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Review Article

Correlation Between the Phenol Content and Antioxidant Efficacy of *Myrtus Communis* (L.) Leaf Extracts

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Abstract

In the present work, we have evaluated the antiradical efficacy of ethanol, methanol and *n*-hexane extracts from leaves of *Myrtus communis* L. to find potential sources of natural antioxidants. Total phenolic content was assessed using the Folin-Ciocalteau assay and Total Flavonoid Content was assessed using Aluminum Chloride (AlCl$_3$) colorimetric method with quercetin as standard flavonoid. On the other hand, antioxidant activities of these extracts were determined spectrophotometrically at ambient temperature and 517nm using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), and evaluated following the kinetics of the DPPH reduction. The Total Phenol Content of extracts ranged between 122.72 ± 1.16 to 137.46 ± 0.35 mg GAE/g extract, while the flavonoid content ranged between 18.41 ± 0.43 and 31.24 ± 1.23mg QE/g extract. In addition, the *n*-hexane extract showed more activity than ethanol and methanol extracts. The antiradical capacity (IC$_{50}$) parameter and its T$_{IC50}$ time equivalent have an influence on the antiradical capacity of extracts. The antiradical efficiency is considered to be a stronger parameter to select antioxidants compared to IC*50*. The present study revealed that *M. communis* represents an important source of natural antioxidants to ensure food safety.

Keywords

Introduction

Oxidation reaction can be defined as the transfer of electrons from one atom to another. It is an important reaction in human living cells because it represents an essential reaction aerobic metabolism. During the aerobic oxidation process, Oxygen molecules are the key electron acceptor in the electron flow system for producing energy in the form of ATP. However, when the electron flow becomes uncoupled (transfer of unpaired single electrons), some problems could arise generating so-called free radicals reactive [1]. On the other hand, an antioxidant is a compound that reacts to free radicals, neutralizing them and thereby preventing or reducing their damaging effects on the human body [2]. The resulted oxidative stress is involved in the pathogenesis of many related diseases such as cancer and inflammation. In fact, the oxidation of lipids is responsible for the deterioration of fats and oils resulting in change in color, flavor and nutritive value [3].

To prevent oxidation, particularly in food industry, the addition of some synthetic molecules to fats, fatty food, and cosmetics is a common practice. These synthetic antioxidant compounds were revealed to have several side effects including carcinogenicity [4,5]. Consequently, synthetic antioxidants used in products for human application are being restricted, considerably increasing interest in antioxidants of natural origin [6,7].

Medicinal plants have long been used for their pharmacological properties. Several extracts and essential oils are tested for their biological effects such as antibacterial, antioxidant, antitumor and antileishmanial activities [8-12]. Indeed, these plants possess different secondary metabolites, some of which are potent antioxidants, including polyphenols and flavonoids contents [1,6]. The antioxidant activity of phenolic compounds is attributed to their redox properties which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers and metal chelators [13]. Many *in vitro* studies confirm that phenolic compounds such as flavonoids and phenolic acid can have a considerable antioxidant activity that depends on the number and position of phenolic hydroxyls in the aromatic ring moieties [14]. Generally, monophenols are less effective than catecholic phenols and phenolic aglicons have higher antioxidant activity than their respective glycosides [14].

Myrtle (*Myrtus communis* L.) is a medicinal plant endemic to the Mediterranean area and has been used local populations for its culinary and medicinal properties since ancient times [15-18]. Its use is a well-established tradition in many countries, including Morocco. Organic extracts and essential oils produced by *M. communis* contain numerous bioactives compounds that possess multiple biological properties, especially antioxidant activity [2,19-22]. However, studies in the field have been limited to the determination of the antioxidant capacity.

The aim of the present study is to examine the *in vitro* antiradical activities of methanol, ethanol, and *n*-hexane of *Myrtus communis* leaves extracts using DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging capacity and therefore determine the antioxidant efficacy by studying the antiradical power of *M. communis* extracts depending on kinetic reaction.

Materials and Methods

Collection of plant material and preparation of extracts

The leaves of *Myrtus communis* used in this research were collected in July 2015 from the Ouezzane province. Samples were air dried in the shadows and milled into powder using an electric grinder, then the investigated dried powdered plant materials were extracted by maceration. The powder (25g) of leaves was placed in an Erlenmeyer flask containing 100mL ethanol, methanol, and n-hexane for 72h. The plant extracts were filtered with a filter paper (Whatman. No.1) and the combined filtrate was then steam-dried using a rotary evaporator (Heidolph Collegiate, LV28798826, New Jersey, USA) at a temperature not exceeding 45°C. All extracts were stored in a dark bottle at 2 - 8°C until analysis.

Determination of Total Phenolic Content (TPC)

The concentration of the phenolic compounds in the plants extracts was determined using the Folin Ciocalteu assay [23], with some modifications. In brief, the extract was diluted to the concentration of 1mg /mL, and aliquots of 100μl or a standard solution of gallic acid (20, 40, 60, 80 and 100mg/L) were mixed with 500μl of Folin Ciocalteu reagent (previously diluted 10-fold with distilled water) and 400μl of Na_2CO_3 (7%). After 40 min of incubation at room temperature (23 ± 2°C), the absorbance was measured at 760nm using a Spectro-photometer against a blank sample. The Total Phenolic Content was calculated using a calibration curve for gallic acid (R^2 = 0.998). The results were expressed as milligram of gallic acid equivalent per gram of dry weight of extract (mg of GAE/g extract). All samples were analyzed in triplicate.

Determination of Total Flavonoid Content (TFC)

The Total Flavonoid Content of the extracts was determined using the Aluminum Chloride ($AlCl_3$) colorimetric method described by Brighente et al., with minor modifications [24]. In a nutshell, 1mL of the extract (1mg/mL in methanol) or a standard solution of quercetin (20, 40, 60, 80 and 100mg/L) were mixed with 1mL of 2% $AlCl_3$ in methanol. After 40 min of staying at room temperature (23 ± 2°C), the absorbance against blank was measured at 430nm using a Spectrophotometer. The Total Flavonoid Content was calculated using a calibration curve for quercetin (R^2 = 0.985). The results were expressed as milligram of quercetin equivalent per gram of dry weight of extract (mg of QE/g extract). All samples were analyzed in triplicate.

Evaluation of the antioxidant activity

The antioxidant activity of *M. communis* leaves extracts is measured by using DPPH scavenging method. It is reduced to the form of hydrazine (no radicular) by accepting a hydrogen atom.

DPPH radical scavenging capacity assay: The reduction

capability of DPPH radicals was determined by the antioxidant-induced decrease in its absorbance at 517nm, which is visually noticeable as a discoloration from purple to yellow [25]. This method has been widely used to evaluate the free-radical scavenging effects of various antioxidant substances [26]. This average absorbance was used to calculate the DPPH scavenging capacity. Therefore, DPPH was used as a substrate to evaluate the antioxidant activity of methanol, ethanol and n-hexane extracts of $M.$ $communis$ at various concentrations compared to standards such as ascorbic acid. The ability of the plant extracts to scavenge DPPH free radicals was assessed using the standard method with some modifications [26]. In sum, Aliquots (0.2mL) of various concentrations (62.5 - 1000μg/mL) of the plant extracts samples were added to 1.8mL of a 0.004% methanolic solution of DPPH prepared daily.

The absorbance at 517nm was measured at different time intervals (in seconds) until the reaction reached a plateau. The concentration of DPPH (mg/L) in the reaction medium was calculated starting from a calibration curve constructed with the following concentrations of DPPH [0.01, 0.015, 0.02, 0.025, 0.03, 0.035mg/l] by a linear regression.

A_{517} nm = a × $[DPPH]_t$ + b

Where $[DPPH]_t$ was expressed as mg/L.

The percentage inhibition of DPPH by the extracts of phenolic compounds was calculated using the formula:

$$\% \text{ inibition} = \frac{Abs(blank) - Abs(sample)t}{Abs (blank)} \times 100$$

Where Abs (blank) is the absorbance of the control at t = 0 and Abs (Sample)$_t$ represents the Abs in the presence of antioxidants at a time t, which varies according to the concentrations. Ascorbic acid was used as positive control.

The results obtained for absorbance versus time do not allow for the calculation of the antioxidant activity. Consequently, it is necessary to transform these data using the residual fraction of DPPH in the methanolic solutions versus time.

This fraction DPPH was calculated on the basis of the following formula:

$$\% \text{ DPPH} = [DPPH]_t /[DPPH]_t = 0$$

Where, $[DPPH]t = 0$ is the initial concentration of the stable radical without the antioxidant and $[DPPH]_t$ is its remaining concentration at the reaction plateau step.

Determination of antioxidant capacity (IC$_{50}$) and Equilibrium Time (T$_{IC50}$): The antioxidant capacity (IC$_{50}$) is defined as the concentration of a compound reducing the initial DPPH level by 50%. It was calculated from the graph of inhibition percentage plotted against the extract concentration and it was generally used to measure the antioxidant power respective antioxidant activity [27-29], which rose at low IC$_{50}$ values, and vice versa [30].

The parameter T$_{IC50}$ is defined as the time necessary to reach the steady state at a concentration corresponding to IC$_{50}$. It was calculated from the graph of times at steady state conditions versus the concentrations for each extract.

Determination of antiradicular efficiency: The two factors IC$_{50}$ and T$_{IC50}$ were combined in order to obtain the parameter of Antiradical Efficiency (AE). It was calculated using the flowing formula:

$$AE = (1/IC50 \times TIC50)$$

Data analysis

All data presented are results of three replicates along with standard deviations. The statistical analysis was performed using a multi-factorial ANOVA and the Tukey's test with the general linear model of SPSS, version 21.0, for Windows software package. Differences are considered significant when $p<0.05$. Correlation coefficients between antioxidant capacity and total phenolic content or flavonoid content were determined using Prism 3 statistical program packages.

Results and Discussion

Total phenol content

phenol content was estimated using the Folin-Ciocalteu colorimetric method in comparison to standard gallic acid and the results were expressed in terms of mg GAE/g extract using an equation obtained from a calibration curve. Among the extracts investigated, total phenolic content ranged from 122.72 ± 1.16 to 137.46 ± 0.35 Gallic Acid Equivalents (GAE mg/g) of dry weight of extract (Table 1). The amounts of phenolic compounds in all extracts were high and no significant difference between the solvents extracts is detected ($p>0.05$). Other studies reported that leaves extracts of $M.$ $communis$ are rich in phenol components. Amensour et al., stated that the total phenol components of ethanol, methanol, ethyl-acetate, and water extracts ranged between 7.81 to 35.56mg GAE/g extract [24]. This difference in total phenol components is likely due to the geographical situation of collected plants.

Indeed, Tawaha et al., estimated that a total phenolic content higher than 20mg GAE/g dry weight is considered as very high [31]. Accordingly, all ours extracts of leaves of *M. communis* are considered as highly important sources of phenolic compounds.

Extract	TPC (mg GAE[a]/g extract)	TFC (mg QE[b]/g extract)
EtOH	126.41 ± 1.03	18.41 ± 0.43
MeOH	137.46 ± 0.35	27.24 ± 0.83
n-Hexane	122.72 ± 1.16	31.24 ± 1.23

Table 1: The TPC and TFC of *M. communis* extracts.

[a]Gallic Acid Equivalents
[b]Quercetin Equivalents

The phenolic compounds of *M. communis* leaves extract were investigated by several works [22,32-34]. Remarkably, phenolic composition may significantly vary, depending on plant organ used for extraction. Indeed, leaf extracts showed significantly higher amount of total phenolic compounds than berry extracts [22,32-36]. Moreover, the contents of total phenols, tannins, flavonoids and proanthocyanidins vary among myrtle parts [22].
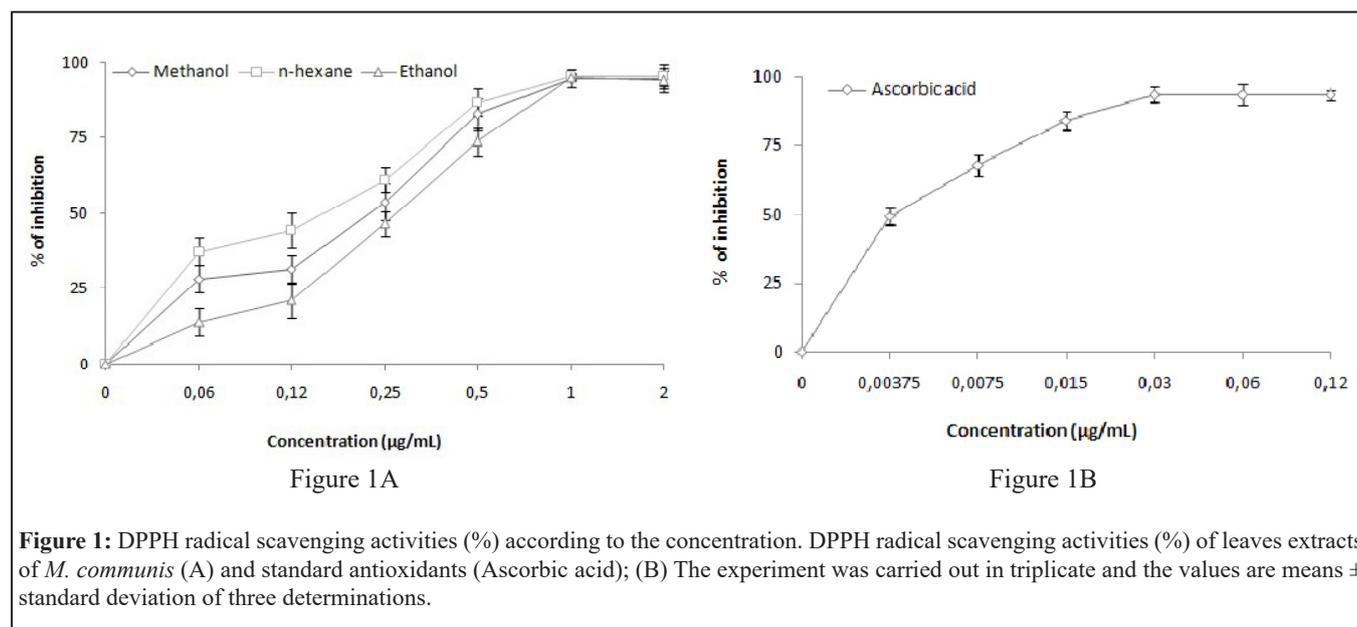
Total flavonoid content

The total flavonoid content was estimated using a colorimetric method with quercetin as standard flavonoid. Results were

methanol extracts showed higher flavonoids content than ethanol extract ($p<0.05$).

According to Aidi Wannes W et al., leaf extracts of *M. communis* showed important charges in flavonoid contents [32]. Flavonoids in *M. communis* leaves extract are detected in relatively large amounts [32], with the exception of quercetin derivatives and phenolic acids, which were found only in small amounts [34]. Except myrtle plant parts, final extract composition depends also on extraction solvent used for extract preparation, mainly because of its polarity [37].

Antioxidant activity

Reduction in the absorption of radical DPPH is explained by its reduction in the presence of methanol, ethanol, n-hexane, and control extracts. This reduction results from the change of the violet color towards yellow during the formation of the DPPH-H radicals, starting by the release of phenolic hydrogen atoms from the extracts and their acceptance by radical DPPH. The results of the antioxidant activity expressed in percentages of inhibition are illustrated in figures 1A and B. Ascorbic acid revealed about 93.42% of inhibition increase at 0.06mg/L concentration. For the three plant extracts tested, the ability to inhibit methanolic solutions of DPPH was almost complete with a high concentration. It is possible to obtain an effect of the scavenging the DPPH at 100%. The percentages of inhibition for methanol, ethanol and hexane varied from 28.24 - 94.65%,

Figure 1A

Figure 1B

Figure 1: DPPH radical scavenging activities (%) according to the concentration. DPPH radical scavenging activities (%) of leaves extracts of *M. communis* (A) and standard antioxidants (Ascorbic acid); (B) The experiment was carried out in triplicate and the values are means ± standard deviation of three determinations.

expressed in terms of mg GAE/g dry extract using an equation obtained from a calibration curve; their values ranging from 18.41 ± 0.43 to 31.24 ± 1.23 Quercetin Equivalents of dry weight of extract (QE mg/g) (Table 1). The n-hexane and

13.82 - 94.09%, and 37.24 - 95.53%, respectively.

Fraction of the residual DPPH solution plotted versus time: Reduction in the absorption of radical DPPH is explained by

its reduction in the presence of methanol, ethanol, *n*-hexane and control extracts (Figures 2A,B). This reduction results from the change of the violet color towards yellow during the formation of the DPPH-H radicals, starting by the release of phenolic hydrogen atoms from the extracts and their acceptance by radical DPPH. The evaluation of the residual DPPH percentages at steady state conditions according to the concentrations is summarized in table 2. In the case of fast kinetics, samples with high concentrations react quickly and reach steady state conditions immediately. On the contrary, slow kinetics and low concentrations take longer to reach the steady state. Methanol extract was revealed to possess a higher potential to scavenge DPPH radical, compared to ethanol and hexane extracts at concentrations of 0.25mg/L (Figures 2A,B). The concentration of each antioxidant correlated with its total antioxidant capacity, and correlation coefficients were >9 in all cases. Concentration increase of the samples revealed a proportional reduction in percentage of residual DPPH. In the case of 0.25mg/L, the steady state was reached after 3 min for methanol, 5 min for ethanol, 7 min for *n*-hexane, and 1 min for ascorbic acid.

($p<0.05$) between n-hexane extract and the other two extracts (methanol and ethanol) was detected. On the other hand, no significant difference has been noted between ethanol and methanol extract ($p>0.05$). T_{IC50} was the time necessary to reach the steady state at a concentration corresponding to IC_{50}. It was obtained through the modeling of times at steady state conditions versus the concentrations for each extract. The values of T_{IC50} are generally different from one tested extract to another (Table 2). This variability depended on the concentration of each extract ($p<0.05$). The results obtained in our studies concerning the antioxidant capacity are in consonance with these found in literature with some variability that may be due the difference in chemical compounds and the methods used to reveal the antioxidant effect [34-36].

Antiradical efficiency: Low IC_{50} values combined with low T_{IC50} resulted in high AE values. The difference in T_{IC50} between these antioxidants indicates that IC_{50} values do not show time dependence (Table 3). The antiradical efficiency of ascorbic acid is higher (6.9×10^{-3}) than that of *n*-hexane (0.87×10^{-3}), methanol (0.3×10^{-3}), and ethanol extracts (0.18×10^{-3}). AEs
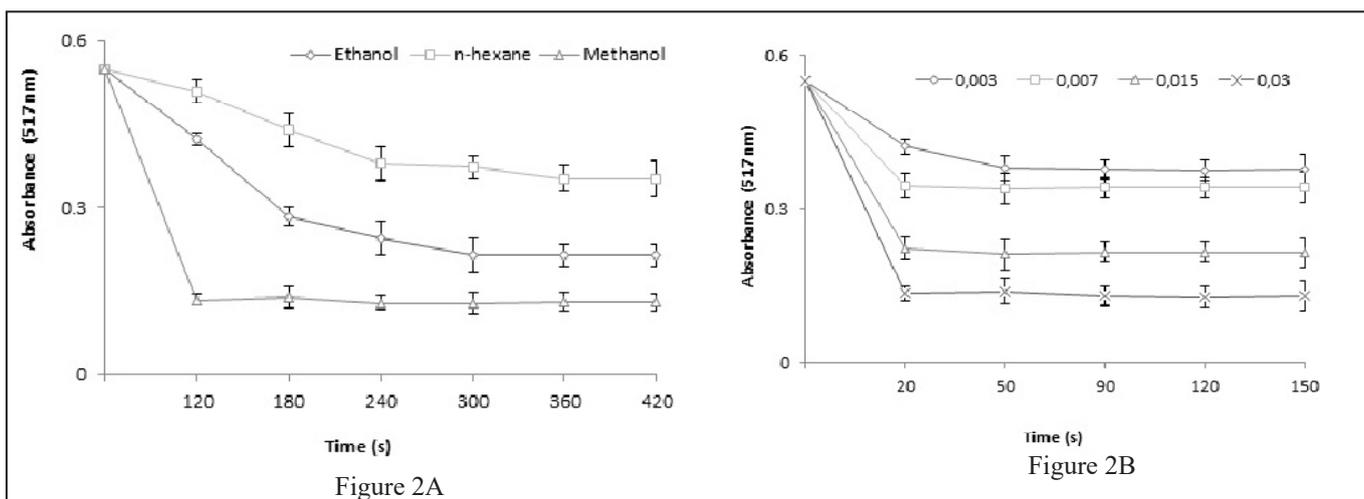

Figure 2: Activity antioxidant of extracts and control: (A) Reduction of the absorption of methanol, ethanol, and *n*-hexane extracts at 0.25g/L versus time; (B) Reduction of the absorption of ascorbic acid versus time. Values are means ± standard deviation of three determinations

Fraction of the residual DPPH versus the concentration of antioxidants: The IC_{50} values (Table 3) were calculated from the residual percentages of DPPH plotted versus the concentrations of the phenolic compound extracts (Figures 3A,B), expressed as mg antioxidant per g DPPH using an exponential equation. According to the obtained results, low IC_{50} values (*n*-hexane 317.51, methanol 514.64 and ethanol 633.07mg/g of DPPH) corresponded to short reaction times. The value for ascorbic acid was 80.51mg/g of DPPH. The comparison of these values to control indicated that *n*-hexane has scavenging activity on the DPPH radical. A significant difference in IC_{50}

of the samples are seeded in the following order: ascorbic acid > *n*-hexane > methanol > ethanol. This shows that AE remains a powerful parameter in the evaluation of the antioxidant activity, compared to IC_{50}. The obtained results reveal that ascorbic acid has a higher antioxidant power for scavenging of DPPH compared to Qian and Nihormbere [30] (0.80×10^{-3}) and lower than Moreno et al., [27] (11.44×10^{-3}) and Benhammou et al., (41.74×10^{-3}) [38]. Ascorbic acid reacts immediately to radical DPPH, and reaches the steady state in 15 seconds [39]. In the present study, this phase is reached after 1.8 min, compared to 1.15 min mentioned by Moreno

et al., [27] and 0.606 min by Benhammou et al., [38]. The antioxidant power of ascorbic acid is also cited by other authors [28,40]. The antioxidant activity of *n*-hexane, methanol, and ethanol extracts is close to that of gallic acid [41]. It has been shown that the antioxidant capacity of *M. communis* extract is mainly due to phenol content.

the undeniable necessity of flavonoids in the ability to scavenge DPPH. The correlation coefficients between the time to reach IC_{50} and the contents of total phenols and flavonoids are $R^2 = 0.086$ and $R^2 = 0.928$, respectively. This result clearly shows the role of flavonoids in this activity. On the other hand, correlation coefficients established previously, the coefficients

Antioxidant	Concentration (g/L) of antioxidants	Concentration (mg/g DPPH')	Time at steady state (min)	% of DPPH at the steady state	Correlation Coefficient (R^2)
MeOH					
	0.06	156.25	14	71.76	0.974
	0.12	312.5	8	68.46	0.985
	0.25	625	3	46.17	0.993
	0.5	1250	5	17.06	0.989
	1	2500	1	5.34	0.997
n-hexane	0.06	156.25	11	62.75	0.984
	0.12	312.5	6	55.63	0.979
	0.25	625	7	38.81	0.994
	0.5	1250	2	13.09	9.997
	1	2500	2	4.51	0.962
EtOH	0.06	156.25	13	86.18	0.991
	0.12	312.5	9	78.73	0.932
	0.25	625	5	53.44	0.987
	0.5	1250	6	26.24	0.956
	1	2500	2	4.86	0.974
Ascorbic acid	0.015	39	3	67.64	0.983
	0.03	78.125	2	51.24	0.979
	0.06	156.25	1	29.28	0.996
	0.12	312.5	0.8	4.34	0.938
	0.25	625	1	4.59	0.959

Table 2: Correlation between the antioxidant capacity and concentrations of antioxidants (extracts and control).

Antioxidant	IC_{50} (mg/g of DPPH)	R^2	Range of times at the steady state (min) for different concentrations	T_{IC50}	R^2	AE ($\times10^{-3}$)
MeOH	514.64	98.34	41640	6.7	0.964	0.3
EtOH	633.07	99.47	41306	5.3	0.981	0.18
n-hexane	317.51	96.17	43406	3.6	0.855	0.87
Ascorbic acid	80.51	99.29	0.8-3	1.8	0.998	6.9

Table 3: Determination of the Antiradical Efficiency (AE) of the samples.

Relationships between phenols content and antioxidant activity: The relationship between the levels of phenols in the extracts and antiradical capacity (IC_{50}), time to reach this capacity and antiradical efficiency, are established. The antiradical ability is related to the flavonoids by a correlation coefficient $R^2 = 0.877$, while this correlation for the total polyphenols was found to be $R^2 = 0.141$. These results reveal

between the antiradical effectiveness and the phenol and flavonoids content are $R^2 = 0.642$ and $R^2 = 0.385$, respectively. The total phenols are not correlated to the IC_{50} and T_{IC50}, but are moderately correlated to EA. On the contrary, flavonoids showed a strong correlation with the IC_{50} and T_{IC50}, but low correlation with obtained EA.
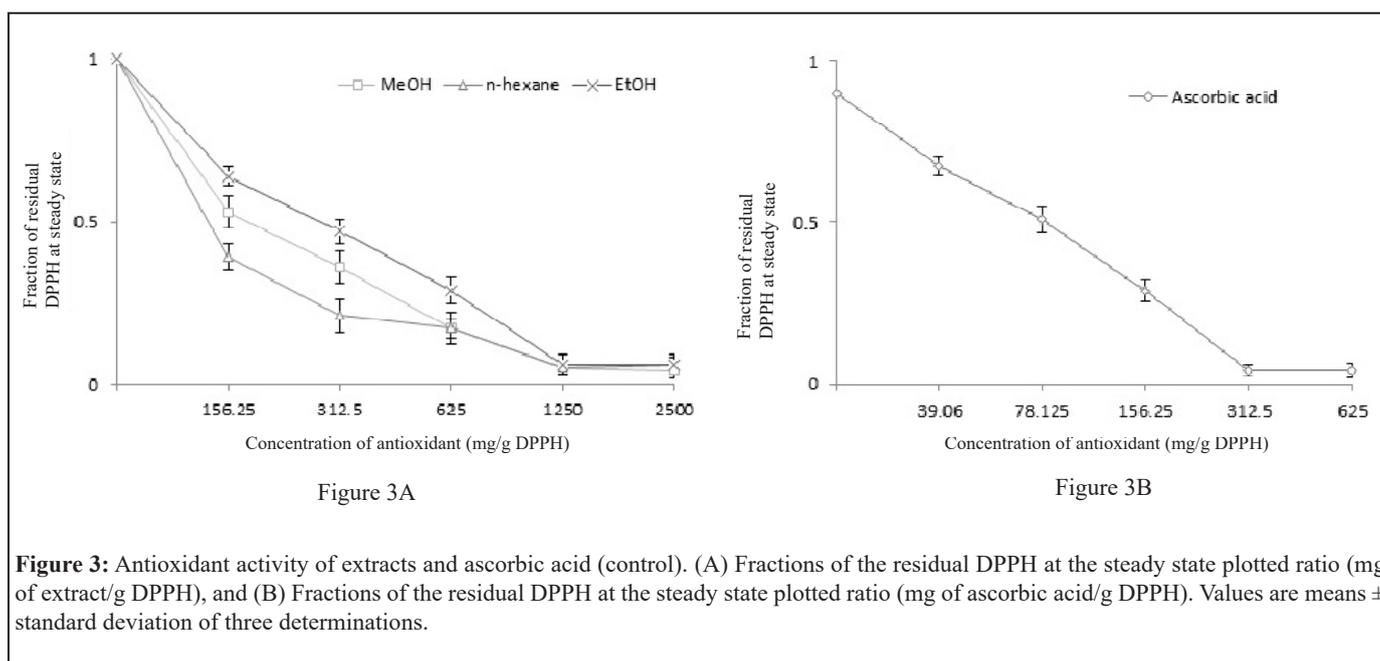
Figure 3: Antioxidant activity of extracts and ascorbic acid (control). (A) Fractions of the residual DPPH at the steady state plotted ratio (mg of extract/g DPPH), and (B) Fractions of the residual DPPH at the steady state plotted ratio (mg of ascorbic acid/g DPPH). Values are means ± standard deviation of three determinations.

This result shows that Anti-Free-Radical Efficiency is a parameter that depends not only on the ability of an antioxidant to scavenge DPPH radical, but al soon other factors including the reaction kinetics and the time required to reach this activity. On the basis of obtained results, we assume that the high antioxidant activity was not limited to phenolics or flavonoids content, as it may also be due to the presence of other antioxidative secondary metabolites, such as volatile oils, carotenoids, lignans, alkaloids, and vitamins [42,43].

Conclusion

This study determined the total phenols and flavonoids contents and examined the kinetics of free radical DPPH scavenging by three extracts (methanol, ethanol and *n*-hexane) of Moroccan *Myrtus communis*. Extracts obtained showed high antioxidant activity, and important phenolic and flavonoid contents. This capacity is reverse when compared to concentrations of IC$_{50}$. Therefore, the parameter of antiradical efficiency is considered more adequate and better compared to IC$_{50}$, in the classification of various antioxidants. The ascorbic acid showed a higher antioxidant capacity than ours extracts. In addition, the efficiency of free radical scavenging increases depending on concentration, and this capacity depends on both the amounts and types of phenolic compounds in the extracts. The industrial exploitation of the extracts of the phenolic compounds from *M. communis* appears to be promising. These species can be used as supplements of natural antioxidants to avoid autoxidation, and the phenomenon of degradation of foodstuffs. Moreover, the free radical scavenging activity is also useful as a preventive property against diseases. The

obtained results suggest that phenolic compounds are the major contributors to the antioxidant activities of *Myrtus communis*, that can be used as an easily accessible source of natural antioxidants and as a possible food supplement or in the pharmaceutical industry.

Acknowledgement

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