP assay, the scavenging activities decreased from ethyl acetate to water extract in *T. garganica* leaves, thus, the polarity of solvents has an indirect function in the extraction process, because it can raise the solubility of antioxidant compounds [80].

IV.3 ABTS$^{\bullet+}$ scavenging activity

ABTS assay is based on the antioxidant ability of the extracts to react with ABTS$^{\bullet+}$ radical cation generated in the system. The averages values obtained for ABTS assay are given in **Table 2**. ABTS (blue–green chromophore) is mixed with the different extracts of *T. garganica* that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of their color [81]. Solvent used for phenolic compounds extraction had a significant effect on antioxidant activity. The highest level of scavenging activity was measured in the ethyl acetate extract (1.41 ± 0.32 Mm TE/g DW) followed by the methanolic fraction is about 1.32 ± 0.07 Mm TE /g DW. Similarly, in another investigation by [43]

methanol extract was found to be the solvent extracting the most efficiently antioxidants. Hexanoic and aqueous fractions have moderate activity (0.69 ± 0.12 and 0.32 ± 0.24 Mm ET/g DW, respectively). The scavenging effect of different extracts of *T. garganica* on the $ABTS^{\bullet+}$ radical decreased in following order: ascorbic acid >BHT >ethyl acetate >methanol>Hexane>water extracts. In comparison with another study, the ABTS activity of *Coriandrum sativum* is about 14.9 mM TE/g extract) and [82] which is much higher than the results obtained in the present study.

IV.4 Reducing power

In the present study, the reducing power of chemical compounds extracted from *T. garganica* leaves by methanol, hexane, ethyl acetate and water were determined and shown in Table 2.

Table 2: Effects of extracts of T. garganica on the in vitro free radical (DPPH, FRAP, ABTS and reducing power).

Extracts	Antioxydant activity				
	DPPH (%)	IC_{50} (mg/ml)	FRAP (Mm TE/g DW)	$ABTS^{\bullet+}$ (Mm TE/g DW)	Reducing power (700 nm)
Ethyl acetate (0.4 mg/ml)	94.43	0.16 ± 0.02	1.12 ± 0.01	1.41 ± 0.32	0.871
Methanol (0.4 mg/ml)	83.85	0.24 ± 0.04	1.08 ± 0.21	1.23 ± 0.07	0.776
Hexane (0.4 mg/ml)	67.35	0.27 ± 0.06	0.74 ± 0.02	0.69 ± 0.12	0.83
Water (0.4 mg/ml)	76.60	0.36 ± 0.01	0.48 ± 0.01	0.32 ± 0.24	0.574
BHT (0.4 mg/ml)	94.32	0.14 ± 0.03	1.85 ± 0.02	1.75 ± 0.04	0.957
Vit C (0.4 mg/ml)	99.83	0.11 ± 0.02	1.96 ± 0.01	1.91 ± 0.012	0.996
F values		21.8***	36.41***	72.12**	32.42***

Values are means ± SD. n = 3; *** Correlation is significant at the 0.001; ** Correlation is significant at the 0.01.

The reducing power of extracts and standard antioxidants decreased in the order of Vit C>BHT>ethyl acetate extract>hexane extract> methanol extract>aqueous extract, in presence of 0.4 mg/ml test sample. According to results obtained in the present study, both ferric reducing power and total phenolic content of ethyl acetate extract were higher than those of the hexane extract. Significant difference (***$p < 0.001$) in reducing power activity was noted in all of the investigated extracts. Total phenolic content and ferric reducing power are related with each other. Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action [83]. According to [84] at 0.4 mg/ml, the absorbance values of ethyl acetate fraction, butanolic fraction, methanolic extract, at 700 nm were 0.13; 0.11; 0.06, respectively in leaves from *Atriplex halimus*. Compared to the present study, *Atriplex halimus* reveals an antioxidant activity less important than *T. garganica.*

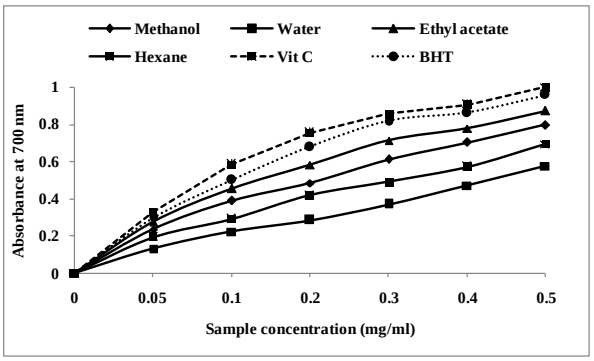

Fig. 6. *Reducing power of methanol, ethyl acetate, hexane and water extracts of T. garganica leaves.*

V. Correlation between phenolic compounds and antioxidant activity

Several studies have reported on the relationship between total phenol and antioxidant activity. Some authors have found a strong correlation between the phenolic contents and the antioxidant activity [85, 86, 87], others have found nothing [88, 89, 90]. In this study, the results have shown a relationship between antioxidant activity and total phenolic contents. Table.3 showed the Pearson's rank-correlation between the antioxidant activity and the phytochemical composition of the different extracts. The findings of this study indicate that total phenolics present high and positive correlations with ABTS scavenging capacity and FRAP ($r = 0.946$, $p<0.05$; $r = 0.956$, $p<0.05$). While a high and positive correlation was obtained between flavonoids and DPPH scavenging activity ($r = 0.953$, $p<0.05$). In fact, it's known that only flavonoids with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and show radical scavenging activity [91]. Furthermore, the extracts are very complex mixtures of many different compounds with distinct activities [91]. Anthocyanins are strongly correlated with DPPH scavenging activity, ABTS and FRAP ($r = 0.992$, $p<0.05$; $r = 0.839$ and $r = 0.793$ respectively). Contrary to the condensed tannins that has shown only a correlation with DPPH scavenging activity ($r = 0.721$, $p<0.05$).

Table 3: Pearson's correlation coefficients (r) of the phenolic compounds and the antioxidant activities

	Total phenolic	Flavonoids	Condensed tannins	Anthocyanins	DPPH scavenging	ABTS	FRAP	Reducing power
Total phenolic	1							
Flavonoids	0.689	1						
Condensed tannins	0.002	0.707	1					
Anthocyanins	0.627	0.953	0.630	1				
DPPH scavenging	0.540	0.953	0.721	0.992	1			
ABTS	0.946	0.842	0.213	0.839	0.771	1		
FRAP	0.956	0.782	0.111	0.793	0.714	0.994	1	
Reducing power	0.935	0.543	-0.057	0.388	0.310	0.785	0.792	1

$p<0.05$

VI. Conclusion

The recovery of phenolic compounds and antioxidant activity was dependent on the solvent used to extract the bioactive compounds of *T. garganica* leaves. Ethyl acetate was proved to be the best solvent for extracting bioactive compounds from *T. garganica* leaves due to the highest phenolics and flavonoids content and the strongest antioxidant activities. Phenolics were the major antioxidants presented in *T. garganica* leaves for their high correlation coefficients with antioxidant activities. Thus, this study was performed useful to evaluate the antioxidant capacity in various extracts that supports the initial study for its potential sources of potent natural antioxidant.

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