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Research Article

Evaluation of Radical Scavenging Activity, Total Phenolics and Total Flavonoids Contents of *Cistus* Species in Syria

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ABSTRACT
There is now an expansion of interest in plants and phytochemicals as new sources of natural antioxidants, many of these plants including *Cistus* lack scientific reports. To support their importance, aqueous and methanolic extracts of two *Cistus* species from Syria (*Cistus creticus* and *Cistus salvifolius*) were evaluated for antioxidant activity using 2,2-diphenyl-l-picrylhydrazyl (DPPH) assay. Total polyphenol and flavonoid contents were determined using Folin-Ciocalteu and aluminum chloride colorimetric methods respectively. The results showed that *C. salvifolius* methanol extract had the highest value of total phenolic content, which ranged in extracts between (65.99±1. 33 to 75.22±4.79 mg GAE/g DW). While the total flavonoid contents varied from (11.56±0.32 to17.68±0.71 mg RUE/g DW), the aqueous extracts exhibited a slightly higher flavonoids content than the methanolic ones. However, the total phenolic and flavonoids content, presents relatively high values when compared with other species of *Cistus*. Antioxidant activity was expressed as IC_{50} and the obtained results ranged from (IC_{50}= 0.019 to 0.007 mg/ml), the results indicated that three extracts of the four examined were greater in activity compared with BHT. Based on these results of investigation, it could be concluded that the two species of *Cistus* in Syria are rich sources of phenolic and flavonoid compounds.

Keywords: *Cistus creticus*, *Cistus salvifolius*, total phenolic content, total flavonoids content, antioxidant, DPPH.

INTRODUCTION

Medicinal plants have been routinely used for years as a source of traditional treatments of various diseases and conditions[1]. Most of these plants constitute the main source of new pharmaceutical and health care products[2]. A recent survey conducted by the World Health Organization (WHO) reported that globally around 20,000 medicinal plants are being used profusely either in pharmaceutical industry or in folk medicines, but only 1.4% of these used plants do possess well established active constituents[3]. *Cistus* is a genus of flowering evergreen shrubs in the Cistaceae, a medium-sized family consisting of eight genera and 180 species[4-6], the common name is Rock-Rose Family[7], Cistaceae show the highest diversity in the Mediterranean floristic region[6]. *Cistus* comprises about 30 species native to the Mediterranean area[4], among which *Cistus creticus* L. and *Cistus salvifolius* L. are known to be native in the flora of Syria[8]. *C. creticus* is a shrub growing to 100 cm, leaves are ovate-oblong, flowers are red-purple. flowering season is from February to April[8]. *C. salvifolius* is a shrub growing to 60 cm, leaves are ovate-elliptic, flowers are white, flowering season is from March to May[7,8]. Since ancient times the importance of *Cistus* species was reported, the leaves of several species, i.e. *C. creticus* and *C. ladanifer*, are coated with an abundant highly sticky brown aromatic resin, also known as labdanum. Besides perfumery, labdanum has been used to treat colds, coughs, menstrual problems, rheumatism, and diarrhea[9]. In addition to labdanum, a decoction of the leaves of *C. salvifolius* and *C. incanus* ssp. *creticus* are also used as a substitute for tea[5]. A survey of plants used in Jordan demonstrated that a tea prepared from *C. salvifolius* herb has traditionally been used for the treatment of gout[10]. Some interesting folk methods of using *Cistus* sp. leaves were reported in Turkey, such as a bath for the external treatment of rheumatism, and a poultice prepared from the boiled leaves is applied externally on the dorsal part of the body at the location of kidneys for the treatment of urinary inflammation[11]. The investigations about Turkish traditional plants have shown that *C. laurifolius* is highly effective for ulcer treatment[12]. In a recent study both of proanthocyanidin compounds and aqueous extract of *C. salvifolius* herb showed inhibitory effect on COX-1, COX-2[13], supporting the anti-inflammatory traditional usage[5]. Moreover, CYSTUS052®; a rich polyphenolic extract of *C. incanus* demonstrated antiviral activity against influenza A virus infections in humans[14-17]. In the same context, *C. creticus* is considered among phenolic accumulating plants as the one with the most extensive accumulation of phenolic compounds in its leaves[18]. Reactive oxygen species (ROS) are harmful intermediates produced endogenously in our body systems as a result of biological combustion involved in the cellular respiration process, exposure to physicochemical conditions or related to some diseases. They can also be produced exogenously by exposure to radiation, toxic chemicals, cigarette

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Table 1: The yields percentage of solid residue.

plant	Extract	Yield (%)
C. creticus	Methanol 80%	24.62%
	Water	21.65%
C. salvifolius	Methanol 80%	21.27%
	Water	20.15%

Table 2: Total phenolic contents in the plant extracts.

Plant	Extract	TPC (mg GAE/g DW)
C. creticus	Methanol 80%	69.34±4.68
	Water	65.99±1.33
C. salvifolius	Methanol 80%	75.22±4.79
	Water	70.34±1.52

Each value is the average of five replicates ± standard deviation.

Table 3: Total flavonoids contents in the plants extracts.

Plant	Extract	TFC (mg RUE/g DW)
C. creticus	Methanol 80%	11.56±0.32
	Water	12.41±0.22
C. salvifolius	Methanol 80%	12.50±0.25
	Water	17.68±0.71

Each value is the average of five replicates ± standard deviation.

Table 4: Antioxidant activity of *C. creticus* and *C. salvifolius* extracts.

Plant	Extract	IC_{50}* mg/ml
C. creticus	Methanol 80%	0.009±0.0006
	Water	0.019±0.0015
C. salvifolius	Methanol 80%	0.007±0.0011
	Water	0.011±0.0020
BHT		0.015±0.0010

Each value is the average of five replicates ± standard deviation.

* IC_{50} is the concentration for a 50% inhibition.

smoking and alcohol consumption, and by eating oxidized polyunsaturated fats[19,20]. Excess ROS and oxidants in the body can lead to accumulative damage in proteins, lipids, DNAs and RNAs, and cause aging, neuro-degenerative diseases and other various human diseases[19] resulting in so-called oxidative stress[21]. Nowadays, the most commonly antioxidants used in food and medicines industries are synthetic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone. Though important, there is a widespread concern over the safety of synthetic antioxidants and agreement to replace them with natural antioxidants (plant extracts) because of the potential health risks and toxicity effects of the synthetic ones[21,1,20]. Epidemiological studies have recognized an inverse correlation between consumption a higher content of some fruits and vegetables and a lower risk of cancer, cardiovascular diseases, and mortality from age-related diseases[21,22]. Which could be partly attributed to the presence of plant antioxidants compounds. Plants are enormous source of natural bioactive compounds; These compounds are derived from secondary metabolism of plants[2,23]. Polyphenoles are the most abundant hydrophilic antioxidants in the diet and the most active antioxidant compounds[1,21]. In fact, these dietary biopolyphenols can stimulate cellular defenses and help to prevent cellular components against oxidative damage[21] due to their redox properties which allow them to act either as reducing agents, hydrogen donors, powerful scavengers against free radicals, or inhibitors of the enzymes involved in the initiation reaction[1,22]. Therefore many intensified research efforts to discover and identify new natural resources as active antioxidant compounds and utilize these agents with few side-effects to substitute the chemical therapeutics and synthetic antioxidants[21,24]. In addition, these natural antioxidants can be formulated to give nutraceuticals, which can help to prevent oxidative damage from occurring in the body[24]. As a part of a systematic study of the chemical composition of Syrian flora plants, and since no previous reports on the composition of *Cistus* genus in Syria are available, we aimed in the present study to evaluate and compare the chemical constituents and antioxidant activity of aqueous and methanolic extracts of the only two species of *Cistus* genus in Syria (*Cistus creticus* and *Cistus salvifolius*), by employing the radical-scavenging activity assay on DPPH (2,2-diphenyl-1-picrylhydrazyl). As a previous step to measure the antioxidant activity, the total polyphenols content and total flavonoids content of the extracts were quantified, as this values correlate extremely well with antioxidant power.

MATERIALS AND METHODS

Chemicals and Equipments

Chemicals: Methanol GR (Eurolab, UK), Folin-ciocalteu phenol reagent (Sigma-Aldrich, Switzerland), Sodium Carbonate anhydrous (PAREAC QUIMICA SAU, Spain), Gallic acid (Titan biotech LTD., India), Rutin (Extrasynthese Genay, France), Aluminum Chloride Hexahydrate (Scharalau Chemie, Spain), DPPH and BHT (Sigma-Aldrich, USA). Distilled deionized water (dd. H2O).

Equipments: Sensitive balance (Sartorius TE214, Germany), Rotary evaporator (Heidolph Instruments, Germany), UV-1800 spectrophotometer (Shimadzu, Japan), Ultrapure TM water purification system (Lotun Co., Ltd., Taipei, Taiwan).

Plant Material

Fresh aerial parts (stems, flowers and leaves) of *Cistus creticus* and *Cistus salvifolius* were randomly collected in May 2014 from wild plants growing in Al-Qadmus region (35° 5' 35" N - 36° 10' 29" E, Tartus, west of Syria). The plant materials were authenticated by an expert at Faculty

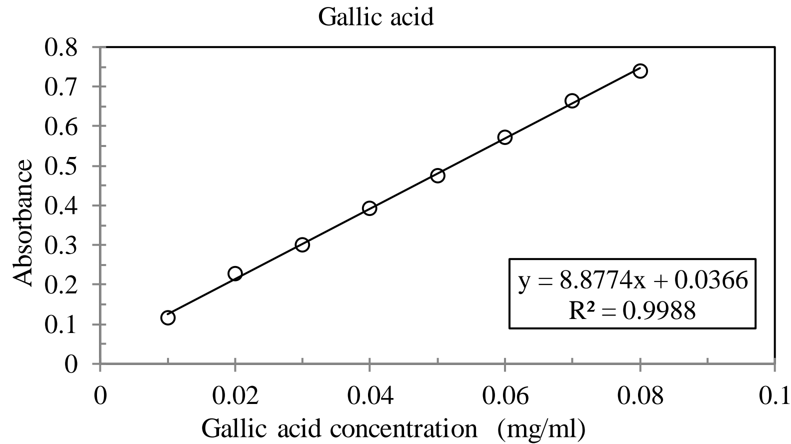

Figure 1: Calibration curve of Gallic acid for determination of TPC.

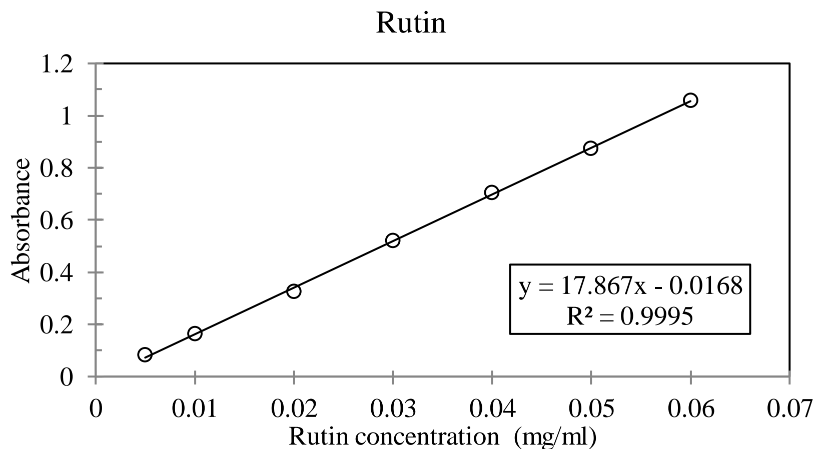

Figure 2: Calibration curve of Rutin.

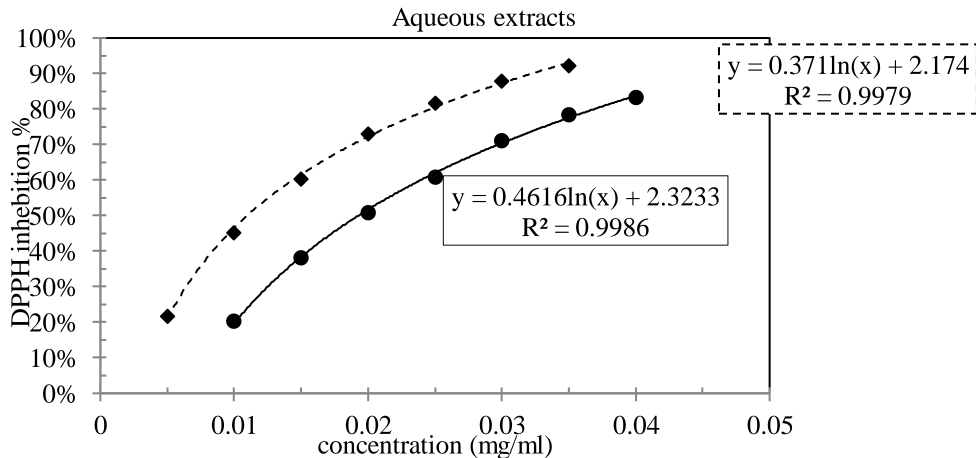

Figure 3: Scavenging activity of the *C. creticus* and *C. salvifolius* methanolic extracts.

of Agriculture - University of Aleppo, Syria. The aerial parts were washed under running tap water, shade dried, then powdered using mechanical grinder and kept in airtight glass container until use.

Preparation of extracts

The extraction method was adapted from[25] and in accordance with[26] as following; a weighed portion of each powdered two plants was extracted by maceration for three days at room temperature with two different solvents (methanol: water 80:20 (v/v), distilled water 60°C), with gentle agitation from time to time. The plant: solvent ratio was 1:10 (w/v). The extract solutions were filtered through Whatman No. 1 filter papers in a sintered glass Büchner funnel, and the residual material was re-extracted three times using the same procedure. After that, the combined extracts were evaporated to dryness in a rotary evaporator at 40°C and under reduced pressure to remove the solvent, the solid residues were kept at 4°C until use[13]. The yield

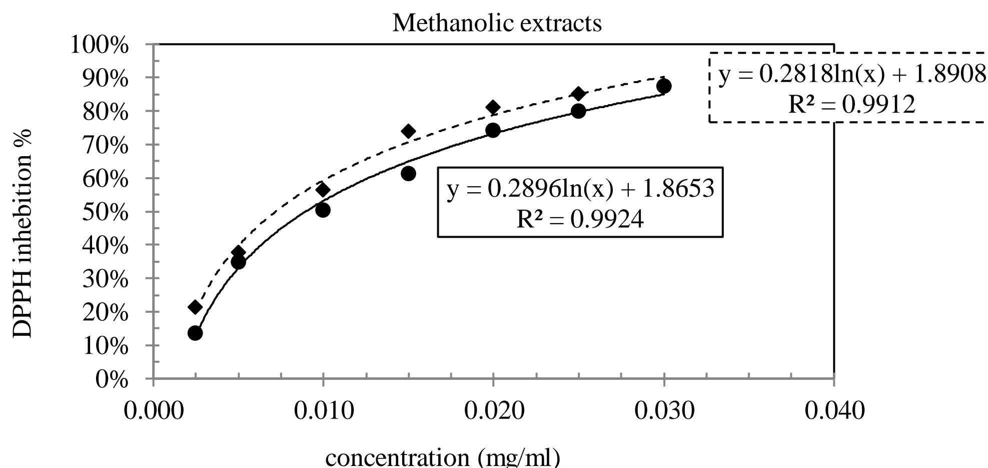

Figure 4: Scavenging activity of the *C. creticus* and *C. salvifolius* aqueous extracts.

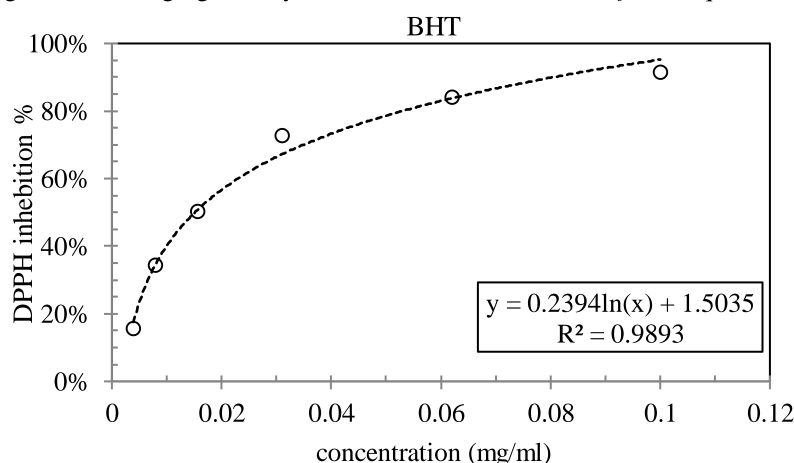

Figure 5: Scavenging activity of the standerd BHT.

percentage was then calculated using the following equation:

Yield (%) = W_{ex} / W_p * 100

Where W_{ex} is the weight of the dried extract and W_p is the weight of the plant material. In addition, the aspect of the smell and the color of the extracts were noticed[2].

Determination of total phenolic contents

The total phenolic content (TPC) in plants extracts was determined spectrophotometrically using Folin-Ciocalteu's reagent according to Stanković[27] with some modifications. Briefly, 0.5 ml of each extract solution of the concentration (0.1 mg/ml in solvent; methanol or water) was mixed with 2.5 ml of 10% Folin-Ciocalteu's reagent diluted in distilled water (dd H_2O), placed for few minutes, and then 2.5 ml of 7.5% Na_2CO_3 was added. The samples were incubated in a water bath at 45°C for 45 min. After that, the absorbance was determined using spectrophotometer at λ_{max}=765 nm against a blank solution prepared by replacing the plant extract with an equal volume of the solvent (methanol or water). The samples were prepared in quintuplicate for each analysis and the mean value of five absorbances was obtained. The same procedures were repeated for the standard solution of Gallic acid in dd H_2O as standard series (0.01 to 0.08 mg/ml) and the liner calibration was construed. Based on the measured absorbance, the concentration of phenolic was calculated from the calibration line; then, the content of phenolics in extracts was expressed in terms of milligrams of gallic acid equivalent per gram of plant's dry weight (mg GAE/g DW).

Determination of total flavonoid content

The total flavonoid content (TFC) in the examined plant extracts was determined using spectrophotometric method[27]. Each plant sample was prepared by dissolving a weighed amount of crude extract in methanol and diluted to obtain the concentration of (0.1mg/ml). 1 ml of previous solution was mixed with the same volume of 2% $AlCl_3$ solution dissolved in methanol. Absorptions at λ_{max}=415 nm were taken after an hour incubation at room temperature. The blank sample consists of 1 ml extract solution with 1 ml methanol without $AlCl_3$. The samples were prepared in quintuplicate for each analysis and the mean value of five absorbances was obtained. The same procedures were repeated for the standard series solution of Rutin in methanol (0.005 to 0.6 mg/ ml) and the calibration line was construed. Based on the measured absorbance, the content of flavonoids was read from the calibration line; then, the content of flavonoids in extracts was expressed in terms of milligrams of rutin equivalent per gram of plant's dry weight (mg RUE/g DW).

Evaluation of antioxidant activity

DPPH radical scavenging assay

The DPPH radical scavenging assay is an easy, rapid and sensitive method for the antioxidant screening of plant extracts[28]. The methanolic and aqueous extracts of the *Cistus* two species were evaluated according to[20,29] using the stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH). A solution of 0.135 mM DPPH in methanol prepared daily was used. In a test tube 1 ml of this solution was mixed with 1 ml of an ascending concentration series of plant extract in methanol or the standard solution. The reaction mixture was left in the dark at room temperature for 30 min. BHT was used as reference standard. The changes in color from deep-violet to light-yellow were measured at λ_{max}=517 nm, using methanol as a control solution. In order to compare the results obtained in the different treatments, the inhibition percentage of free radical DPPH was calculated from the following equation:

DPPH radical scavenging activity % = $[(Abs_{control} - Abs_{sample}) / (Abs_{control})] \times 100$

Where $Abs_{control}$ is the absorbance of DPPH radical + methanol, Abs_{sample} is the absorbance of DPPH radical + sample (plant extract or standard). A logarithmic curve was plotted of percent inhibition versus extract concentration, then, the concentration of sample required for 50% inhibition was calculated and expressed as IC_{50} values. All the tests were carried out in quintuplicate and averaged.

Statistical analysis

All the experiments were performed in quintuplicate, the results were expressed as mean values ± standard deviation (SD). Statistical analysis was carried out using the Statistical Package for the Social Science SPSS Version 19; wherein the data was subjected to T test. Differences were considered significant at probability (p) value < 0.05.

RESULTS

Extraction yield

The extracts were obtained as described in the methods section. The crude dried extracts obtained were brown, nicely aromatized, and the yield percentage of solid residue of *C. creticus* extracts were higher than *C. salvifolius* ones. *C. creticus* methanolic extract (*C. creticus* MeOH) produced the highest yield percentage while the lowest extraction yield was for *C. salvifolius* aqueous extract (*C. salvifolius* Aq), as shown in (Table 1).

Total phenolic content (TPC)

The total phenolic content in the examined plants extracts reacted with the Folin-Ciocalteu's reagent was calculated according to the equation of calibration curve for Gallic acid (figure 1), ($y=8.8774x + 0.0366$, $R^2=0.9988$). The values obtained for the extract's concentration of total phenols were expressed as mg of GAE/g of plant's dry weight. (*C. salvifolius* MeOH) showed significantly high content. The results ranged from (75.22± 4.79 to 65.99±1.33 mg GAE/g DW). (Table 2).

Total flavonoids content (TFC)

The total flavonoids content of the two plants extracts was determined using aluminum chloride colorimetric method and calculated according to the equation of calibration curve for Rutin (figure 2), ($y=17.867x - 0.0168$, $R^2 = 0.9995$). The results were expressed as mg of RUE/g of plant's dry weight. The plants' aqueous extracts are significantly higher in total flavonoids content comparing with the methanolic ones. (Table 3).

Antioxidant activity using DPPH

The assessment of antioxidant activity showed that the examined extracts were able to scavenge DPPH radical. The radical scavenging activities of *C. creticus* and *C. salvifolius* were estimated by comparing the IC_{50} value of the extracts and BHT, considering that, the lower the IC_{50} value, the higher is the antioxidant activity, IC_{50} values were constructed from the equation of logarithmic curve of each extract and BHT. (figures 3,4,5). The lowest IC_{50} value was found for (*C. salvifolius* MeOH) which reveals high scavenging activity. Among the four examined extracts, three showed strong antioxidant activity higher than these of BHT (IC_{50BHT}= 0.015mg/ml). (Table 4)

DISCUSSION

As phenolic (including many flavonoids) contain polar phenolic hydroxyl group/s, their high extraction into methanol and water is quite reasonable, and the extraction percentage yields supports this.

Total phenolics content

The mechanism of TPC assay is that a phenol loses an H^+ ion to produce a phenolate ion, which reduces Folic-Ciocalteu reagent. The changes is monitored by spectrophotometre[30]. The highest phenolic content was observed in *C. salvifolius* MeOH (75.22 ±4.79 mg GAE/g DW), *C. creticus* MeOH extract showed (69.34±4.68 mg GAE/g DW), this results exceeds the ones reported by Nicolettia from Italy[9] about methanolic extracts of other *Cistus* species from Italy and Tunisia; *C. villosus*, *C. libanotis*, and *C. monspeliensis*, where TPC were reported as (32.51, 40.51 and 33.16 mg GAE/g DW) respectively. Aqueous extracts of studied plants showed less (TPC) than those reported by[31] of *C. ladanifer* and *C. populifolius*, but more than the results of Dudonné et al[21].

Total flavonoids content

The basis of the total flavonoid assay is the fact that aluminum ion (Al_3^+) forms complexes with C-4 keto and either C-3 or C-5 hydroxyl, or with ortho hydroxyl groups in the A or B ring of flavonoids structure[30]. In this study, aqueous extracts of *C. salvifolius* and *C. creticus* showed (17.68 ±0.71, 12.41 ±0.22 mg RUE/g DW) respectively, which are higher in TFC than methanolic ones (12.50 ±0.25, 11.56 ±0.32 mg RUE/g DW), however methanolic extracts still higher than those of *C. villosus* (9.84 mg RUE/g DW)[32], and other *Cistus* species methanolic extracts which showed (5.59, 9.44, and 8.49 mgCE/g DW) for *C. monspeliensis, C. villosus* and *C. libanotis* respectively[9]. In each solvent, the TPC is higher than the TFC, supporting the fact that most flavonoids are also phenolic[30]. The variation in total phenols and flavonoids content among species could be due to various intrinsic and extrinsic factors, one of such factors may be the genetic potential of individual species for polyphenol biosynthesis. Apart from the genetic background, the environment and maturation stage may also be critical in this respect[9,33].

DPPH radical scavenging activity

The DPPH free radical assay has been extensively used for screening antioxidant activity because it can accommodate many samples in a short period and it is sensitive enough to detect active ingredients at low concentrations[1]. This assay is based on the principle that (DPPH) radical is able to decolorize its purple color when mixed with a solution of substrate that can donate a hydrogen atom. The color turns to yellow as the molar absorptive of the DPPH radical at 517 nm reduces, when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The degree of discoloration indicated the scavenging potential of the extracts in terms of hydrogen donating ability[20,34]. In this study methanol extracts exhibited higher antioxidant activity than aqueous ones, the highest was displayed by *C. salvifolius* methanolic extract (IC_{50}= 0.007 mg/ml), which is 2.14 times higher than of BHT. Three extracts among the four examined showed strong antioxidant activity higher than the synthetic antioxidant BHT whilst *C. creticus* Aqueous came at last as follows; (IC_{50}: *C. salvifolius* MeOH= 0.007> *C. creticus* MeOH= 0.009> *C. salvifolius* Aq= 0.011> BHT= 0.015> *C. creticus* Aq= 0.019 mg/ml). These results showed that the antioxidant activity was greater in methanol extracts, which means phytochemicals by methanol possess a stronger potential to scavenge DPPH free radical than those extracted by water, methanolic extracts also had higher levels of polyphenols, supporting the opinion that plant extracts have a potent antioxidant activity mainly due to their richness of phenolic compounds. Generally, plants with great amount of phenolic compounds have a very strong antioxidant capacity. This radical scavenging activity could be affected by substitution of hydroxyl groups on phenolic aromatic ring, because of their hydrogen donor ability, forming resonance-stabilized phenoxyl radicals[8,31]. In fact, the antioxidant activity increases by increasing the degree of hydroxylation[21,35]. These results agree with the literature data which reported that high antioxidant activity of *Cistus* species from Libya, Tunisia and Italy[9,19], the IC_{50} values obtained from *C. incanus, C. villosus* and *C. libanotis* were (IC_{50}= 17.75, 22, 28 μg/ml) respectively, which were much lower compared to our values, in the same studies, *C. parviflorus* and *C. monipeliensis* recorded (IC_{50}= 4.75, 3.0 μg/ml) respectively, which were higher values than our results. In this study, we have identified a promising source of natural antioxidant compounds from plants poorly studied; *Cistus*, so they may be recommended for use in the fields of food and drugs preservation and to be included in nutraceutical formulations. Additional studies are needed to characterize the active compounds and biological activities of these plants extracts.

CONCLUSIONS

The results of this study of *Cistus* species in Syrian flora conclude that; *C. salvifolius* is richer than *C. creticus* in total phenolic and total flavonoids. Both plants revealed prominent free radicals scavenging capacity. Methanolic extracts were more potent even than BHT. This study supports the idea that *Cistus* species may be good sources of natural antioxidants to be used by the food and drug industry as natural sources of antioxidants compounds.

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