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KINETIC PARAMETERS OF THE ANTIOXIDANT ACTIVITIES OF EXTRACTS AND ESSENTIAL OIL OF OLIVE LEAVES

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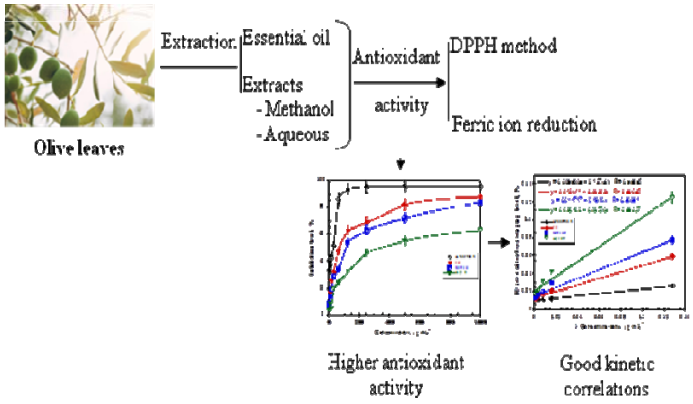
The present study was conducted to determine the kinetic parameters of the antioxidant activity of essential oil, methanol and aqueous extracts of olive leaves. Antioxidant potential was determined by 2,2-diphenyl-2-picryl-hydrazyl (DPPH) and ferric ion reduction power. Result showed that the essential oil and methanol extract exerts higher scavenging activities of DPPH and significant ability for iron reduction at concentration of 1000 µg/mL. Lower values of IC50 were obtained by essential oil and methanol extract and determined by two tested methods. The kinetic parameters showed that the maximal rates of DPPH scavenging were obtained by essential oil and methanol extract. The lower value of KM obtained indicated the good affinity of essential oil and methanol extract to free DPPH radical. The maximal rate of iron reduction (r max) was obtained by essential oil and methanol extracts and the optimal concentration determined was 62.5 µg/mL. The results indicate that the essential oil and methanol extract exerts a strong antioxidant activity can be used as valuable source for commercial exploitation.



INTRODUCTION

Oxidative stress has been one of the main interests of the scientific community for centuries. During the cell metabolism, a series of reactive oxygen species (ROS) are synthesized.[1] The redundant free radicals can damage cells by destructing protein and resulting in the development of inflammation and lipid oxidation.[2] The imbalance between antioxidant defense system and ROS may lead to patho-biochemical mechanisms caused by the development of different diseases.[3] Antioxidants are vital agents that possess the capacity to protect the body from all damages caused by free radical induced oxidative stress and retard the advancement of several chronic diseases.[4] Recently, the synthetic antioxidant agents are restricted because they are suspected to have some toxic effects and even possibly carcinogens.[5] Hence, the studies on natural antioxidants have gained intensively greater importance. Many antioxidant agents, naturally occurring in plant source have been identified as potential antioxidants.[6] Due to its richness in different bioactive phenolic compounds, plants have been used for alternative medicine, pharmaceutical industries, natural therapies and food preservation.[7]

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It has long been acknowledged that some plant essential oils exhibits antioxidant activity and it is necessary to investigate those plants scientifically.[8]

The olive tree (*Olea europaea* L.) deriving from the Mediterranean region, belongs to the Oleaceae family, is one of the oldest fruit tree cultivated for its edible fruits as table olives and for extraction of olive oil.[9] Olive fruit extracts are used as food additives, nutrition supplements and medicinal ingredients.[10] The total polyphenol content in olive leaves is higher than that in olive fruits, however, the use of olive leaves is lower, compared to that of olive fruits.[1] During olive tree cultivation, the pruning step generates an important quantity of olive leaves, which are commonly used as animal feed, and which could also be used as a source of antioxidants.[11] Several methods were used for evaluation of the antioxidant activity; however, the most utilized approaches are based on scavenging DPPH level %, variation of optical density or calculation of IC50 (concentration of substrate that inhibits 50% of the DPPH radicals present in the reaction medium). To the best of our knowledge, the present study is the first to report the determination of the kinetic parameters to explain the affinity of the antioxidant to inhibit the free radical and mechanism of action of the tested natural antioxidant. Hence, present study was carried out to determine the antioxidant activity of essential oil of olive leaves, methanol and aqueous extracts of olive leaves and determination of the kinetic parameters of the reactions.

EXPERIMENTAL

Chemicals substances: All chemicals and solvents (analytical grade) were purchased from Sigma Aldrich (Munich, Germany), unless otherwise specified.

Plant material: Olive leaves used in current study were collected from an olive orchard located in the region of Mohammedia, Mascara (North-West of Algeria) during Mars 2020. The olive leaf was the most used in traditional medicine in the region. The specie was identified taxonomically and authenticated by botanist at SNV faculty, University Mustapha STAMBOULI of Mascara. The leaves were washed with tap water to remove all impurities and then with distilled water. The samples were dried in darkness at room temperature and chopped into small particles to increase the surface of diffusion.

Extracts preparation: The dried leaves were made into fine powder of 40 mesh size using the pulverizer. 10 grams of leaves were macerated in hydromethanolic solution (methanol/water 70:30 v/v) at ratio of 1:10 (w/v) with magnetic stirring overnight at room temperature.[12] This soaking was repeated three-folds by renewing the solvent every 24 hours and then maceration of each solvent was combined. The hydromethanolic extract was recovered after filtration through Whatman No.41 filter paper and concentration in a rotavapor (Buchi Labortechnink AG, Postfach, Switzerland). For preparation of aqueous extract, 10 grams of leaves powder were macerated in 100 mL of distilled water at ratio of 1:10 (w/v) with magnetic stirring overnight at room temperature. This operation was repeated three-folds and then combined. The aqueous extract was recovered after filtration through Whatman No.41 filter paper. The two concentrated extracts were used for *in vitro* determination of antioxidant activity.

Isolation of essential oil: 100 grams of olive leaves in 500 mL of distilled water were submitted to hydro-distillation for 3 hours, using a Clevenger-type apparatus (ST15 OSA, Staffordshire, UK) until total recovery of oil. The extracted essential oil was dried over anhydrous sodium sulfate. In order to preserve it original quality, the oil was stored at 4 °C until tested in an opaque glass bottle sealed. The essential oil yield was evaluated by gravimetric method and expressed in terms of % (w/w ratio between the weight of the obtained oil and the weight of the leaves to be treated). The purity of essential oil was assessed by measurement of physic-chemical indices were evaluated according to the European Pharmacopoeia.[13] The pH was measured by using a digital pH meter apparatus and density by density meter. The relative density (d_{20}^{20}) was determined, which refers to the ratio of mass of the liquid sample and the mass of water, both at 20 °C. Pycnometers were used according to the amount of the essential oil available. The refraction index was evaluated in a refractometer using sodium light of wavelength of 589.3 mm (D ray), which was adjusted with distilled water (refraction index of 1.3330), the samples being kept at 20 °C. The rotatory power was measured by using a polarimeter. The miscibility with ethanol is the volume of ethanol needed to solubilize one volume of the essential oil (v/v). For evaluation of the acid index, a solution of m in (mg) of essential oil was mixed with 5mL of 95% ethanol and 5 drops of phenolphthalein 0.2%. This solution was neutralized with an ethanolic solution 0.1 M of KOH. The titration is over when the pink color begins to appear and persists for at least 15 seconds, we note the volume V in (mL) of the KOH solution that causes this color change and it was calculated by using the following formula:

$$Ia = \frac{5.61xV}{m} \quad (1)$$

where: Ia (acid index), V (volume in mL of the used KOH solution), m (mass of the sample test in gram).

For determination of the saponification index, in a 250 mL monocolumn flask equipped with a reflux condenser, the test sample m (mg) was mixed with 25 mL of 0.5 M alcoholic potassium hydroxide and a few glass beads. The refrigerant was adapted and refluxed for 30 minutes and 1 mL of phenolphthalein was added to the solution. The latter was titrated immediately with 0.5 M hydrochloric acid (sample test). The blank test was carried out under same conditions (blank test). The saponification index was calculated by using the following formula:

$$Is = \frac{(Vt - Ve)xC.HClxM.KOH}{m} \quad (2)$$

where: (Is) saponification index, (Vt) volume in mL of HCl 0.5 M poured (blank test), (Ve) volume in mL of HCl 0.5M

poured (sample test), (C.HCl) concentration of HCl in mol/L, (M.KOH) molar mass of KOH (56.10 g/mol, (m) mass of the sample test in gram. The ester index (Ie) was calculated from the saponification index (Is) and the acid index (Ia) according to the formula:

$$Ie = Is - Ia \quad (3)$$

For determination of iodine value, in a 500mL volumetric flask, one gram of oil sample was mixed with 15mL of carbon tetrachloride and 25mL of Wijs solution. The flask was stoppered and swirled to ensure complete mixing. The sample was then placed in the dark for 30 minutes at room temperature. The flask was removed from storage and 20 mL of 10% potassium iodide (KI) solution added, followed by 150mL of distilled water. The mixture was titrated with 0.1N thiosulphate ($Na_2S_2O_3$) solution adding gradually and with constant and vigorous shaking until the yellow colour had almost disappeared. 1.5mL of starch indicator solution was added and the titration was continued until the blue colour disappeared. A blank determination was conducted simultaneously. The peroxide present was evaluated by titration against thiosulphate in the presence of KI using starch as an indicator.

Evaluation of the antioxidant activity

DPPH free radical-scavenging activity: The antioxidant potential was evaluated in terms of hydrogen donating or radical-scavenging capacity, using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a reagent. The capacity of the sample to scavenge DPPH was assessed by using the method as described by Shen *et al.*[14] with some modifications. The samples to be tested for their antiradical activities were prepared in methanol to achieve the concentration of 1 mg/mL. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99, and 0.97µg/mL. A volume of 50µL of each solution was added to 1950µL of methanol solution of DPPH (6.10^{-5} M) as free radical source. The mixtures were stirred vigorously for 30 seconds and then incubated in the dark for 30 min at room temperature. The absorbance was measured using UV/Vis spectrophotometer model Hitachi 4-2000 at 517 nm against pure methanol. Ascorbic acid was used for comparison as positive control. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The inhibition level, % of DPPH was determined using the equation:

$$DPPH\ radical\ scavenging\ activity, \% = \frac{A\ control - A\ sample}{A\ control} . 100 \quad (4)$$

where: A control: absorbance of the control containing all reagents except the oil or extract, A sample: absorbance of the sample (presence of the essential oil or extract).

The IC50 was estimated from the inhibition level, % versus concentration plot, using a non-linear regression algorithm.

Ferric ion reduction: The ferric ion reduction power of tested samples was determined by using the potassium ferricyanide-ferric chloride method described by Yildirim *et al.*[15] 1mL of each tested sample at different concentrations was mixed with 2.5 mL of 0.2 M phosphate buffer pH 6.6 and 2.5 mL of potassium ferricyanide solution $K_3Fe(CN)_6$, 1 %. After incubation for 20 min at 50 °C, 2.5 mL of trichloroacetic acid 10% was added and the reaction mixture was centrifuged for 10 min at 3000 rpm (Sigma labrzentrifugen D-37620 Osterode am Harz, Germany). An aliquot of 2.5 mL of the supernatant from each mixture was mixed in a test tube with 2.5 mL of distilled water and 0.5 mL of ferric chloride solution (0.1%) prepared freshly in distilled water. After 20 min of reaction time at 35 °C, the absorbance was recorded at 700 nm against a blank that contains all reagents except the essential oil (or extract) solutions and ferric chloride. The control was achieved by different concentrations of ascorbic acid. Higher absorbance of the reaction mixture indicated higher ion reduction power. The concentration providing 0.5 of absorbance (IC50) was estimated by plotting absorbance at 700 nm against the corresponding sample concentration.

Evaluation of the kinetic parameters

Kinetic parameters for DPPH assay: This approach consists to considering the reaction of inhibition of the free radical DPPH by an antioxidant as a chemical reaction similar to the enzymatic reaction. In an enzymatic reaction the maximum reaction rate (r max) and the affinity of the substrate for the enzyme (Km) are determined graphically by the relationship of Lineweaver and Burk. In the reaction of DPPH with the antioxidant, the free radical DPPH inhibition level, % can be considered as the initial rate of reaction. So the plotting of the curve of 1/free radical DPPH scavenging, %= f (1/concentration of tested antioxidant) linear regression makes it possible to graphically determination of the kinetic parameters (r max and Km).

$$\frac{1}{Free\ radical\ DPPH\ scavenging\ \%} = \frac{Km}{r\,max} . \frac{1}{Concentrations\ of\ antioxidant} + \frac{1}{r\,max} \quad (5)$$

Kinetic parameters for ferric reduction power assay: The ferric reducing power assay is often used to estimate the capacity of tested antioxidant to donate an electron or hydrogen to form more stable product. The evaluation of the ferric reducing antioxidant was based on the reduction of Fe^{+3}/ferriccyanide complex to the ferrous form in the presence of antioxidants in the tested samples. The Fe^{+2} were then monitored by measuring the formation of Perl's Prussian blue at 700 nm. In fact, it is widely accepted that higher absorbance at 700 nm is correlated to reducing power. In this method the intensity of the absorbance is proportional to the iron reduction reaction. Plotting the optical density (OD) = f (concentration of antioxidant) curve indicates the power of an antioxidant to reduce iron. The first derivative of this curve gives the rate of the reduction of iron. To have this rate we draw the curve: difference of optical density (dOD) versus difference of concentration of antioxidant plot (dC), using a non-linear regression algorithm.

$$dOD = f(dC) \quad (6)$$

$$OD2 - OD1 = f(C2 - C1) \quad (7)$$

Data analysis: All determinations were conducted in triplicates and results for each measured parameter were expressed as mean ± SD. Data were statistically determined by analysis of variance ANOVA using the level significance ($P<0.05$) using Microsoft Excel and SPSS statistics software 8.1.

RESULTS AND DISCUSSION

Physic-chemical characterization of olive leaves essential oil: Olive leaves essential oil was characterized by a limpid liquid aspect, volatile, mobile, with light green colour, strong and fresh smell. This oil was marked by a yield of 0.9 ± 0.3%. The physic-chemical characterization (pH 5.1 ± 0.2, density at 20 °C 0.84 ± 0.02 g/mL, relative density 0.91 ± 0.01 g/g, refractive index at 20 °C 1.355 ± 0.002, rotatory power +5.32 ± 0.12, miscibility with ethanol at 96% 1:9 (v/v), acid index 17.362 ± 0.010 mg KOH/gEO, ester index 115.171 ± 0.030 mg KOH/gEO at 20 °C, saponification index 134.20 ± 0.04, peroxide index 12 ± 0.3 meq O2/KgEO, carbonyl index 146.31 ± 0.02, and iodine number 2.9 ± 0.3) showed the good quality of the studied oil.

Antioxidant capacity of extracts and essential oil of olive leaves

DPPH free radical-scavenging capacity: DPPH is free radical that provides information about the ability of the antioxidant agent to inhibit oxidative cell damages by preventing reactive radical species from attacking key bio-compounds in biological and food systems.[16] It is frequently used to evaluate the antioxidant activity. As shown in Fig 1, the antioxidant potential was directly correlated to the concentrations used. The ascorbic acid (positive control) displayed the higher scavenging activity of DPPH radical 96.3 ± 0.2% at concentration of 1 mg/mL, folowed by essential oil 88.0 ± 0.1%, then methanol extract 84.0 ± 0.2% and aqueous extract 63.2 ± 0.3% at same concentration.

Recent studies have conducted to evaluate the antioxidant compounds derived from leaves and fruit of olive trees, numerous fruits and vegetables, as well as medicinal and aromatic plants and spices.[17] Free radical scavenging capacity is strongly related to the abundance of polyphenol compounds. Recent research showed that the main constituents of phenolics compounds of olive leaves extracts determined by UPLC-MS/MS were as follows: apigenin, diosmetin, hydroxytyrosol, luteolin, oleanolic acid, oleuropein, and quercentin.[18] Similar results obtained by using FPLC coupled with photo diode array detection (DAD) revealed six major polyphenolic compounds: oleuropein, verbascoside, luteolin-7-O-glucoside, apegenin-7-O-glucoside, hydroxytyrosol and tyrosol.[19] The methanol extract of olive leaves content a large quantity of phenol content 2.06 CAE.[20] This phenol content was mainly represented by polyphenols 41.77%, flavonoids 30.01%, soluble proteins 20.40% and free amino acids 0.09%. [21] According to the literature, the oleuropein and phenols are the main constituents present in olive leaves possessing high antioxidant activity.[22] The antioxidant capacity of hydroxyl compounds in olive leaf extract could be related to the abundance of the hydroxyl groups in their structure such as oleuropein, hydroxytyrosol, and luteolin-7-o-glucoside acid.[23] Presented results are in line with other studies suggested that olive leaves extracts are a source of antioxidants acting as radical scavengers.[16,17,22] Similar results were obtained by several authors who describe that for a same plant organ, extracts obtained with methanol possess a higher antioxidant potential than those prepared with water.[20,24] Certain studies suggests that the olive phenolics compounds of olive leaves exhibits a synergic behavior in the ability of elimination of free radical when mixed in the form of extract, superior to the vitamin C and E6.[25]

Ferric ion reduction: As shown in Fig 2, the reducing ability of tested samples increased in a concentration-dependant manner. According to the results, we note that significant ability for reducing iron, values observed by optical density (OD) of 2.07 ± 0.20 for ascorbic acid, followed by 1.90 ± 0.10 for essential oil, then 1.75 ± 0.10 for methanol extract and 0.99 ± 0.20 for aqueous extract of olive leaves in the concentration of 1 mg/mL. The present work reveals that the essential oil and methanol extract of olive leaves possesses strong antioxidant capacities presumably due of its phytochemical constituents such as polyphenols and oleuropein.

The DPPH scavenging capacities of tested samples showed a good correlation with its reductive activities. These facts justify the medicinal use of the leaves for treatment of various diseases. This can be of valuable source in preservation of foodstuffs, drug products and cosmetics, where free-radical-mediated chain reactions result in lipid oxidation. These natural substances may also prove to have therapeutic capacity, as free radicals are believed to be involved in the pathogenic cascade of events in many maladies. Several authors have observed a direct correlation between antioxidant capacities and reducing power of certain plant extracts.[26, 27] The reducing capacity is generally associated with the presence of reductones. The antioxidant activity of reductones is based on the breaking of the free radical chain by donating a hydrogen atom.[28] The marked antioxidant potential may be attributed to the abundance of polyphenols which

may act as reductones to convert free radical into more stable products and terminate free radical chain reaction. Reductones are reported to react with certain precursors of peroxide, thus preventing peroxide formation.

IC50 values: The IC50 parameter frequently used to estimate the antioxidant capacity and is necessary for each sample to reduce 50% of DPPH radical concentration in a defined period of time, a low value of IC50 corresponds to a higher antioxidant capacity. It was used to classify antioxidant activity of tested sample in comparison with the standard.

As shown in Fig. 3, the ascorbic acid displayed a lower values of IC50: 30 ± 0.2 µg/mL (determined by DPPH method) and 50 ± 0.1 µg/mL (evaluated by iron reduction), followed by essential oil (70 ± 0.1 µg/mL, 60 ± 0.2 µg/mL), then mehanol extract (110 ± 0.1 µg/mL, 80 ± 0.3 µg/mL), and aqueous extract (400 ± 0.4 µg/mL, 220 ± 0.3 µg/mL). Presented data of DPPH IC50 values are lower to those found by Abaza *et al.*[22], who report that methanol extract exhibited IC50 of 170 µg/mL and water extract the value of 970 µg/mL. Several studies have demonstrated that the iron reduction ability of bioactive compounds is related to its antioxidant capacity.[29, 30]

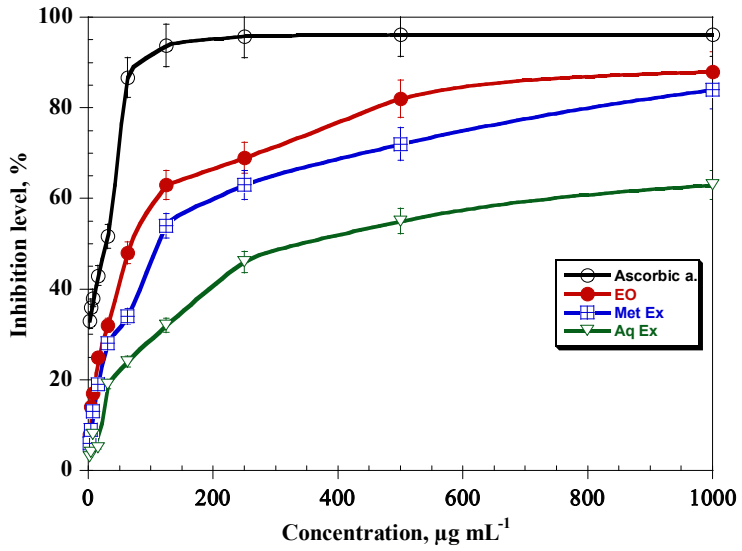

Fig. 1 – Free radical-scavenging activities of ascorbic acid, essential oil (EO), methanol (Met Ex) and aqueous extracts (Aq Ex) of olive leaves. Values represent Mean ± SD; n=3; Confidence level $p \leq 0.05$.

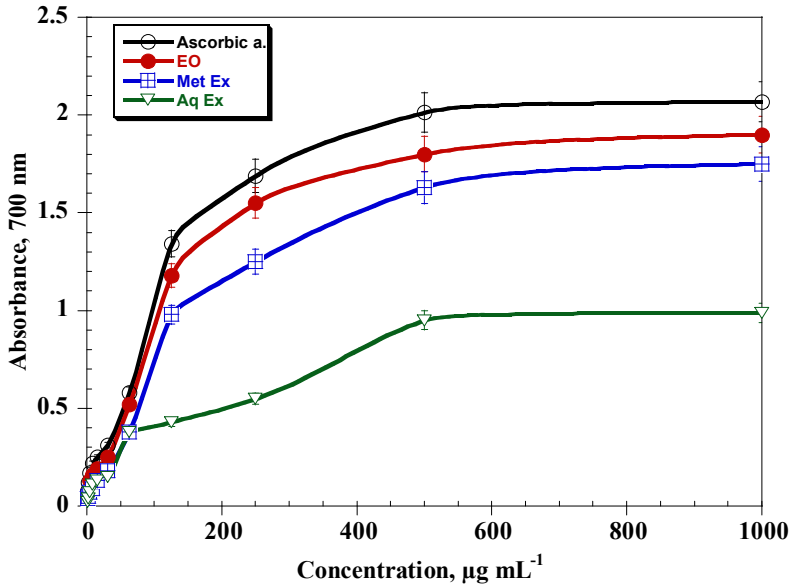

Fig. 2 – Ferric reducing ability of ascorbic acid, essential oil (EO), methanol (Met Ex) and aqueous extracts (Aq Ex) of olive leaves. Values represent Mean ± SD; n=3; Confidence level $p \leq 0.05$.

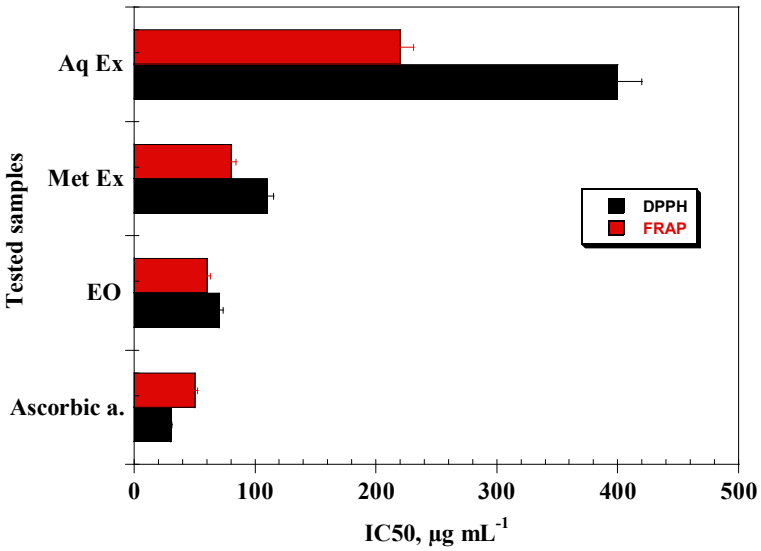

Fig. 3 – Values of IC50 obtained by ascorbic acid, essential oil (EO), methanol (Met Ex) and aqueous extracts (Aq Ex) of olive leaves using DPPH and Ferric ion reduction assays. Values represent Mean ± SD; n=3; Confidence level $p \leq 0.05$.

Kinetic parameters of antioxidant activities: As shown in Fig. 4 and Table 1, the ascorbic acid displayed the higher rate of DPPH scavenging (100%), followed by essential oil with rate of 79.107%, then methanol and aqueous extracts of olive leaves 68.948% and 49.014%. The affinity of tested antioxidant to the DPPH was estimated by determination of the KM. Ascorbic acid presents a lower concentration of Km (12.768 µg/mL) indicated higher affinity, followed by essential oil 28.718 µg/mL, then methanol extract 33.417 µg/mL and aqueous extract 40.464 µg/mL. Ascorbic acid, EO and methanol extract exhibited a higher rate of DPPH scavenging and have a good affinity to the DPPH compared to the aqueous extract.

Table 1

Kinetic parameters (r_{max} and Km) of tested antioxidants against free radical DPPH

Attributes	Ascorbic acid	Essential oil	Methanol extract	Aqueous extract
r_{max}	100	79.107	68.948	49.014
Km	12.768	28.718	33.417	40.464

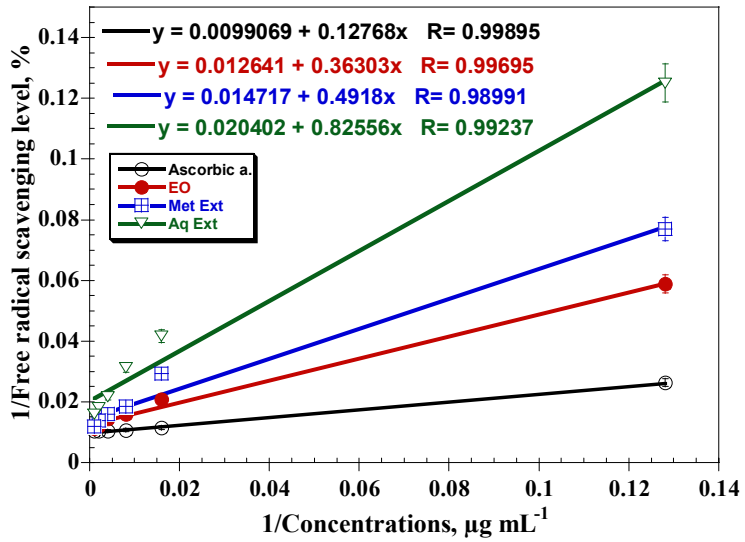

Fig. 4 – Correlation between (1/Free radical scavenging level, %) and (1/Concentrations of antioxidant agent, µg/mL) of ascorbic acid, essential oil, methanol and aqueous extracts of olive leaves.

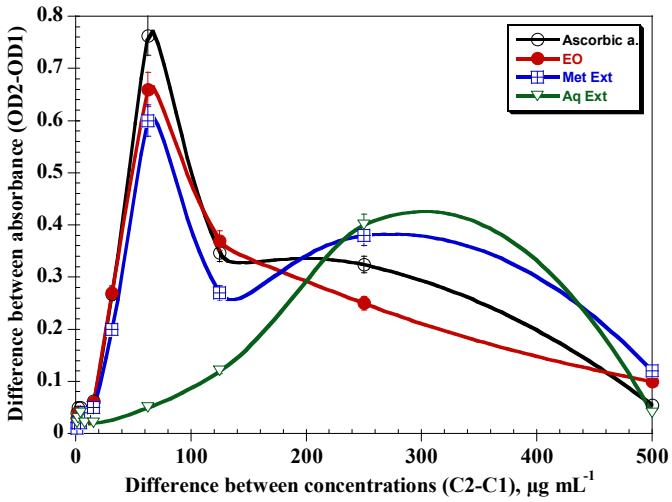

Fig. 5 – Rate of iron reduction by tested antioxidant (ascorbic acid, essential oil, methanol and aqueous extracts of olive leaves).

Table 2

Kinetic parameters (r_{max}) of tested antioxidants for iron reduction.

Attributes	Ascorbic a.	EO	Met Ext	Aq Ext
Maximal rate of iron reduction (r_{max})	0.763	0.660	0.600	0.400
Optimal concentration of antioxidant used (µg/mL)	62.5	62.5	62.5	250.0

As shown in Fig. 5 and Table 2, the concentration of 62.5 µg/mL of antioxidant is able to exert a maximal rate of iron reduction by ascorbic acid (0.763), essential oil (0.66) and methanol extract (0.6). For aqueous extract of olive leaves, the higher concentration (250 µg/mL) provokes only the rate of 0.4.

CONCLUSIONS

The results from this study demonstrated clearly that essential oil and methanol extract of olive leaves exerts a strong antioxidant potential determined by two methods: DPPH free radical scavenging and ferric ion reduction. Our results denoted that methanol is the most efficient extraction solvent compared to the water solvent. It may provide a valuable source of natural antioxidants for commercial valorization and exploitation. The determination of kinetic parameters of antioxidant activity (r max and Km for DPPH assay, optimal concentration and rate of reduction for FRAP method) gives a new approach and a good explanation of mechanism of action of these tested antioxidants. Furthermore studies on the isolation and quantification of bioactive compounds presents in essential oil and methanol extract are required to help research concerning the bioavailability of these substances for pharmaceutical and clinical studies.

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