Voltammetric determination of antioxidant character in *Berberis lycium* Royel, *Zanthoxylum armatum* and *Morus nigra* Linn plants

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Abstract: The antioxidant activity potential of three different plant extracts was investigated against superoxide anion radical while employing cyclic voltammetry technique. The plants *Berberis lyceum* Royle, *Morus nigra* Linn and *Zanthoxylum armatum* were selected because of their potential use in the traditional medicine. The voltammetric response of the electrochemically generated superoxide anion radial in DMSO was monitored in the absence and presence of the plat extracts. The decrease in the current was interpreted in terms of antiradical activity of the added extract. The thermodynamic feasibility of the radical scavenging by extracts was accounted in terms of antioxidant activity coefficient (K_{ao}) and standard Gibbs free energy (ΔG°). The values of K_{ao} and ΔG° ranged from 1.0 x 10^2 to 57 x 10^2 L^{-1} and -18 to -27 $kJmol^{-1}$, respectively. The possible mechanism of the antioxidant reaction was regarded as E_rC_i mechanism i.e. reversible electron transfer followed by hydrogen atom transfer- an irreversible chemical reaction.

Keywords: Antioxidant activity, superoxide anion radical, cyclic voltammetry, *Berberis lycium-Royle*, *Morus nigra Linn*, *Zanthoxylum armatum*.

INTRODUCTION

Traditional herbs and plants are in use for centuries for the treatment of a variety of diseases, in almost all parts of the world. Unani and homeopathic medicine systems highly depend upon the herbs and plants, therefore, they use their different parts for the treatment of various diseases. Though, with the advancement of the science and technology, the traditional way of medication has decreased but the quest to know the scientific reason behind such, years tested, medication is continuously increasing. It is being observed that from the last few decades there is a growing interest in the field, not only to obtain useful compounds from plants but also to investigate the underlined science and make use of it in future. The in depth knowledge of structure-function relationship, of the useful chemicals present in plant extracts, will definitely be helpful to reduce the side effects and toxicity of the synthetic drugs (Ratty and Dos, 1988).

Out of several others Berberidaceae, Rutacea and Moracea plant families are famous for their medicinal potential. One plant from each of these families is selected for the present study. Berberidaceae family has many plants which are used for the treatment of various diseases in Indian and British pharmacopeias. Few out of them relevant to this very study are referred below. *Berberis lycium-Royle* (BLR) (local name Sumble) a semi deciduous thorny shrub having 2 to 4 meter height, is a traditional medicine of Himalaya region (Ahmad *et al*., 2009a). It is being used for the treatment of stomach pain, chronic diarrhea, jaundice, diabetes mellitus, inhibition of melondialdehyde (MDA) and as antihyperlipidemic agent (Ahmad *et al*., 2009a; Khan *et al*., 2010; Ahmed *et al*., 2009b; Srivastava *et al*., 2006). This plant also shows the wound healing activity (Asif *et al*., 2007). An alkaloid Berbamine, obtained from BLR has hypotensive effect (Ahmad *et al*., 2009b). The leaves of this plant are used as a tea substitute and the ripe fruit is eaten either fresh or dried to use later as such or in cooked form. The fruit is 8 mm long and has slight acidic flavor. Locally the plant is used as remedy for swollen and sore eyes, broken bones, internal injuries, ulcers, jaundice and preparation of dyes (Khan *et al*., 2010).

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Zanthoxylum armatum DC (Rutacea) (local name Timber) is an ever green shrub or small tree of 3-4m height. It is used against sore throats, cold, cough, chest pains, ulcers and for treatment of poisonous snake bites. This plant also shows hepatoprotective activity, reduces lipid peroxidation and increases the antioxidant enzymatic level and also has hypotensive, anticholinergic and antistaminic effects (Tiwarya *et al*., 2007). The same authors also mentioned that the bark is pungent and used to clean teeth, for remedy of toothache and as an insecticide for mosquito vector. The fruits and seeds are employed as an aromatic tonic in fever, scabies (Jain, *et al*., 2001), cholera and dyspepsia, and to expel roundworms (Akhtar *et al*., 2009). The reported compounds of Z. armatum are asarinin, fargesin, α and β-amyrins, β-sistosterol-β-D-glucoside, berberine, dictamnine, xanothplanine and armatumamide (Narendra *et al*., 1999; Ranawata 2010).

The black mulberry, *Morus nigra* Linn. (local name Toot) belongs to the family Moracea, and is used as a traditional medicine in different parts of the world. It is a 5-10 m tall deciduous tree growing in tropical region of many continents. It is reported that Morus genus contains various phenolic compounds which exhibit antioxidant and free radical scavenging activities (Padilha *et al*., 2009). Almost all parts (root, leaves, and fruits) of the mulberry tree have pharmacological applications e.g., diabetes, cancer, AIDS, high blood pressure etc. (Kumar and Chauhan 2008). Fresh fruit is used as an edible or in the production of jam, marmalade, wine and ice cream (Pawloska *et al*., 2008). It is a common observation that the juice of the ripped fruit of *Morus nigra* Linn. is used in the preparation of different kinds of the syrups, while the dried ripped fruit is used in the winter season as a warming agent and its fine powder is added to flour for bread. Its fruit is used in natural medicines for the ailment of sore throat, cough, asthma, fever, inflammation, hypertension, dysentery and anemia, to relieve pain and stop bleeding (Naderi *et al*., 2004; Yang *et al*., 2010). Previous literature shows the presence of vitamin C, minerals, phenolics, flavonoids, some organic acids, anthocynins, fat and fatty acids in the fruit of the *Morus nigra Linn* (Pawloska, *et al*., 2008). Data is documented in a detailed analysis about the nutritional constituents in the riped fruit of *Morus nigra* Linn (Kumar and Chauhan 2008).

These three plants were selected for the present study because of their extensive use as 'local medicines' for several ailments e.g., digestion problem, internal injuries (ulcer), throat pain, etc, in Pakistan. Despite the significant documented literature as mentioned above, there is rarely a scientific report regarding their use as free radical scavenger i.e. as an antioxidant. To the best of our knowledge no electrochemical studies has been made so far on the subject matter. Keeping in view the aforementioned points the present work was aimed to evaluate, for the first time, the antioxidant activity of the extracts of these plants. For the purpose superoxide anion radical ($O_2^{\bullet-}$) was selected as target radical because of its presence in human body and perilous nature; as it has longer half life and is capable of generating other harmful radicals such as hydroxyl radical. The method used to determine antioxidant activity is advantageous as it also furnishes mechanistic insight about the phenomenon under investigation.

MATERIALS AND METHODS

Instrumentation

Cyclic voltammetric measurements were carried out using Eco Chemie Autolab PGSTAT 302 potentiostat/ galvanostat (Utrecht, The Netherlands) along with the software GPES 4.9. All the experimentation was made in a double walled electrochemical cell (Model K-64 PARC) and conventional three electrode system was employed. Glassy Carbon (GC) electrode having area 0.013 cm^2 was used as a working electrode. Electrode surface was polished before each measurement. Platinum wire was used as a counter electrode and silver-silver chloride (Ag/AgCl, 3MKCl) of Metrohm Company with a plastic tip was used as a reference electrode.

Reagents

Dimethyl sulfoxide (DMSO) of analytical grade purchased from LAB-SCAN/Analytical Sciences was used as solvent without further purification. Tetrabutyl ammonium perchlorate (TBAP) of electrochemical grade (99%) from Fluka Company, was used as supporting electrolyte and its concentration was kept 0.1 M.

Plants Sources and identification

The three plants with respective herbarium voucher number include; *Berberis lycium-Royle* (85091 Isl.), *Morus nigra Linn* (92066 Isl.) and *Zanthoxylum armatum* (70753 Isl.). All the three plants were gathered from a local village Rehar of Mansehra district, KPK, Pakistan. The identification of the plants was done with the help of Dr. Mushtaq Ahmad, Assistant Professor Department of Plant Sciences, Quaid-i-Azam University, Islamabad. The local name along with the botanical name of each plant is given below in table 1.

Table 1: Information about the plants used

S. No.	Local name	Botanical name	Part of Plant used
1	Toot	*Morus nigra* Linn	Fruit
2	Sumble	*Berberis lycium* Royel/*Berberis pseudoumbellata*	Fruit
3	Timber	*Zanthoxylum armatum* DC/*Zanthoxylum Alatum* DC/ *Zanthoxylum planispinum* Sieb	Stem

Table 2: Volume of extract used for cent percent decrease of radical anodic current, binding constant (K_b) change in free energy of reaction (ΔG°) and antioxidant capacity K_{ao} for the extracts

Extract of	Volume of extract for 100% decrease in I_{pa} (μL)	$K_b \times 10^{-2}$ $(L)^{-1}$	$-\Delta G^\circ$ (kJ/mol)	$K_{ao} \times 10^{-2}$ $(L)^{-1}$
Sumble	100	414	26.6	57
Toot	150	195	24.7	2.4
Timber	2500	12	17.8	1.0

Extraction of herbs

The fruit of toot (ripe), sumble (ripe) and bark of stem of the timber were completely dried in air and then 5 g of each was soaked separately in 50 mL of DMSO. After one weak the extract was purified by repeated filtration and was used as such for cyclic voltammetric measurements. The calculated percentage yields (w/w) of the above mentioned plant extracts were 7.2 %, 6.5% and 11.5%, respectively.

Procedure

Superoxide anion radical was generated in DMSO containing 0.1 M TBAP. The scan rate was kept 20mV/s and potential window was -1.0 V-0.0 V. The atmospheric solubility of oxygen in DMSO was 2.1 mM. The additions of an extract were made volumetrically as the concentration of the compounds of extracts was not possible to be calculated. The extract was added incrementally to the *in situ* generated radical and resultant behaviour was recorded. From the change in the shape of the voltammograms the antioxidant activity was assessed qualitatively and quantitated using pertinent mathematical formulations.

RESULTS

The superoxide anion radical was generated by one electron reduction of the atmospheric molecular oxygen (O_2) dissolved in DMSO at room temperature (28±1˚C) and the resultant CV response is presented in Fig.1. Next, the voltammetric performance of the three extracts was investigated one by one in the DMSO + 0.1 M TBAP. The observed voltammetric responses of Toot and Timber showed no indication of any peak in the potential window of -2.0 V to +2.0 V. The extract of Sumble was found electroactive and the obtained response is shown in fig.2.

The extracts of Toot (*Morus nigra* Linn), Sumble (*Berberis lycium* Royel) and Timber (*Zanthoxylum armatum* DC) were added incrementally to investigate their effect on superoxide anion radical. The corresponding voltammograms are presented in fig. 3.

Quantitation of the scavenging of the superoxide anion radical by added plant extracts was done through thermodynamic parameters namely, binding constant (K_b), standard Gibbs free energy (ΔG˚) and antioxidant coefficient (K_{ao}). The calculated results are shown in table 2.

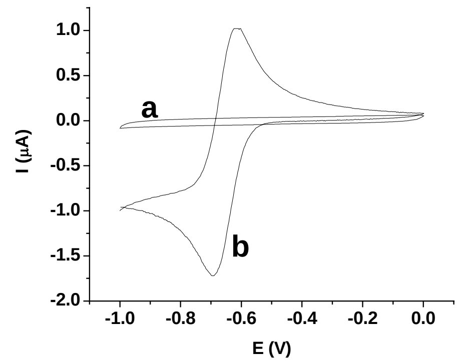

Fig. 1: Cyclic voltammogram of: (a) medium (DMSO+ TBAP) (b) $O_2^{\bullet-}$ in DMSO + 0.1M TBAP, on GC as working electrode vs. Ag/AgCl as reference at 28°C with scan rate of 20 mV/s.

DISCUSSION

Electrochemical generation and voltammetric behaviour of superoxide anion radical

The cyclic voltammogram of superoxide anion radical showed one electron reversible process (fig. 1), having well developed and clear oxidation and reduction peaks with peak separation (ΔEp) value of 66±3mV, well in agreement with the reported data (Bourvellec, *et al*. 2008). The height of the voltammogram corresponds to

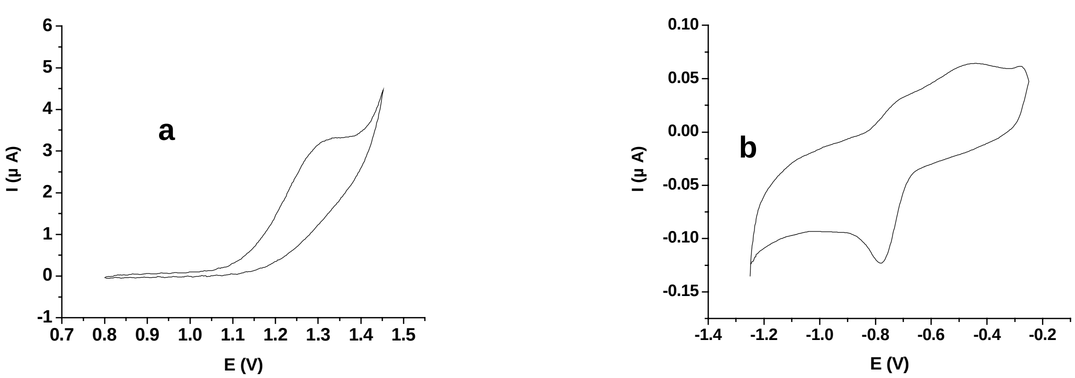

Fig. 2: Cyclic voltammograms of 40μL Sumble extract in DMSO + 0.1 M TBAP on GC as working electrode vs. Ag/AgCl at 28°C with scan rate of 20 mV/s for; (a) positive region, (b) negative region.

the solubility (concentration) of the oxygen. As far as the stability of the $O_2/O_2^{\bullet-}$ in an unperturbed solution is concerned, $O_2^{\bullet-}$ is stabilized by the ion pair formation and solvation process with tetra butyl ammonium per chlorate cation. Here the oxygen radical can act as a strong Bronsted base, a nucleophile and as a one electron donor. The estimated values of heterogeneous charge-transfer rate constant (k_s) and diffusion coefficient (D) for the reduction of oxygen to superoxide anion in DMSO +0.1 M TBAP were about 3×10^{-2} cm s^{-1} and 5×10^{-5} cm^2 s^{-1}, respectively. The values impart on the reversibility and stability of the radical as shown in Fig.1. The selection of $O_2^{\bullet-}$ as target radical has many advantages; several pathological effects are attributed to it, *in situ* electrochemical generation of the $O_2^{\bullet-}$ is cost effective, no involvement of chemical i.e. green chemistry and above all the online monitoring of the behaviour of the added compound.

Voltammetric behaviour of the extracts

The observed behaviour of the Toot and Timber shows the electro-inactivity of the extracts in the selected potential range (-2.0 V to +2.0 V). Fruit of Toot is sweet and contains carbohydrates (mono, di or polysaccharides) apart from other constituents. Glucose which is a monosaccharide also showed similar behaviour like Toot. The voltammograms of Sumble extract showed two peaks; one in negative potential region i.e. reduction (E_{pc}=-0.774±0.002) and one in positive region i.e. oxidation (E_{pa} =1.288±0.003 V) (fig. 2). Most of the flavonoids; phenolic acids and poly phenols oxidize or reduce in negative region (Yang, *et al.,* 2001), the observed electrochemical behaviour of the Sumble points towards the presence of such type of flavonoids in the extract.

Effect of extracts on the superoxide radical

The obtained results (fig. 3) show that in all the three cases the addition of the extract causes a proportional decrease in anodic current while the effect on the cathodic current appears to be negligible. The observed systematic depletion in the anodic current is interpreted in terms of the scavenging of the radical which was formed while going towards negative potential. The intactness of the cathodic wave and systematic decrease in anodic current, upon further addition of the extract, ensures the consumption of the $O_2^{\bullet-}$. No change in the reduction wave also imparts that there is no interaction between the extract and the molecular oxygen, as was expected. As for as the effect of added extract on the peak potential is concerned, a very small positive shift (towards less negative potential) was observed nearly in all of the cases which is a usual observation in such type of systems (Ahmed *et al.,* 2007).

The decrease in the anodic current of superoxide anion radical is attributed to the presence of some active components (antioxidants) in the extracts which react with superoxide radical and decrease its concentration at/around the electrode surface. Sumble and Toot showed effect at lesser volume additions than Timber. This points either the presence of the high concentration of the antioxidants or potential flavonoids in Sumble and Toot than in Timber. The results are understandable as extracts of Sumble and Toot were obtained from their fruits while that of Timber was obtained from the bark of stem. It is reported that the juice of *Morus nigra Linn* possesses compounds, which have hydroxyl groups in their structure (Ercisli and Orhan, 2007) and thus can act as free radical scavengers; therefore, such compounds react with superoxide anion radical more efficiently than others and decrease its concentration in the solution.

The added volume of the extracts did not increase the volume of the cell more than 5% in case of Sumble and Toot while it was increased in case of Timber (about 2.5mL, 25%). As the experimentation was done upto the complete scavenging of the anodic peak of $O_2^{\bullet-}$ therefore it was essential to do the same for Timber as well. Apparently it appears that the decrease in anodic peak may be because of this dilution but a close look negates this hypothesis. Firstly, it is not correct for the oxygen because it was dissolved from the atmosphere and the cell was opened to the atmosphere before each measurement. Second evidence, which is even stronger, is the absence of such effect on the cathodic current. Another aspect, though small in magnitude, is the potential shift which is characteristic of the scavenging behaviour and interaction between substrate and the radical under investigation (Ahmed and Shakeel, 2011).

The CV technique was also helpful to determine the mechanism of free radical scavenging. The observed changes in the shape of the cyclic voltammograms are same, as reported elsewhere (Ahmed and Shakeel, 2011) for some commercial flavonoids. There it was designated as H-atom abstraction from the flavonoid by the superoxide radical. The identified compounds of the Toot (fruit) have more than one –OH in their structure therefore it is assumed that the hydrogen of OH group is responsible for the interaction with superoxide. The voltammograms are identical in all the three cases which indicates the presence of polyhydroxyl compounds and thus make the operating mechanism same for all the extracts. Based upon this analogy, the proposed mechanism is thus hydrogen atom abstraction by the superoxide anion radical from hydroxyl group present in the extract and can be represented as;

$$O_2 + e \text{ (from electrode)} \rightarrow O_2^{\bullet-} \quad (1)$$

$$ROH + O_2^{\bullet-} \rightarrow [RO\text{--}H\text{--} O_2^{\bullet-}]^{\#} \rightarrow RO^{\bullet} + HO_2^{-} \quad (2)$$

$$HO_2^{-} + ROH \rightarrow H_2O_2 + RO^{\bullet} \quad (3)$$

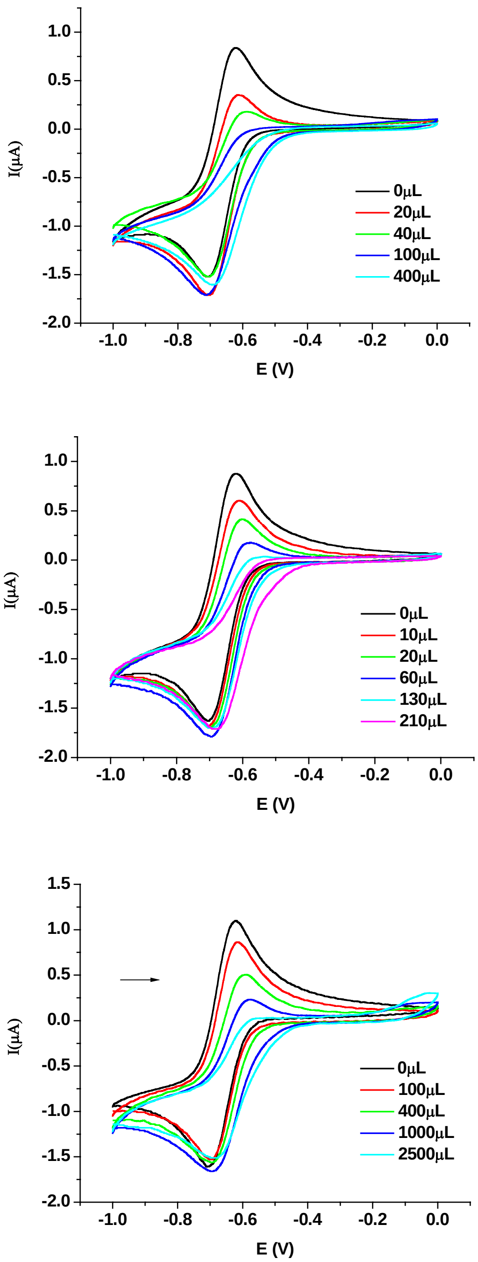

Fig. 3: Cyclic voltammograms of $O_2^{\bullet -}$in the presence of different volumes of (a) Sumble (b) Toot and (c) Timber, in DMSO + 0.1 M TBAP on GC as working electrode vs. Ag/AgCl at 28°C with scan rate of 20mV/s.

The $RO^{\bullet}$ produced is assumed to be of very low toxicity because of bulky groups attached and also because of high probability of the dimerization. As the whole process is taking place in the electrochemical cell, therefore, the overall mechanism can be pictured as reversible electron transfer (formation of stable $O_2^{\bullet -}$ from molecular oxygen) followed by an irreversible coupled chemical reaction (H-atom transfer from the extract to the radical). Electrochemically it can be written as E_rC_i mechanism i.e. reversible electron transfer followed by irreversible chemical reaction.

Thermodynamic Parameters

To quantify the results, the strength of interaction, between superoxide anion radical and the probable antioxidant in the extract, was estimated in terms of binding constant K_b. Based on the decrease in peak current, the binding constant (K_b) was calculated using following equation (Feng *et al*., 1997).

$$\log\left(\frac{1}{[AO]}\right) = \log K_b + \log\left(\frac{I_p}{I_{po} - I_p}\right) \qquad (4)$$

Where, I_{po} and I_p are the peak currents of superoxide anion radical in the absence and presence of additives, respectively, [AO] is the concentration of the antioxidant. As [AO] is not known, therefore, this term was replaced by the volume of the extracts (ΔV_{ext}). It is noticeable that the volume of the solution containing $O_2^{\bullet -}$ is fixed thus addition of volume increments of the extract is proportional to the addition of more number of moles (i.e. concentration) of the compound(s). Another thermo-dynamic parameter, standard Gibbs free energy (ΔG°) was calculated using the measured K_b.

The additive which gives high value of binding constant shows strong interaction with the radical. From Table2 it is evident that the % decrease of the anodic current of superoxide anion radical and binding constant (K_b) values follow the following order:

Sumble > Toot > Timber

The obtained K_b values of the extracts are higher than the synthetic flavonoids used in a similar study (Ahmed and Shakeel 2010) which confirm the strong affiliation of the compounds present in extracts with the radical and indicate the high probability of the presence of more than one compound in the extract. Sufficiently large negative value of ΔG°, indicates not only the spontaneity of the antiradical reaction but also points towards the stability of the newly formed species, which in turn is a strong evidence of the effectiveness of the extracts for the consumption of free radical.

Antioxidant Activity (AOA)

The relative capacity of flavonoids to scavenge the target radical was determined as antioxidant activity coefficient (K_{ao}). The constant K_{ao} is defined as the ratio of current density values, with and without the addition of substrate to the free radical. To quantify the effect equation (5) was (Feroci and Fini 2007; Korotkova *et al*., 2004) employed with little modification.

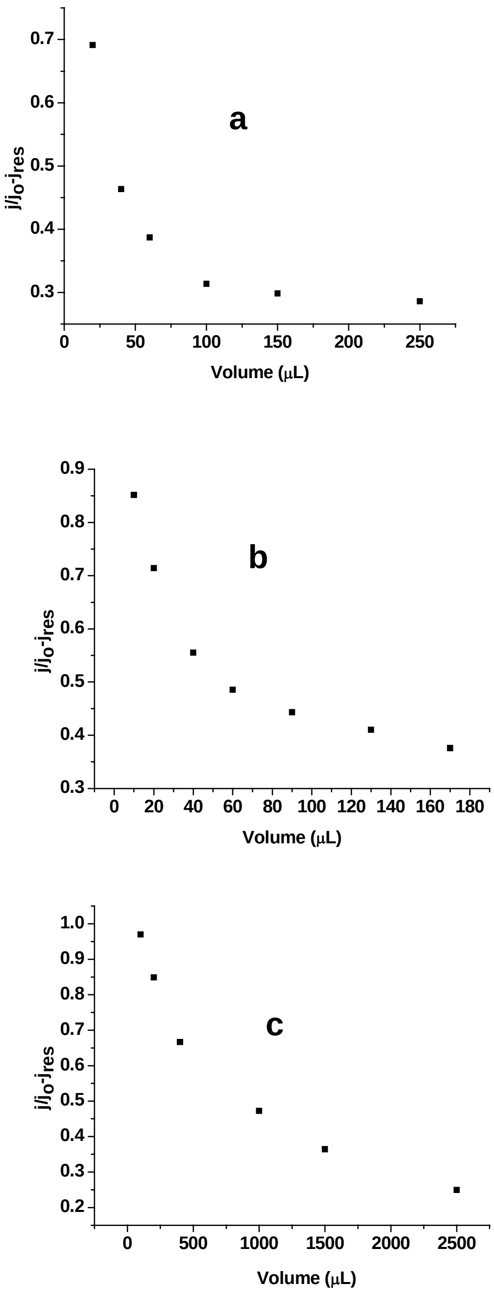

Fig. 4: Relative change of the $O_2^{\bullet-}$ current density vs. change in extract volume (proportional to extract concentration in the radical solution) for anodic peak of (a) Sumble, (b) Toot and (c) Timber.

$$K_{ao} = \frac{\Delta j}{\left(j_o - j_{res}\right)\Delta C} \qquad (5)$$

Where, Δj is the change in the oxygen current density with the addition of the substrate, j_o is the limiting current density of oxygen without the substrate in the solution, j_{res} is the residual current density of the oxygen and ΔC is the change in the concentration of the substrate in mol/L. The equation was employed only for the region in which there was a linear change in the value i.e. at low concentration of the additives (fig. 4). Antioxidant activity of the extracts is calculated with the modified equation i.e. ΔC is replaced with ΔV_{ext}. The tabulated data (table 2) shows that the observed antioxidant activity trend of the extracts is; Sumble > Toot > Timber. The K_{ao} values confirm the presence of flavonoids or antioxidants as main components of the extracts against superoxide radical. For example it is reported (Naderi *et al*., 2004) that mulberry contains bioflavonoids, therefore, it is expected that these powerful antioxidants may be responsible for the scavenging of the free radicals. The isolation and identification of the chemical compounds present in these extracts needs a separate study. However, this additional work has secondary significance on the results reported on the basis of work carried out in this very project.

CONCLUSION

The current work was aimed to investigate the antioxidant character of three plant extracts, namely, *Berberis lycium-Royle*, *Morus nigra Linn* and *Zanthoxylum armatum* against superoxide anion radical while employing cyclic voltammetric method. The outcome of the work can be summarized into three main points. **(i)** All the three extracts cause a decrease in the anodic current of superoxide anion radical which evidently demonstrates their potential antioxidant activity. Quantification, in terms of binding constant (K_b), standard Gibbs free energy ($-\Delta G^\circ$) and antioxidant activity coefficient (K_{ao}), fully complements the radical scavenging ability of the given extract. **(ii)** All the cases followed electron transfer followed by hydrogen atom transfer i.e. (E_rC_i) mechanism. **(iii)** The results reveal the existence of potential antioxidant compounds in the extracts of chosen plants. Further, the proposed mechanism indicates towards the presence of polyhydroxyl group containing compounds in the used extracts. The isolation and identification of such species could be an interesting future study.

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