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Evaluation of Antioxidant Activity by Electrochemical and Chemical Methods, Kinetics and Thermodynamic Parameters of Superoxide Anion Radical towards *Cupressus sempervirens* L. Extracts

Zineb Rahmani[1*], Assia Belfar, Tatou Touahria[1], Chayma Bensaci[1], Zehour Rahmani[1,2], Messaouda Dekmouche[1,2], Mokhtar Saidi [1,2] and Ali Douadi[2,3]

[1]Laboratoire de Valorisation et Promotion des Ressources Sahariennes (LVPRS)
[2]Univ Kasdi Merbah, Department of Chemistry, Faculty of Mathematics and Matter Sciences, BP 511 Route Ghardaia, Ouargla, Algeria - 30000
[3]Pollution & Waste Treatment Laboratory

Corresponding Author Email: zhorrahmani@gmail.com

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ABSTRACT

For the first time in this report, we determined the antioxidative capacity of *Cupressus sempervirens* extracts by using two methods such as inhibition of superoxide anion ($O_2^{\bullet-}$) made by alkaline pyrogallol, and electrochemical generation of this radical. We have studied the $O_2/O_2^{\bullet-}$ redox couple on the GC in DMF. We obtained well-resolved quasi-reversible and reproducible cyclic voltammograms for the $O_2/O_2^{\bullet-}$ redox couple. A $\frac{i_{pa}}{i_{pc}}$ value of 0.96 and ΔE_p value of 160 mV for scan rates 0.1 V/s were obtained. In addition, the standard electrochemical rate constant $k°$ is 2.31 x 10^{-3} cm s^{-1}; all these characteristics clearly show that the system is quasi-reversible. Furthermore, we have used the cyclic voltammetric to study the antioxidant capacity of *Cupressus sempervirens* extracts. The thermodynamic feasibility of the radical scavenging by extracts was accounted in term of standard free energy ΔG°, which ranged from -8.934 to 0.042 kJ/mol.

1. INTRODUCTION

Usually, molecular oxygen in the mitochondria, and through electrons, result in the production of energy, which allows our cells to stay alive. Nevertheless, this operation is not perfect: 1 to 5% of the oxygen concerned can remain free and be at the origin of the formation of reactive oxygen species (ROS) [1].

Free radicals are chemical species (atoms or molecules) that have one or more single electrons (unpaired electron) on their outer shell and capable of existing independently. They can be derived from oxygen (ROS) or other atoms such as nitrogen (RNS). All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage.

Tissue destruction and degeneration can result in increased oxidant damage, by such processes as metal ion release, phagocyte activation, and disruption of mitochondrial electron transport chains (so that more electrons "escape" to oxygen to form $O_2^{\bullet-}$). It follows that almost any disease is likely to be accompanied by increased formation of reactive oxygen species[2].

Under normal physiological conditions, the concentrations of ROS are subtlety regulated by antioxidants, which can be either generated endogenously or externally supplemented[3].

Natural antioxidants are extracted, usually in a mixture of several compounds, from variable sources[1]. Among these plants a cypress that affiliate to *Cupressaceae* family, which is widely used in traditional medicine. *Cupressaceae* is a family of gymnosperm plants. It contains about 19 genera and 130 species of trees and shrubs. *Cupressus sempervirens* are endemic to the Mediterranean area.

Popularly, it is known as "sarwel" that is considered to be a medicinal tree as its dryish leaves are used for stomach pain as well as to treat diabetes. This plant is known to anti-inflammatory and astringent[4]. Essential oil of *Cupressus sempervirens* showed antiviral activity against HSV-1 virus[5].

In previous article[4], we reported that all extracts have high reductive activity better than butylated hydroxyanisole (BHA), whereas in α-amylase enzyme inhibitory activity, ethyl acetate fraction of fruits showed the highest α-amylase inhibitory activity.

The other aim of our work was to determine the antioxidant activity especially inhibition of superoxide anion of *Cupressus sempervirens* extracts by chemical and electrochemical methods.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Butylated hydroxyanisole (BHA), ascorbic acid, Gallic acid and Pyrogallol were provide by Sigma-Aldrich, Sodium dihydrogen phosphate, Di-sodium-hydrogenand and N,N-Dimethylformamide (DMF HPLC-grade) were provide by Biochem chemopharma. Tetrabutyl ammonium tetrafluoroborate ($TBABF_4$) (electrochemical grade 99 %;

Sigma-Aldrich). Molecular oxygen was provided from a cylinder (research grade (99.99 %); Linde gaz Algeria). All other reagents used were of analytical grade.

2.2. Preparation of plant extracts

In previous article[4], we reported the protocol of preparation of plant extracts.

2.3. Inhibitory capacity of the radical superoxide anion

2.3.1. Chemical method

The auto-oxidation of pyrogallol will be used as an anion source. Their anionic form like phenolate in alkaline medium is oxidized and gives quinone accompanied by oxygen consumption. Superoxide anion scavenging activity of the extract of *C. sempervirens* were assessed by the pyrogallol auto-oxidation method[6]. 10 µL of 45 mM pyrogallol mixed with 4.5 mL of Phosphate Buffered Saline (pH 8.2) and 0.5 mL of 10^{-3} g L^{-1} of the extract or water at 25 °C. The absorbance of the samples was read at 320 nm every 30 seconds; the percentage of inhibition of $O_2^{\bullet -}$ is calculated by the following equation:

$$I = \left[\frac{\Delta A^{\circ} - \Delta A}{\Delta A^{\circ}}\right] \times 100 \quad (1)$$

ΔA° is rate of auto-oxidation of pyrogallol in the absence of antioxidant

ΔA is rate of auto-oxidation of pyrogallol in the presence of antioxidant

2.3.2. Electrochemical method (cyclic voltammetry)

The cyclic voltammetry belongs to the electrochemical methods most usually used. It constitutes a method of choice to acquire simply and quickly information on the processes redox, or the reversibility of the studied electrochemical system. This method is also used to estimate the antioxidant power of plant products[7]. The cyclic voltammetry method is carried out according to Le Bourvellec et al[8].

First, we apply this method to study the electrochemical behavior of radical $O_2^{\bullet -}$ then study the electrochemical behavior of the plant extracts with this radical.

Cyclic voltammetry method is carried out by a Potentionstat-Galvanostat device PGZ301 Voltalab 40 controlled by VoltaMaster 4 software of the radiometric analyzer, a 25 mL electrochemical cell which contains the electrodes: working electrode is a glassy carbon with a diameter of 3 mm, auxiliary electrode is a platinum electrode and reference electrode is saturated calomel electrode (SCE). The medium used is DMF and Support electrolyte $TBABF_4$ with concentration 0.1 M.

Experimental conditions: Glassy carbon electrode; 25 mL of DMF/$TBABF_4$ 0.1 M; scan rate 0.1 V s^{-1}; 25 °C. The solutions were saturated with high purity commercial oxygen for 15 min prior to each experiment.Volumes of extract are added to 25 mL of solution (DMF/$TBABF_4$), each time. Then, the voltammogram is recorded after each addition. The extracts used in this electrochemical part are *n*-butanol, ethyl acetate fractions for the fruits and leaves and Ascorbic acid, Gallic acid were used as standard.

The radical anion superoxide inhibition was expressed as a percentage of inhibition and is calculated according to voltammogram by equation (2):

$$I = \frac{i_0 - i_s}{i_0} \times 100 \quad (2)$$

Where:

I is percentage of inhibition

i_{pa0} is density of the anode current in the absence of sample

i_{pas} is density of the anode current in the presence of sample

The IC_{50} index is defined by the concentration of antioxidant, which is capable of inhibiting 50 % of the radical anion superoxide.

Statistical Analysis

Statistical software analysis was performed using Origin Pro 8 software. Data are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to compare any significant differences between extracts and organs. P values < 0.05 were regarded significant.

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3. RESULTS AND DISCUSSION

3.1. Superoxide anion scavenging activity by chemical method

Measurement of superoxide anion scavenging activity of *C. sempervirens* is estimated by using the auto-oxidation reaction of pyrogallol occurring under alkaline condition, percentages of trapping the radical of all extracts varied between (71.47 ± 0.45 % and 17.24 ± 0.36 %).

Figure 1 shows the inhibition effects of different samples at the same concentrations (10^{-3} g L^{-}). The greatest inhibition superoxide anion was found at ethyl acetate fractions of leaves and fruits (71.163 ± 0.06 % and 71.47 ± 0.45 % respectively), whereas that the lowest percentages of trapping the radicals was recorded at dichloromethane fraction of fruits (17.24 ± 0.36 %). We reported that ethyl acetate leaves is strong inhibition of $DPPH^{\bullet}$[4].

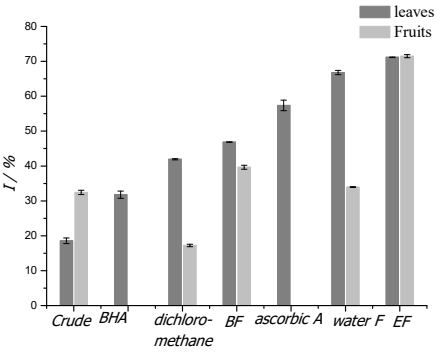


Figure 1. Inhibition effects of different samples at 10^{-3} g L^{-1} by spectrophotometric method

3.2. Superoxide anion scavenging activity by electrochemical method

The cyclic voltammetry technique is used to generate the superoxide anion radical $O_2^{\bullet -}$ in the layer of diffusion of electrode CV by the reduction of oxygen to an electron in the DMF. It is known that the radical $O_2^{\bullet -}$ is stable in medium aprotic like DMF[9]. The superoxide anion radical was generated by one electron reduction of the molecular oxygen O_2 dissolved in DMF at room temperature 25 °C. The voltammogram was recorded firstly in the absence of substrate to determine the i_{pa0} value (the anodic oxidation current density of $O_2^{\bullet -}$. The reduction potential of $O_2/O_2^{\bullet -}$ is -0.93 V vs. SCE, and the oxidation potential of $O_2/O_2^{\bullet -}$ is -0.77 V vs. SCE. Moreover, the cathodic and anodic peak heights (current density values) are 67.61 µA cm^{-2} and 70.24 µAcm^{-2}, respectively. We have obtained an ΔE_p value of 160 mV for a scan rate 0.1 Vs^{-1}, corresponding to the quasi-reversible one electron reduction of oxygen to produce superoxide anion radical. In DMSO, DMF and pyridine, the cathodic/anodic peak separation on typical cyclic voltammograms is of 100 to 200 mV, regardless of the nature of the electrode. This value, which deviates from the "classical" 60 mV expected for a 1-electron process, is attributed to the conjunction of the slow charge transfer kinetics, the difference in the diffusion coefficients of oxygen and superoxide, and uncorrected or underestimated ohmic drop[10].

Based on the theory by Matsuda and Ayabe [11] the experimenter can estimate the electron transfer rate constant k^0 and determine the electrochemical system according the following data:

$k^0 > 0.35 \cdot v^{1/2}$	reversible
$0.35 \cdot v^{1/2} > k^0 > 3.5x10^{-4} \cdot v^{1/2}$	quasi-reversible
$k^0 < 3.5x10^{-4} \cdot v^{1/2}$	irreversible

The peak potential is expressed as:

$$Epc = E° - 2.3\frac{RT}{\alpha nF}\left[\log\left(\frac{D_{ox}^{\frac{1}{2}}}{k°}\right) + \log\left(\frac{\alpha nF}{RT}\right)^{\frac{1}{2}} + 0.34\right] \quad (3)$$

We calculated the relative charge transfer rate, $k°$ from the equation (3) with the following data:

$D_{ox} = D\,o_2^{\bullet -} = 1.3 \times 10^{-5}$ cm^2 s^{-1}; [12] $\alpha = 0.5$; $v = 0.1$ V s^{-1};

$E° = \frac{(Epa + Epc)}{2} = -0.85$; $R = 8.31$ J mol^{-1} K^{-1}; $T = 298$ K.

Therefore, we found $k^0 = 2.31x10^{-3}$ cm s^{-1}, this value sufficiently expresses that the electrochemical system of reduction of oxygen is quasi-reversible.

In our work the half-peak potential: $E_{1/2}$ of $O_2/O_2^{\bullet -}$ redox couple in DMF and $TBABF_4$ is -0.828 V vs. SCE, some of the experiments were performed using different supporting salt and DMF as solvent also glassy carbon electrode. The results show that the half-peak potentials remained identical within 0.078 V. For example, $E_{1/2}$ of $O_2/O_2^{\bullet -}$ redox couple in $TBAClO_4$ is - 0.78 V vs. SCE[13], another study shows that, $E_{1/2}$ of $O_2/O_2^{\bullet -}$ redox couple in Bu_4NPF_6 is - 0.75 V vs. SCE[14].

The half-peak potential depends on the relative diffusion coefficients for oxygen and superoxide, and on the reaction kinetics. In addition, in such high-permittivity solvents, the nature of the anion associated to the TBA^+cation (ClO_4^-, PF_6^- or BF_4^-) and the overall salt concentration also play minor role on the first reduction of oxygen[15].

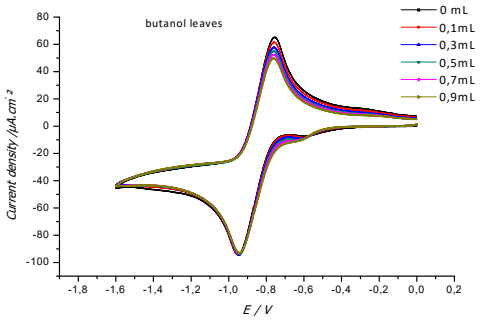
The half-wave potential of $O_2/ O_2^{\bullet -}$ is somewhat more negative in $TBABF_4$ than in $TBAClO_4$, showing that the BF_4^- ions solvate more strongly in DMF than ClO_4^-.

In summary, the data obtained in $TBAClO_4$ (- 0.78 V) and $TBAPF_6$ (- 0.75 V) based electrolytes reveal that the anion has little on the redox processes.

This weak but noticeable oxygen reduction dependence on the counter ion is evident by these shifts. The oxygen reduction reaction in $TBABF_4$ solution is slightly negative by 85 mV (E_{pc} is - 0.855 V for $TBAClO_4$[16], in $TBABF_4$ is - 0.93 V), indicating that oxygen reduction in the presence of BF_4^- is to some extent slightly polarized. This may be due in part to the less coordinating nature of the BF_4^-, allowing the larger TBA^+ ion to interact with dissolved oxygen. Generally, the electrolyte/electrode interface is affected by the nature of the counter ion.

The voltammograms of the reduction of $O_2$was recorded in the presence of an antioxidant (extracts) in order to evaluate the antioxidant capacity of the molecule in search for its reactivity towards$O_2^{\bullet -}$. The increase in the concentration of the antioxidant substrate leads to a concentration decrease of $O_2^{\bullet -}$ which corresponds to a decrease in anodic current density: i_{pas} while cathodic current density: i_{pcs} is not significantly modified (Figure 2).

According to the values of IC_{50}, ethyl acetate fractions of leaves (EF) and fruits (EFF) were recorded the greatest scavenging activity with 0.136 and 0.427 g L^{-1} respectively while the *n*-butanol fractions of leaves (BF) and fruits (BFF) exhibited the less inhibition of $O_2^{\bullet -}$ radical (Figure 3). Cyclic voltammetry is suitable technique for evaluating the antioxidant activity by determining the mechanisms the inhibition of free radicals. According to the cyclic voltammograms the ethyl acetate fractions of leaves and fruits is the more active to trap$O_2^{\bullet -}$radicals whence this scavenging power can be attributed to bioactive compounds found in these parts in spite of this fractions have contents of flavonoids, phenols less than contents of flavonoids, phenols which found in butanol fractions. The interaction of flavonoids with many radicals has been used in several studies to define the major elements of antioxidant activity. Flavonoids (Flav-OH) are thermodynamically capable of reducing oxidative free radicals (R•) such as superoxide, peroxyl radical, alkoxyl radical and OH• by hydrogen transfer.



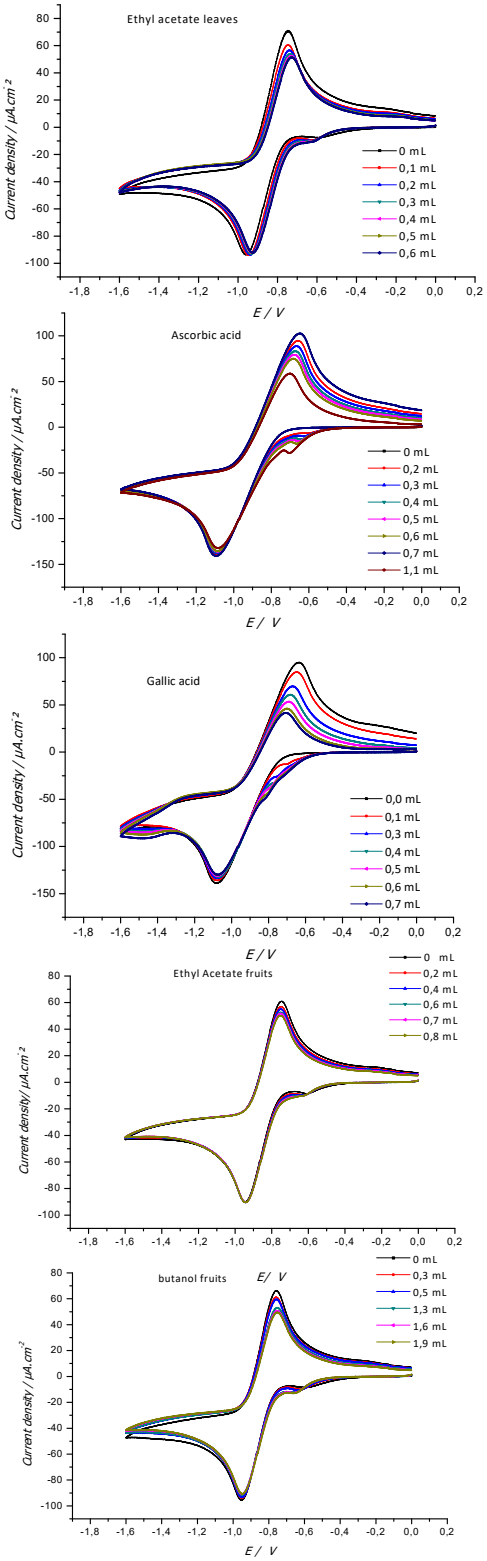


Figure 2. Cyclic voltammograms of oxygen-saturated DMF/0.1 M $TBABF_4$ on a GC electrode in presence of *C. sempervirens* extracts, Gallic acid and Ascorbic acid; Scan rate 0.1 V s^{-1}; 25 °C

The resulting aryl radical (Flav-O•) can react with another free radical to form a stable quinone structure. In addition, the aroxyl radical can interact with oxygen to give a quinone and a superoxide anion. Therefore, the ability of flavonoids to act as antioxidants depends not only on the redox potential of the Flav-O• / Flav-OH pair, but also on the reactivity of the aroxyl radical[4].

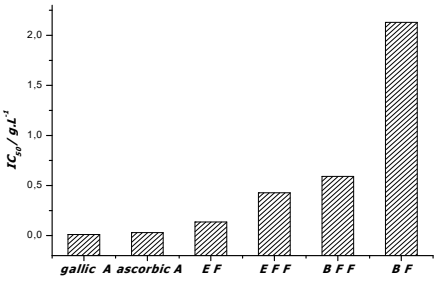


Figure 3. Values of IC_{50} for Inhibition of $O_2^{\bullet-}$ of *C. sempervirens* extracts, Gallic acid and Ascorbic acid by cyclic voltammetry method

3.2.1. Antioxidant activity coefficient K_{ao}

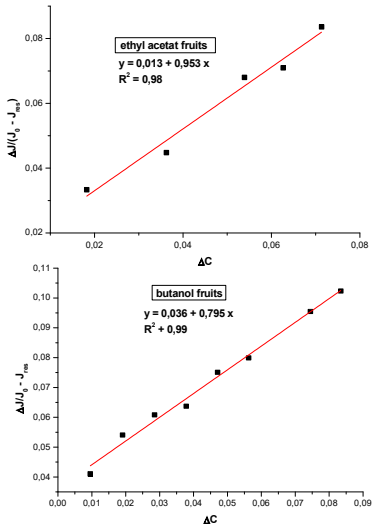
The relative superoxide scavenging capacity was calculated as the antioxidant activity coefficient K_{ao}[17] and data shown below in Figure 4. The constant K_{ao} is expressed as the ratio of current density values in the presence and absence of substrate to the electrochemically generated superoxide free radicals. The relative antioxidant capacity of each compound was quantified by the following equation (4):

$$Kao = \frac{\Delta J}{(J0 - Jres)\Delta C} \qquad (4)$$

Where:

ΔJ is the change in current density with the addition of antioxidant

J_o is the limiting current density without antioxidant J_{res} is the residual current density of the dissolved oxygen ΔC is the change in the concentration of the antioxidant in g L^{-1}



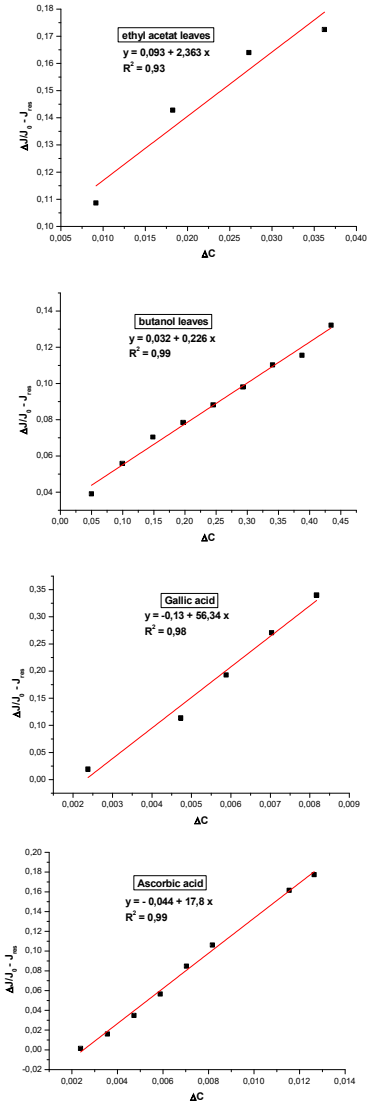


Figure 4. Plots to determine antioxidant activity coefficient K_{ao} using equation $\frac{\Delta J}{(J_0 - J_{res})}$ vs ΔC for *C. sempervirens* extracts, Gallic acid and Ascorbic acid in DMF/0.1 M $TBABF_4$

3.2.2. Thermodynamic parameters

Binding constant and free energy

The addition of x mL of antioxidant in DMF to oxygen-saturated DMF/0.1 M $TBABF_4$ solution shows a decrease in peak current, (Figure 2). The interaction between superoxide radical and antioxidant was estimated in terms of the binding constant K_b. Based on the decrease in the peak current, the binding constant K_b of superoxide radical with the additive was calculated using (5) [12]:

$$\log\left(\frac{1}{[OA]}\right) = \log Kb + \log\left(\frac{Ip}{Ip0 - Ip}\right) \qquad (5)$$

Where:

I_{po}, I_p are peak currents of superoxide anion radical in the absence or presence of antioxidant, respectively.

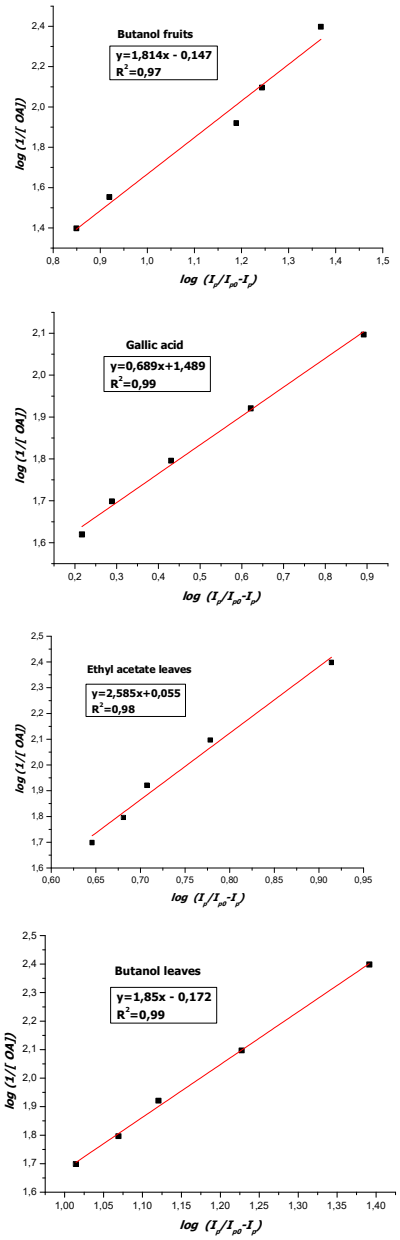
[AO] is concentration of antioxidant, since the antioxidants are plant extracts, we calculate the concentration by volume / volume (vol. extract/25 mL).

The binding energies ΔG were calculated using (6):

$$\Delta G = -RT \ln Kb \qquad (6)$$

Where ΔG is the binding free energy in KJ mol^{-1}, R is the gas constant, 8.32 J mol^{-1} K^{-1} and T is the absolute temperature, 298 K.

Binding constant and binding free energy of studied antioxidant as obtained from Figure 5 and are listed in (Table 2). The effect of the addition of antioxidant to the solution of oxygen-saturated DMF/0.1 M $TBABF_4$ on the wave voltammetry is changed. The current drops on the addition of antioxidant owing to the binding of $O_2^{\bullet -}$. The peak potential shifted to a more negative value in the presence of standards antioxidant.



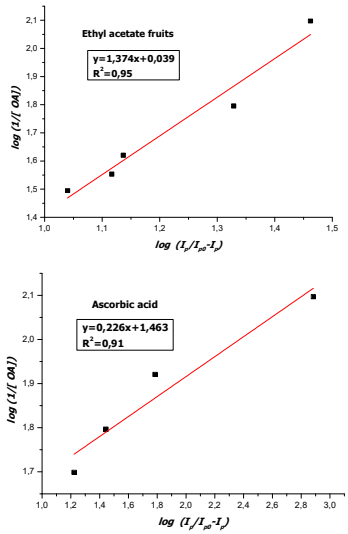


Figure 5. Plots to determine binding constant K_b using equation log (1/[OA]) vs log (I_p/I_{p0}-I) for *C. sempervirens* extracts, Gallic acid and Ascorbic acid in DMF/0.1 M $TBABF_4$

The obtained K_b values of the extracts are lower than the Gallic acid and ascorbic acid, which confirm the strong affiliation of these synthetic compounds with the radical and indicate the high probability of the presence of more than one compound in the extract that play an anti-synergistic role.

Table 1. Binding constant: K_b, change in free energy of reaction: ΔG° and antioxidant activity constant: K_{ao} for the antioxidants

Antioxidant	K_b*	ΔG°(kJ mol^{-1})	K_{ao} (L g^{-1})*
Ethyl acetate fruits	1.095	-0.225	0.953
Ethyl acetate leaves	1.130	-0.302	2.363
n-butanol fruits	0.706	0.862	0.795
n-butanol leaves	0.983	0.042	0.226
Gallic acid	36.88	-8.934	56.342
Ascorbic acid	29.05	-8.073	17.797

Values followed by * are significantly different at $p< 0.05$

The addition of different concentrations of antioxidant in DMF to a solution of DMF saturated with commercial oxygen provokes a remarkable decrease in the peak current density, Figure 2. The substantial diminution in peak current density can be attributed to the decrease in free radical $O_2^{\bullet-}$ concentration due to the formation of $O_2^{\bullet-}$ −antioxidant product.

Kinetics

Kinetics of the free radical scavenging was assessed in terms of the second order homogenous rate constant k_2. For this purpose pseudo first order rate constants k_f for the reaction of additives and superoxide radical were calculated using the Nicholson- Shain equation (7) [18].

$$Ep = E_{1/2} - \frac{Rt}{nF}\left[\left(0.78 - \ln\sqrt{\frac{kf}{\alpha}}\right)\right] \quad (7)$$

Where:

$a = nF\,v\,/\,RT$

$E_{1/2}$ is reversible half wave potential of super oxide radical

E_p is peak potential after the addition of a higher concentration of the additive

v is scan rate (V s^{-1})

The values of k_2 for the compounds were calculated from the following relationship (8):

$$k_2 = \frac{k_f}{[AO]} \quad (8)$$

$[AO]$ is the concentration of antioxidants, which was present in large excess in order to obtain the pseudo first order condition. From the k_2 Gibbs energy of activation (ΔG^*) was calculated using the Arrhenius form of the rate constant relationship (9).

$$k_2 = \frac{KT}{h}\exp(-\Delta G / RT) \quad (9)$$

Where:

K is Boltzman constant

R is gas constant

h is Plank's constant

T is Temperature

The data obtained is presented in (Table 2) along with the Gibbs activation energies ΔG^*.

Table 2. Homogeneous rate constant: k_2 and energy of activation: ΔG^* for the antioxidants

Antioxidant	E_p	[AO] *	k_fx10^{-3}	k_2 x10^{-3}	ΔG^*
Ethyl acetate fruits	-0.755	0.032	5.486	171.43	43.10
Ethyl acetate leaves	-0.731	0.024	35.62	1484.2	37.75
n-butanol fruits	-0.756	0.036	5.074	140.9	43.58
n-butanol leaves	-0.764	0.036	2.720	75.55	45.13
Gallic acid	-0.717	0.024	106.06	4419.2	35.05
Ascorbic acid	-0.691	0.044	804.72	18289.1	31.53

Values followed by * are significantly different at $p< 0.05$

It must be noted that the k_2 values are proportional to the K_{ao}. The former tells how fast is the antioxidant behaviour while the latter is an estimate of the scavenging capacity, i.e. an antioxidant having a very high value of the antioxidant activity coefficient K_{ao} also have a very more value of k_2. Significantly, large values of the order of 10^3 to 10^7 indicate a fast reaction between the radical and the antioxidant used.

Small values of K_b and K_{ao} classify the extracts as weak antioxidants but, on the other hand, large k_2 values suggest a fast reaction. Thus, even with a weak antioxidant the effect occurs within a short time interval. It is interesting that a weak antioxidant can be used effectively for certain applications in which the reaction is desired to occur in a short time interval.

The obtained ΔG^* values are positive and in the range of the activation controlled reactions in contrast to the electrochemical production of superoxide anion radical which is purely a diffusion controlled process.

4. CONCLUSION

To the best of our knowledge, this is the first report describing the quasi-reversible system of $O_2/O_2^{\bullet-}$ and scavenging activity of anion superoxide by *Cupressus sempervirens* extracts. Furthermore, it is also proposed, that extracts can scavenge

$O_2^{\bullet-}$ by ErCi mechanism. All the extracts cause a decrease in the anodic current of superoxide anion radical which evidently demonstrates their potential antioxidant activity. In addition, all extracts show an important percentage inhibitory of ($O_2^{\bullet-}$) made by alkaline pyrogallol, this method is sufficient to determine the antioxidant activity. The isolation and identification of *Cupressus sempervirens* could be an interesting future study.

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