Arabian Journal of Chemistry (2017) **10**, S1119–S1128

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Evaluation of antioxidant activity of crocin, podophyllotoxin and kaempferol by chemical, biochemical and electrochemical assays

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Received 7 November 2012; accepted 11 February 2013
Available online 20 February 2013

KEYWORDS

Antioxidant;
Crocin;
Kaempferol;
PTOX;
DPPH;
Cyclic voltammetry;
Superoxide

Abstract The present study was designed to evaluate the antioxidant potential of three natural origin drugs, namely crocin, kaempferol and podophyllotoxin by chemical, biochemical and electrochemical assays. The chemical assay was carried out by DPPH and reducing power assays while the biochemical assay evaluated the lipid peroxidation inhibition capacity, using brain cells as models; the electrochemical characterization was performed by cyclic voltammetry and differential pulse voltammetry using multi-walled carbon nanotube paste electrode (MWCNTPE) in 0.02 M acetate buffer (pH 4.5). The superoxide radical scavenging activity was performed at dropping mercury electrode (DME) in 0.1 M KCl. All the species proved to have antioxidant activity, and particularly, by the electrochemical techniques, it has been shown that these drugs showed scavenging ability on superoxide anion produced by electrochemical reduction of oxygen. The highest scavenging prop-

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.arabjc.2013.02.004

erty of crocin may be due to the hydroxyl and glucose moieties that could provide the necessary component as a radical scavenger.

1. Introduction

Living cells, including those of man, animals, and plants, are continuously exposed to a variety of challenges that exert oxidative stress. Oxidative stress arises in a biological system after an increased exposure to oxidants, a decrease in the antioxidant capacity of the system, or both. It is often associated with or leads to the generation of reactive oxygen species (ROS), including free radicals, which are strongly implicated in the pathophysiology of diseases, such as cancer, rheumatoid arthritis, cirrhosis and arteriosclerosis as well as in degenerative processes associated with ageing. Reactive free radicals may come from both endogenous as well as exogenous sources (Ames et al., 1993; Halliwell and Gutteridge, 2003).

Lipid peroxidation is another type of oxidative degradation of lipids in cell membranes resulting in cell damage by free radical chain reaction mechanism. It generates primary unstable oxidation products. Among them the most common is malondialdehyde (MDA). It reacts with thiobarbituric acid (TBA) reagent to produce a pink colour, known as thiobarbituric acid reactive substances (TBARS). The end-products of lipid peroxidation may be mutagenic and carcinogenic (Marnett, 1999).

Antioxidant supplements or antioxidant-containing foods may be used to help the human body to reduce oxidative damage or to protect food quality by preventing oxidative deterioration (Halliwell and Gutteridge, 2003). In recent years, the restriction in the use of synthetic antioxidants, such as BHA (2-tert-butyl-4-methoxyphenol) and BHT (2,6-di-tert-butyl-4-methylphenol), has caused an increased interest towards natural antioxidant substances (Ames, 1983). Natural antioxidants are being extensively studied for their capacity to protect organisms and cells from damage brought on by oxidative stress, the latter being considered as a cause of ageing and degenerative diseases. The antioxidants contained in foods, especially vegetables, are phenolic compounds (phenolic acids and flavonoids), carotenoids, tocopherol and ascorbic acid that are important protective agents for human health (Gillman et al., 1995). Mushrooms are also rich sources of these compounds (Barros et al., 2007a) and in the last few years several protocols to determine their antioxidant activity based on spectrophotometric techniques have been reported (Barros et al., 2007b; Ferreira et al., 2007). Progressively, electrochemical techniques have been tested and developed as an alternative tool, for the evaluation of different food extracts, expressed in terms of ''antioxidant power'', due to their quickness, simplicity and low cost (Blasco et al., 2005; Cosio et al., 2006).

Antioxidant compounds can act as reducing agents and, in solutions, they tend to be easily oxidized at inert electrodes. Based on this fact, some of the previously cited authors established an interesting relationship between electrochemical behaviour of the antioxidant compounds and their resultant ''antioxidant power'', where ''low oxidation potential'' corresponds to ''high antioxidant power''. Generally there is a relationship between antioxidative and peroxidative activities and oxidative potentials. The lower the antioxidant potential of extracts higher would be the antioxidant capacity. Lower the oxidative potential, higher is the ability to donate electron easily to the system generating free radicals (Arulpriya1 et al., 2010). On the other hand, ascorbic acid and phenolic compounds are common antioxidants in mushrooms. Electrochemical measurement at positive potentials will then correspond to the oxidation of ''total phenolic'' and ascorbic acid, plus all compounds with natural antioxidant properties and electrochemical activity, which are present in foods. Blasco et al. defined an ''Electrochemical Index'' to express the evaluation of the ''total natural antioxidants (Blasco et al., 2005).

In the present work, cyclic voltammetry and differential pulse voltammetry have been used to evaluate antioxidant activity of three natural origin drugs. Moreover, the lipid peroxidation inhibition capacity of the same drugs was accessed by biochemical assays used as models for the lipid peroxidation damage in biomembranes, namely inhibition of thiobarbituric acid reactive substance (TBARS) formation in brain cells. Their antioxidant properties were also evaluated through the reducing power determination and radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. Moreover, a new electrochemical method to evaluate antioxidant activity of a drug has been developed which is based on the scavenging ability of the drug on superoxide anion produced due to the reduction of oxygen at dropping mercury electrode (DME).

2. Materials and methods

2.1. Samples

Corms (roots) and petals of saffron (*Crocus sativus*) were obtained from the fields of saffron in Pampur, Srinagar, Kashmir (Jammu and Kashmir), India. Plant samples of rhizome of *Podophyllum hexandrum* was obtained from the hilly forest areas of village Sugan, District Budgam, Kashmir (Jammu and Kashmir), (India). The plant samples were identified and authenticated in the department of Botany, Dr. Hari Singh Gour University, Sagar, Madhya Pradesh, India.

2.2. Sample preparation

Podophyllotoxin was isolated from the rhizome of *P. hexandrum* (Anthony et al., 1956), crocin from stigmas of *C. sativus* (Dar et al., 2011) and kaempferol from its petals (Farzin et al., 2003). Kaempferol was also isolated from the corms of *C. sativus* using a column containing silica gel using 1:9 (chloroform: methanol) solvent system. The standard stock solution of kaempferol, and podophyllotoxin was prepared by dissolving 0.1 mg of each separately in 100 ml of pure ethanol and 0.1 mg of crocin in 100 ml of water. The supporting electrolyte (0.02 M acetate-acetic acid buffer, pH 4.5) was prepared in triply distilled water.

2.3. Standards and reagents

Pure kaempferol, Crocin and podophyllotoxin and multi-walled carbon nanotube powder (Sigma grade) were used in the present work. Standards L-ascorbic acid, and gallic acid were also purchased from Sigma (St. Louis, MO, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azobis(2-amidinopropane) dihydrochloride were obtained from Himedia and other chemicals used in the present work were either of Anala R or Himedia Laboratories Pvt. Ltd. Mumbai, grade.

2.4. Electrochemical characterization

2.4.1. Instrumentation

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed on a Ω Metrohm 797 VA Computrace (ion analyser, version 3.1). A three-electrode cell was employed incorporating a hand-made working multi-walled carbon nanotube paste electrode (MWCNTPE), an Ag/AgCl (3.0 M KCl) reference electrode and a platinum wire counter electrode. Mass transport was achieved with a Teflon-coated bar at approximately 400 rpm using a magnetic stirrer (KIKA Labortechinik, Germany). A systronics digital μpH metre model-361 was used for pH measurements. All experiments were performed at room temperature and the dissolved oxygen was removed by passing pure nitrogen through the solutions.

Carbon nanotube electrode was prepared in the usual way by hand-mixing graphite powder (Aldrich; 1–2 mm), carbon nanotube powder (40–300 m^2/g, Sigma) and mineral oil (Sigma). The ratio of these three was 60:10:30. The prepared paste was filled into the Teflon well. A copper wire fixed to a graphite rod and inserted into the Teflon well serves to establish electrical contact with the external circuit. A good reproducibility of electrode response was achieved by simply renewing the surface of paste electrode. A new electrode surface was formed by mechanically pressing the paste from the top of the Teflon well smoothening of the electrode surface was done by rolling a smooth glass rod on the electrode surface and finally it was cleaned carefully by distilled water. Each measurement involved fresh carbon nanotube surface. For the measurements of the scavenging ability of the drugs on superoxide anion, a dropping mercury electrode (DME) was employed.

2.4.2. Procedure

All the standard compounds, podophyllotoxin, crocin and kaempferol were studied in 0.02 M acetate-acetic acid buffer/4% methanol (pH 4.5) solutions. For the calibration plots of standards (ascorbic acid and gallic acid), the concentration was set between 0.1 and 10 $\mu g\ ml^{-1}$, and for drug solution, the concentration was also varied between 0.1 and 10 $\mu g\ ml^{-1}$. Cyclic voltammetry was used to characterize the electro-chemical responses between 0 and 1.5 V, at 100 $mV\ s^{-1}$, whereas the antioxidant power was evaluated by DPV, using the operating conditions as 50 mV pulse amplitude and 100 $mV\ s^{-1}$ as scan rate. For the drugs, the oxidation current was plotted as a function of concentration of the drug and compared with those of the standards.

Free radical scavenging activity determination was carried out based on the electrochemical reduction of oxygen (Zheng et al., 2005) Differential pulse voltammetry (DPV) was used under the following conditions: scan rate 25 $mV\ s^{-1}$, pulse amplitude 50 mV, initial potential 0 mV and final potential −1000 mV. The electrochemical cell containing 5 mL 0.02 M KCl (aq.) supporting electrolyte was similar to the cell discussed earlier and consisted of a dropping mercury electrode (DME) in place of the carbon nanotube working electrode. The supporting electrolyte was first saturated with oxygen by bubbling oxygen gas through the solution for 5 min. The potential of the working electrode was then set at 200 mV for 30 s while stirring the solution with a magnetic stirrer. After the stirring was stopped, the potential was scanned in the negative direction and differential pulse voltammogram was recorded that gave a peak current proportional to the amount of oxygen in the solution. This was then followed by adding a known concentration of the drug solution to the electrochemical cell under the similar conditions. The proportional decrease of the oxygen peak current corresponding to concentration of the added drug was measured. These experiments were repeated for solutions of gallic acid and ascorbic acid which were used as standards. All measurements were carried out at room temperature (25 ± 2 °C).

2.5. Chemical assays

2.5.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

The DPPH assay constitutes a quick and low cost method, which has frequently been used for the evaluation of the antioxidative potential of various natural products. Various concentrations of each drug including standard ascorbic acid and gallic acid (0.1–1 ml) were mixed with methanolic solution containing DPPH radicals (6×10^{-5} mM, 2.7 ml). The mixture was shaken vigorously and left to stand for 60 min in the dark (until stable absorption values were obtained). The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolourize in the presence of antioxidants. The DPPH radical contains an odd electron that is responsible for the absorbance at $\lambda = 517$ nm and also for the visible deep purple colour. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolourized, which can be quantitatively measured from the changes in absorbance (Elico SL164 Double beam UV–VIS Spectrophotometer).

The radical scavenging activity (RSA) was calculated as a percentage of DPPH decolourization using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the drug has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The drug concentration providing 50% of radical scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against concentration of the drug. Ascorbic acid and gallic acid were used as standards (Barros et al., 2007a,b; Lillian et al., 2008).

2.5.2. Reducing power

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) is measured by the intensity of the resultant blue–green solution which absorbs at 700 nm. Different volumes of test samples were mixed with sodium phosphate buffer (pH 6.6, 0.2 M, 5 ml) and potassium ferricyanide (1% w/v, 5 ml). The mixtures were incubated

in a water bath at 50 °C for 20 min. After the incubation period, trichloroacetic acid (10%, 5 ml) was added, and the mixture was centrifuged at 1000 rpm for 10 min. The upper layer was separated and its 5 ml was mixed with distilled water (5 ml) and ferric chloride (0.1%, 1 ml), the absorbance was then measured spectrophotometrically at 700 nm. The higher absorbency represents the stronger reducing power. The drug concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at λ_{max} = 700 nm against concentration of the drug. Gallic acid and ascorbic acid were used as standards (Barros et al., 2007; Lillian et al., 2008).

2.6. Biochemical assay (thiobarbituric acid reactive species (TBARS) assay)

The thiobarbituric acid (TBA) test was proposed over 40 years ago and is now one of the most extensively used methods to detect oxidative deterioration of fat-containing foods. During lipid oxidation, malondialdehyde (MDA), a minor component of fatty acids with three or more double bonds, is formed as a result of the degradation of polyunsaturated fatty acids. MDA is a three-carbon dialdehyde with carbonyl groups at the C-l and C-3 positions). It is usually used as an indicator of the lipid oxidation process, both for the early appearance as oxidation occurs and for the sensitivity of the analytical method (Uchiama and Miahara, 1978). In this assay, the MDA is reacted with thiobarbituric acid (TBA) to form a pink MDA–TBA complex that is measured spectrophotometrically at its absorption maximum at 530–535 nm (Tea et al., 2006). It must, however, be noted that alkenals and alkadienals also react with the TBA reagent and produce a pink colour. Thus, the term thiobarbituric acid reactive substances (TBARS) is now used instead of MDA.

A modified TBARS assay (Tea et al., 2006; Juana et al., 1997; Fereidoon and Ying, 2005) was used to measure the potential antioxidant activity using brain homogenates as lipid rich media. Brains were obtained from four rats of body weight 140–160 g, dissected and homogenized with a polytron in ice-cold Tris–HCl buffer (pH 7.4, 20 mM) to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000 rpm for 10 min. An aliquot (0.1 ml) of the supernatant was incubated with different concentrations of sample (drug) solutions (10–500 μg ml^{-1}), prepared immediately before use, which were added to a test tube and made up to 1.0 mL with distilled water. A volume of 0.05 mL (0.07 M) of 2,2′-azo-bis(2-amidinopropane) dihydrochloride solution in water was added to induce lipid peroxidation. Then 1.5 mL of 20% acetic acid in 1.1% (w/v) sodium dodecyl sulphate solution was added and the resulting mixture was vortexed, and heated at 95 °C for 60 min. After cooling, 5.0 mL of butan-1-ol was added to each tube, then vortexed and centrifuged at 1200*g* for 10 min. The absorbance of the organic upper layer was measured using a spectrophotometer at 532 nm. All the values were based on the percentage of antioxidant index ($AI/\%$):

$$AI/\% = (1 - AT/AC) \cdot 100$$

where AC is the absorbance value of the fully oxidized control, and AT is the absorbance of the test sample. The drug concentration providing 50% lipid peroxidation inhibition (EC50) was calculated from the graph of antioxidant activity percentage against drug concentration.

2.7. Statistical analysis

For each of the three drugs, three samples were analysed and also all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The antioxidant activity of the drugs was analysed using a one way analysis of variance (ANOVA) using SigmaStat version 3.1.

3. Results and discussion

3.1. Evaluation of antioxidant properties by electrochemical techniques

Fig. 1a and b shows the cyclic voltammograms of the standard ascorbic and gallic acids, respectively. Both compounds present typical irreversible oxidation processes, as observed in many antioxidant substances (Cosio et al., 2006; Kilmartin et al., 2001) with one anodic peak at E_{pa} = 0.28 ± 0.05 V for ascorbic and two anodic peaks at $E(I)_{pa}$ = 0.41 ± 0.05 V, $E(II)_{pa}$ = 0.71 ± 0.05 V for gallic acid. The same irreversible electrochemical behaviour was observed for natural origin podophyllotoxin with E_{pa} = 1.10 ± 0.05 V (Fig. 1c), and crocin E_{pa} = 0.37 ± 0.05 V (Fig. 1d), and reversible for kaempferol with E_{pa} = 0.38 ± 0.05 V and E_{pc} = 0.28 ± 0.05 V (Fig. 1e). These results showed that both standards are oxidized at less positive potentials than the compounds under study (podophyllotoxin, crocin and kaempferol). The similarity in the oxidation potential between all these drugs studied indicates that they should have an analogous relationship with respect to the antioxidant activity.

Cyclic voltammetry is frequently used for the characterization of electroactive systems. However, when organic substances are present, there are greater chances for adsorption phenomena on the electrode surface, limiting the use of this technique to quantitative measurements. To overcome this constriction and gain quantitative information in the antioxidant capabilities of any compound, differential pulse voltammetry is used (Brett and Brett, 1993) Fig. 2 shows the differential pulse voltammograms for all the species under study. In fact, with this technique the peaks are better resolved than in CV, overcoming the difficulties in accessing a correct baseline. Representative differential pulse voltammograms of one of the standards (gallic acid) and one of the drugs under study (crocin) at several concentrations of gallic acid and crocin are shown in Fig. 3a and b, respectively. As can be seen there is an increase in peak current with the increase in gallic acid and crocin concentrations, which leads to a linear relation between the two parameters. The same behaviour was found for ascorbic acid and other species under study (podophyllotoxin and kaempferol (Fig. 3c and Table 1), although the slopes for the plots peak current vs. concentration of the drug are very different. For this technique, the peak current depends, not only on the concentration, but also on the electron transfer kinetics and the diffusion coefficient of the electroactive species (or the average of several species (Arulpriya1 et al., 2010) preventing the direct comparison between the standards and samples data. The difference between the slopes of the two standards and the drugs under study is reported in Table 1. The data reflect the difference between their diffusion coefficients.

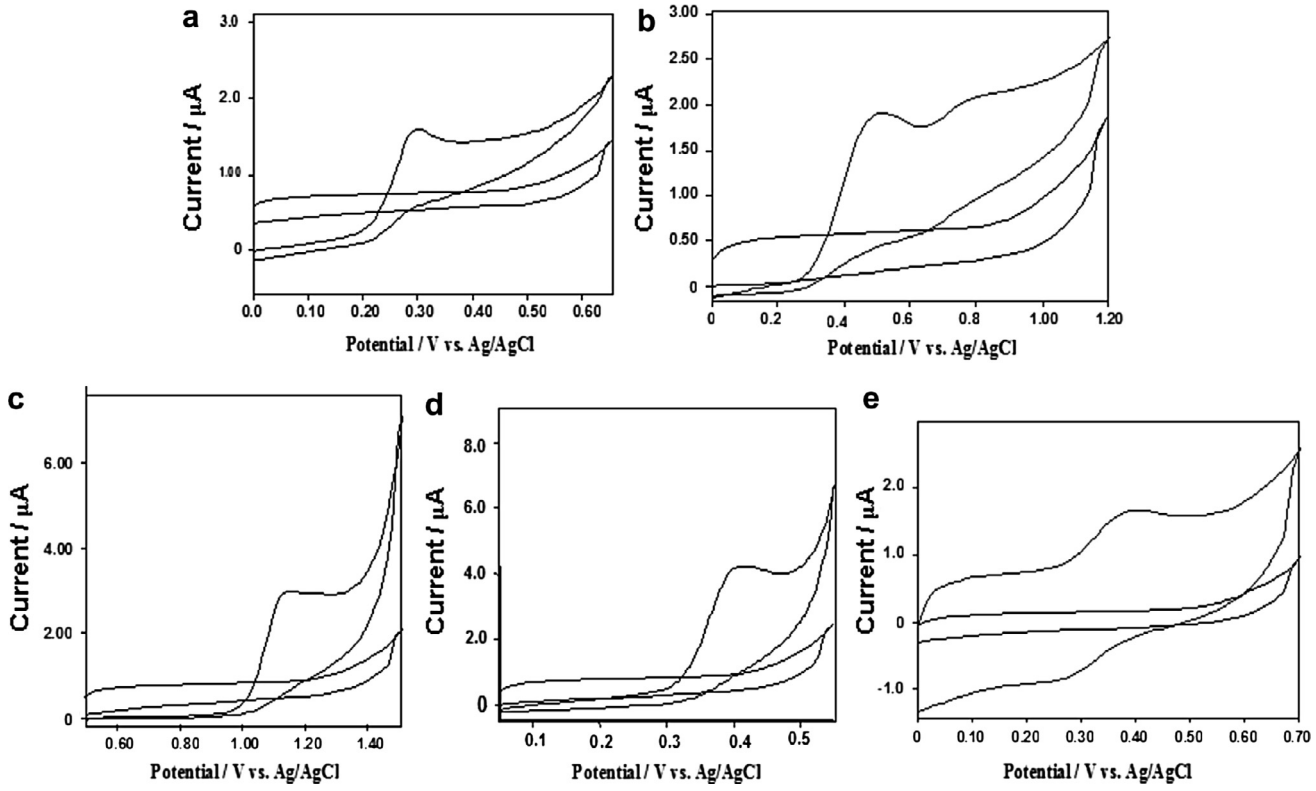

Figure 1 Cyclic voltammograms, at 100 mV s^{-1} of (a) 0.5 µg ml^{-1} ascorbic acid, (b) 0.5 µg ml^{-1} gallic acid, (c) 0.5 µg ml^{-1} PTOX, (d) 0.5 µg ml^{-1} crocin, (e) 0.5 µg ml^{-1} kaempferol in 0.1 M acetate-acetic acid buffer/4% methanol (pH 4.5) solutions.

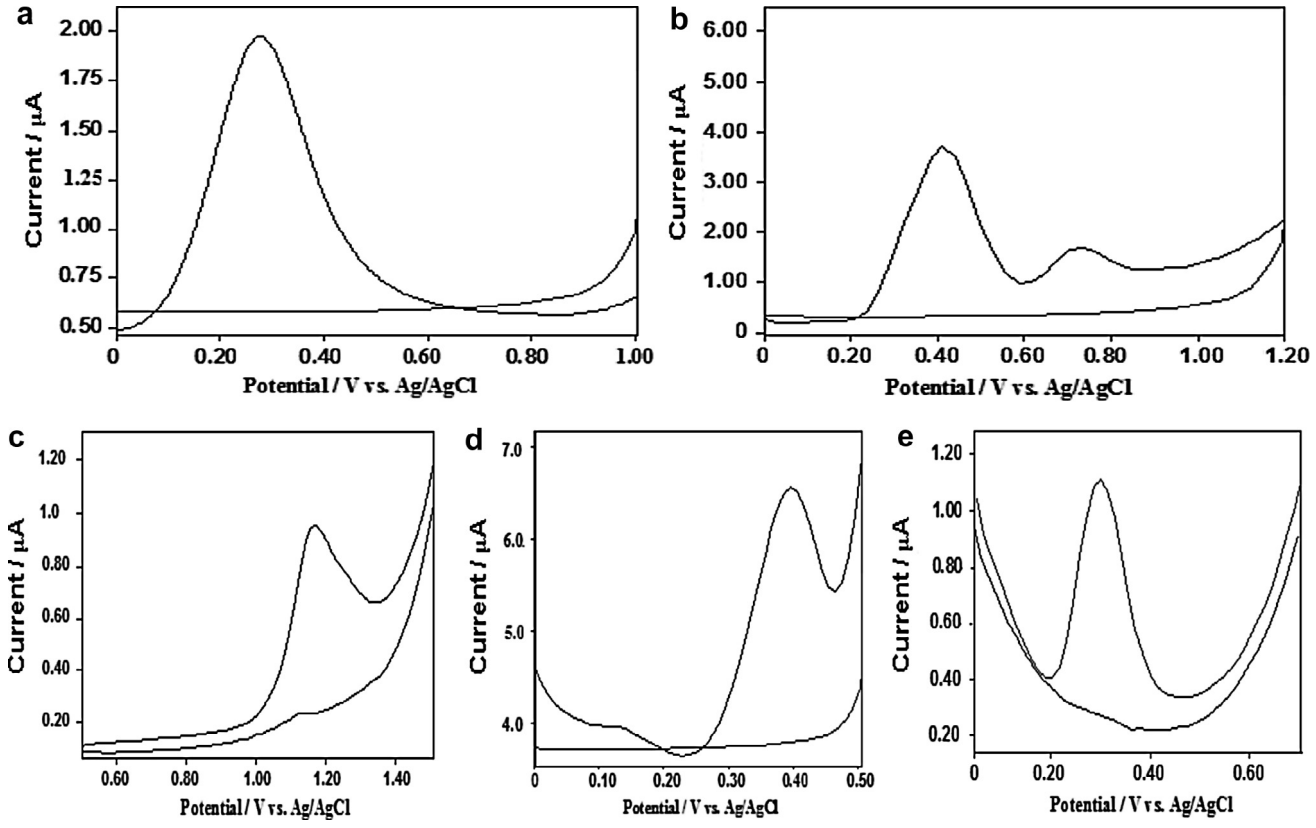

Figure 2 Differential pulse voltammograms in 0.1 M acetate-acetic acid buffer/4% methanol (pH 4.5) of 1 µg ml^{-1} solution of (a) Ascorbic acid, (b) Gallic acid, (c) PTOX, (d) Crocin and (e) kaempferol.

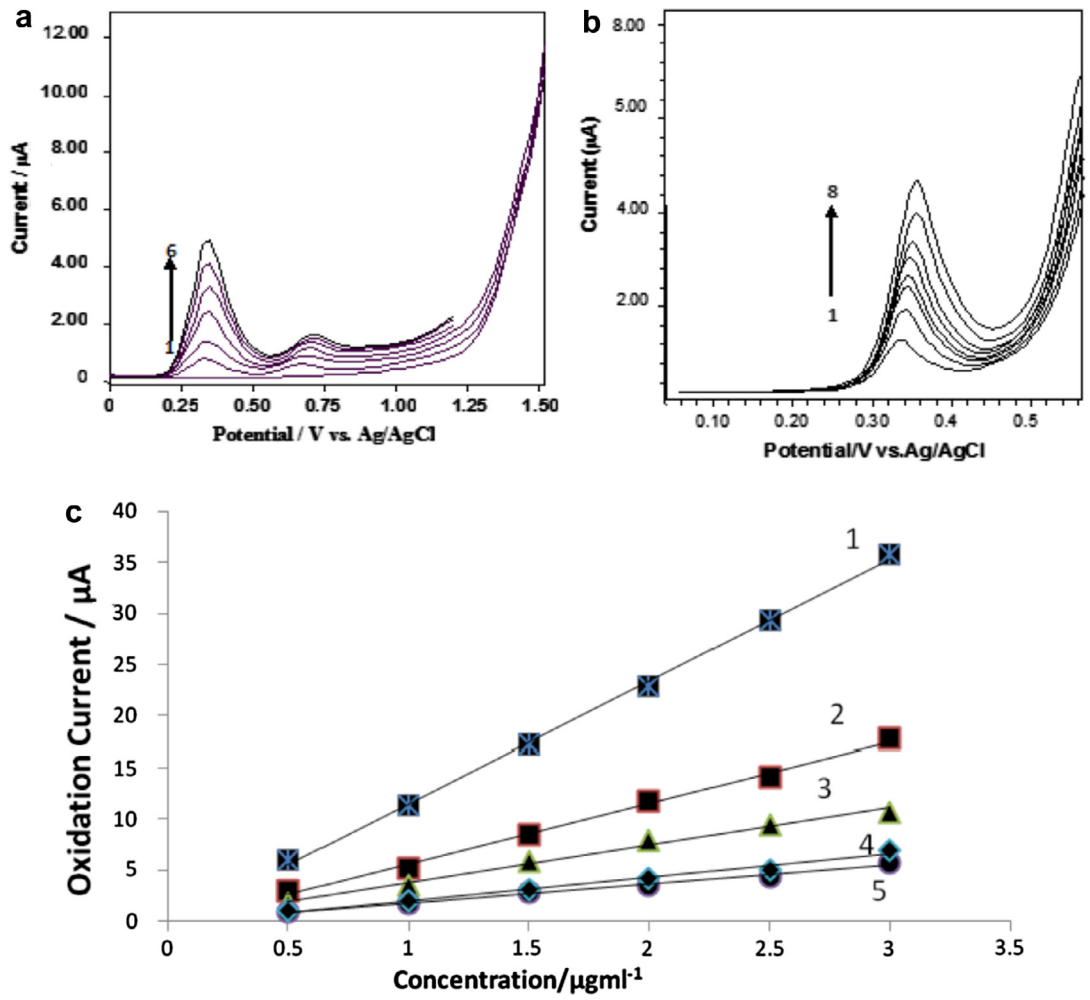

Figure 3 Differential pulse voltammograms of (a) gallic acid and (b) crocin in 0.1 M acetate-acetic acid buffer/4% methanol (pH 4.5) of (1) 0.20 µg ml^{-1}, (2) 0.40 µg ml^{-1}, (3) 0.60 µg ml^{-1}, (4) 0.8 µg ml^{-1}, (5) 1.0 µg ml^{-1}, and (6) 1.2 µg $ml^{-1}$of gallic acid and (7) 1.4 µg ml^{-1}(8) 1.6 µg $ml^{-1}$of crocin. (c) Variation of the peak current in DP voltammograms with concentration of (1) Crocin, (2) Gallic acid, (3) Ascorbic acid, (4) Kaempferol and (5) PTOX. Other conditions as in Fig. 2.

Table 1 Cyclic voltammetric and differential pulse voltammetric results of ascorbic acid, gallic acid, PTOX, Crocin and Kaempferol in 0.1 M acetate-acetic acid buffer/4% methanol (pH 4.5) solutions.

Drug type	E_{pa}/V	Slope/µA mg^{-1} ml	A.P. (AA) (mg/g)	A.P. (GA) (mg/g)
Crocin	0.37 ± 0.05	11.87	3330.0	1857.1
Kaempferol	0.38 ± 0.05	2.30	564.1	314.28
PTOX	1.10 ± 0.05	1.85	487.1	271.42
Ascorbic acid	0.28 ± 0.05	3.69	1000	557.14
Gallic acid	0.41 ± 0.05/0.71 ± 0.05	5.93	1794.8	1000

In order to express the ''antioxidant power'' of the different drugs in equivalent terms we compared the results with those of the standards. We must mention that at very low and high concentrations of the drug there are significant deviations in linearity of current (I) vs. concentration of the drug, most probably due to adsorption phenomena on the electrode. The values are presented in Table 1, and are expressed in terms of either gallic or ascorbic acid. These results show that crocin exhibits the highest ''antioxidant power'' and the podophyllotoxin the least in agreement with the results obtained in the chemical and biochemical assays.

3.2. Scavenging ability on superoxide anion

Free radical scavenging activity determination was carried out based on the electrochemical reduction of oxygen. Dropping mercury electrode was used for the reduction of oxygen. When oxygen is reduced it proceeds at the cathode in several stages with the formation of the active anion-radical of oxygen or superoxide anion ($O_2^{\bullet -}$) as intermediate. Fig. 4 shows selected differential pulse voltammograms of oxygen reduction at DME before and after the addition of different concentrations of each drug. The reduction peak was observed at a potential

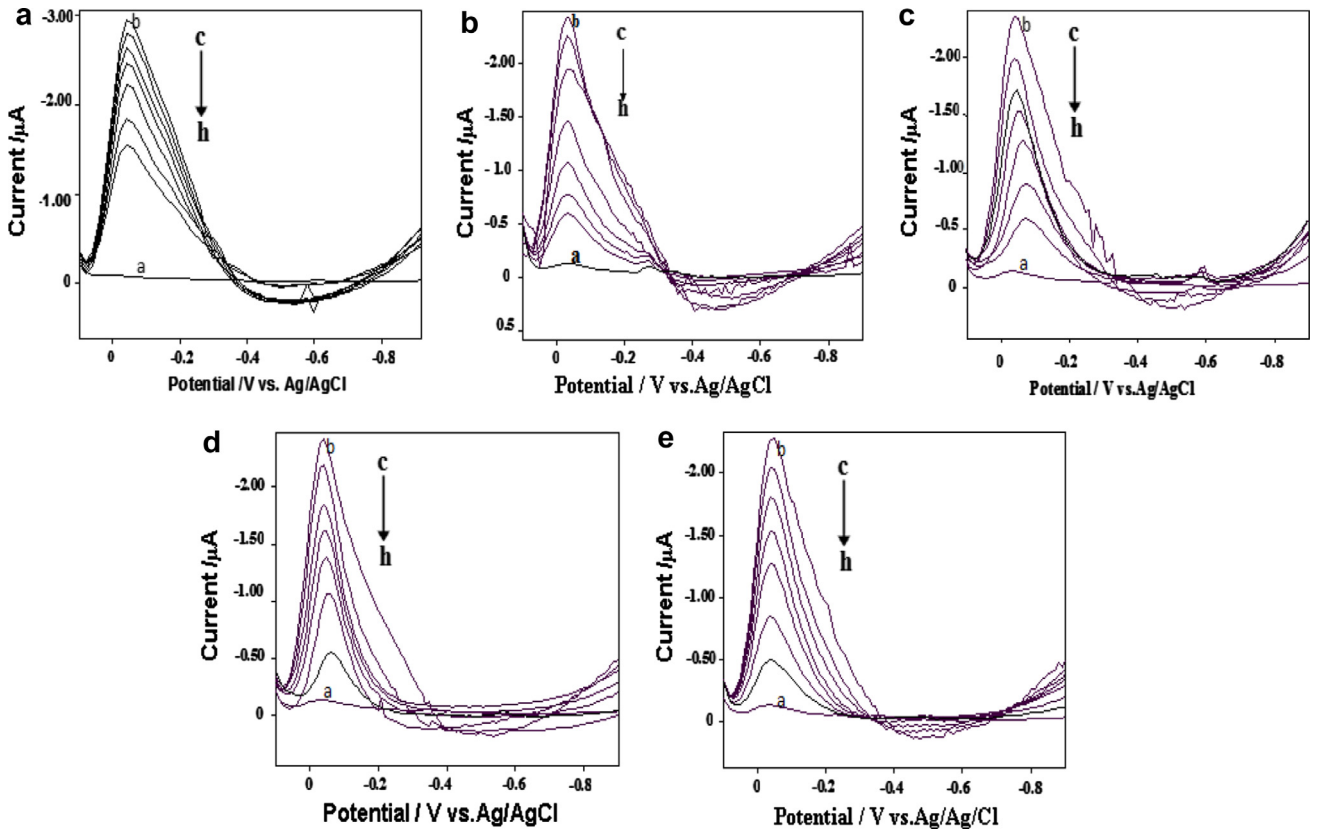

Figure 4 Differential pulse voltammograms for the electrochemical oxygen reduction in 0.1 M KCl supporting electrolyte at DME of (a) deaerated solution with N_2; (b) air saturated solution and varying concentrations of the drugs as (c) 40 μ ml^{-1}; (d) 80 μ ml^{-1}; (e) 120 μ ml^{-1}; (f) 160 μ ml^{-1}(g) 200 μ ml^{-1} and (h) 140 μ ml^{-1} spiked air saturated solution of (a): ascorbic acid, (b): gallic acid, (c): kaempferol, (d) PTOX, (e): crocin.

of about −0.16 V. When the drug was added, the peak current decreased with increasing concentrations of the drug. It is obvious that the drug can scavenge the active oxygen radicals yielded by the cathodic reduction of oxygen. The inhibitory rate which is defined as the percentage of the ratio of the difference of the peak current obtained due to oxygen reduction before and after adding the drug to the peak current obtained due to oxygen reduction before adding the drug was plotted as a function of concentration of the drug. The plot (shown in Fig. 5a) was linear and is described by the following equation.

Inhibitory rate for superoxide ($O^{2\bullet-}$) = 0.324c + 12.90, R^2 = 0.948 (for Crocin).
Inhibitory rate for superoxide ($O^{2\bullet-}$) = 0.283c + 10.65, R^2 = 0.968 (for Gallic acid).
Inhibitory rate for superoxide ($O^{2\bullet-}$) = 0.264c + 4.231, R^2 = 0.945 (for Ascorbic acid).
Inhibitory rate for superoxide ($O^{2\bullet-}$) = 0.237c + 10.73, R^2 = 0.950 (for Kaempferol).
Inhibitory rate for superoxide ($O^{2\bullet-}$) = 0.198c − 9.10, R^2 = 0.934 (for PTOX).

IC_{50}, that is the concentration of the drug when the inhibitory rate reaches 50%, was calculated from the linear curve as 125.3 ± 0.04 μg ml^{-1},146.5 ± 0.07 μg ml^{-1}, 178.5 ± 0.023 μg ml^{-1}, 186.8 ± 0.008 μg ml^{-1}, 275.3 ± 0.06 μg ml^{-1} for crocin, gallic acid, ascorbic acid, kaempferol and PTOX, respectively indicating that crocin has comparably the highest radical scavenging activity and PTOX the lowest. The highest scavenging property of crocin may be due to the hydroxyl and glucose moieties that could provide the necessary component as a radical scavenger.

3.3. Evaluation of antioxidant properties by chemical assays

3.3.1. Reducing power assay

Fig. 5b shows the reducing power of crocin, kaempferol, podophyllotoxin and standard ascorbic acid and gallic acid as a function of their concentration. As can be seen, each drug caused significant elevation of reducing power. In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, measuring the formation of ferric ferrocyanide, $Fe_4[Fe(CN)_6]_3$ complex, commonly known as Perl's Prussian blue at 700 nm can monitor the Fe^{2+}concentration. The reducing power of crocin (0.856 ± 0.08 at 750 μg ml^{-1}) was relatively more pronounced than that of kaempferol (0.617 ± 0.03 at 750 μg ml^{-1}) and PTOX (0.572 ± 0.011 at 750 μg ml^{-1}). The higher absorbance at high concentrations indicates the strong reducing power potential. The figure also shows the reductive capabilities of these drugs compared with gallic acid (0.742 ± 0.008) and ascorbic acid (0.695 ± 0.005)

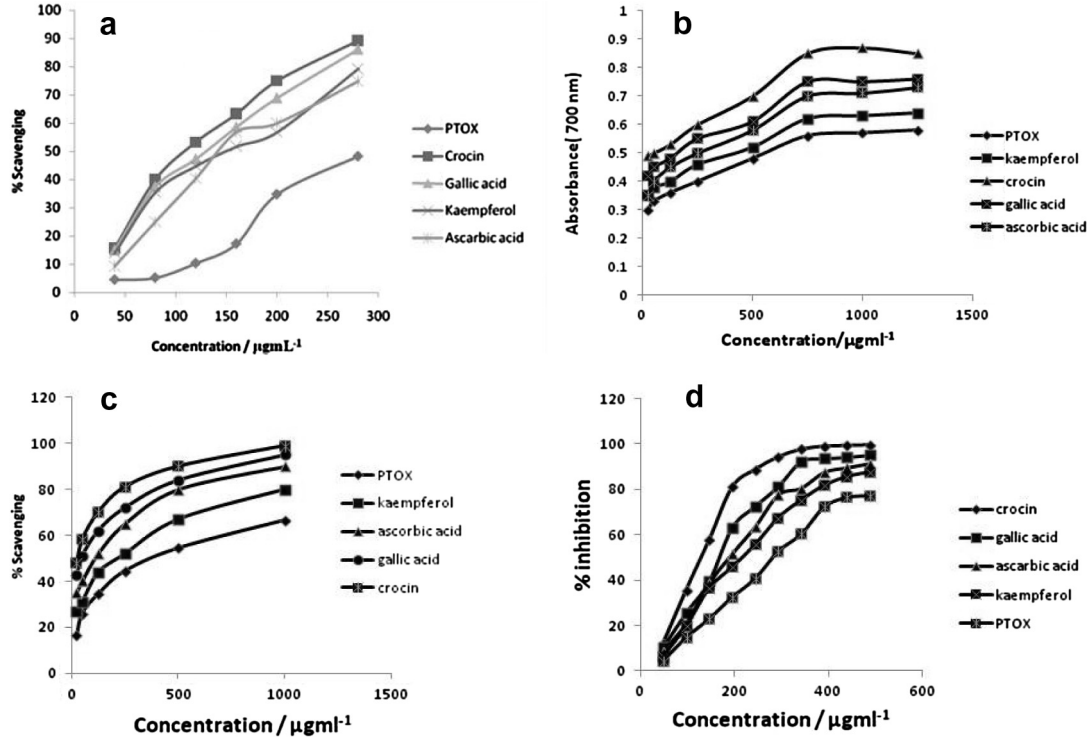

Figure 5 (a) Superoxide anion radical scavenging of crocin, kaempferol and PTOX in comparison with two standard antioxidants ascorbic acid, and gallic acid. (b) Reducing power of crocin, kaempferol and PTOX in comparison with standard (ascorbic acid) and gallic acid at $\lambda = 700$ nm. (c) DPPH radical scavenging activity and IC_{50} values of crocin, kaempferol and PTOX in comparison with standard ascorbic acid and gallic acid. (d) TBARS assay and IC_{50} values of crocin, kaempferol and PTOX in comparison with standard ascorbic acid and gallic acid. Each value is mean ± SD ($n = 3$).

at 750 µg ml^{-1}). The reducing power of these drugs might be due to the poly and mono hydroxyl substitutions in their rings which possess potent hydrogen donating abilities (Amarowicz et al., 2004; Shimada et al., 1992). Accordingly, these reductones can react with free radicals to stabilize and block radical chain reactions.

3.3.2. Radical-scavenging activity (RSA) by DPPH assay

The RSA of drugs was tested using a methanolic solution of the "stable" free radical, DPPH. Unlike laboratory-generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition (Roedig-Penman and Gordon, 1998). A freshly prepared DPPH solution exhibits a deep purple colour with absorption maximum at 517 nm. This purple colour generally fades/disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colourless/bleached product (i.e., 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm. Hence, the more rapidly the absorbance decreases, the more is the antioxidant activity of the drug. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a commonly employed assay in antioxidant studies of specific compounds across a short time scale.

The RSA values of crocin, kaempferol, podophyllotoxin and ascorbic acid and gallic acid used as standards were examined and compared against one another (Fig. 5). Results are expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of drug at 517 nm. Comparison of the antioxidant activity of all the three drugs and the standard ascorbic acid and gallic acid is shown in (Fig. 5c). All these drugs exhibited a significant dose-dependent inhibition of DPPH activity, with a 50% inhibition (IC_{50}) at a concentration of 27.50 ± 0.005 µg ml^{-1}, 244.30 ± 0.01 µg ml^{-1} and 396.75 ± 0.005 µg ml^{-1} for crocin, kaempferol and PTOX, respectively as compared with the standard ascorbic acid (121.20 ± 0.008 µg ml^{-1}) and gallic acid (52.13 ± 0.007 µg ml^{-1}).

3.4. Evaluation of antioxidant properties by biochemical assay

Biochemical assay used to screen the antioxidant properties is inhibition of lipid peroxidation in brain tissue (measured by the colour intensity of MDA–TBA complex). The assays were performed for all the drugs including standard ascorbic acid and gallic acid. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds. These include reactive carbonyl compounds. The most abundant among them is malondialdehyde (MDA), one of the secondary lipid peroxidation products. Malondialdehyde (MDA) levels, as an index of lipid peroxidation, were measured. MDA reacts with thiobarbituric acid

Table 2 IC_{50} and EC_{50} values[c] ($\mu g\ ml^{-1}$) of crocin, kaempferol, podophyllotoxin and standard ascorbic acid and gallic acid obtained in reducing power, DPPH scavenging and TBARS, assays.

Sample	Reducing power assay (EC_{50})[a]	DPPH (IC_{50})[b]	TBARS assay (IC_{50})[b]
Crocin	49.32 ± 0.007	27.50 ± 0.082	124.53 ± 0.092
Kaempferol	456.55 ± 0.054	244.300 ± 0.043	213.49 ± 0.092
Podophyllotoxin	572.30 ± 0.072	396.75 ± 0.021	296.43 ± 0.013
Ascorbic acid	198.53 ± 0.092	121.20 ± 0.043	175.67 ± 0.021
Gallic acid	101.760 ± 0.012	52.13 ± 0.065	151.73 ± 0.061

[a] EC_{50} ($\mu g\ ml^{-1}$): effective concentration at which the absorbance is 0.5.
[b] EC_{50} ($\mu g\ ml^{-1}$): effective concentration at which 50% of DPPH radicals are scavenged.
[c] Values represent mean ± S.D.

(TBA) as a thiobarbituric acid reactive substance (TBARS) to produce a pink coloured complex which has peak absorbance at 532 nm (Miguel et al., 2004). Fig. 5d shows the antioxidant activity expressed as antioxidant index ($AI/\%$) of crocin, kaempferol, PTOX and ascorbic acid and gallic acid as standard antioxidants. All these drugs exhibited a significant dose-dependent lipid peroxidation inhibition activity, with a 50% inhibition (IC_{50}) at a concentration of 124.50 ± 0.02 $\mu g\ ml^{-1}$, 213.49 ± 0.005 $\mu g\ ml^{-1}$ and 296.43 ± 0.04 $\mu g\ ml^{-1}$ for crocin, kaempferol and PTOX, respectively as compared with the standard ascorbic acid (175.67 ± 0.07 $\mu g\ ml^{-1}$) and gallic acid (151.73 ± 0.05 $\mu g\ ml^{-1}$).

In Table 2, we present the EC_{50} values for reducing power, DPPH scavenging effects and TBARS assay (IC_{50}) obtained from each drug along with ascorbic acid and gallic acid as standard antioxidants.

Overall all these drugs revealed good antioxidant activities, namely radical scavenging activity and lipid peroxidation inhibition capacity. Crocin was the most efficient species presenting the lowest EC_{50} values in the chemical and bio-chemical assays, and the highest ''antioxidant power'' in the electrochemical assays. This was also evident in EC_{50} values for DPPH scavenging effect (27.50 ± 0.005 $\mu g\ ml^{-1}$ for crocin, 244.30 ± 0.001 $\mu g\ ml^{-1}$ for kaempferol and 396.75 ± 005 $\mu g\ ml^{-1}$ for PTOX) and from the biochemical assay (124.53 $\mu g\ ml^{-1}$ for crocin, 213.49 $\mu g\ ml^{-1}$ for kaempferol and 296.43 $\mu g\ ml^{-1}$ for PTOX). Further the, EC_{50} values obtained for superoxide anion inhibition capacity was better than for RSA and TBARS assays. This was much more evident in EC_{50} values for superoxide anion inhibition capacity (125.30 $\mu g\ ml^{-1}$ for crocin, 186.80 $\mu g\ ml^{-1}$ for kaempferol and 275.30 $\mu g\ ml^{-1}$ for PTOX).

The highest scavenging property of crocin may be due to the hydroxyl and glucose moieties that could provide the necessary component as a radical scavenger. Further the, EC_{50} values obtained in reducing power assay were better than as observed using RSA and TBARS assays. Further the results of the study infer that these drugs may bring new natural products into the food industry with safer and better antioxidant activity that provides good protection against the oxidative damage, which occurs both in the body and our daily foods.

4. Conclusion

The antioxidant activity of crocin (isolated from stigmas of *C. sativus*), kaempferol (isolated from corms and petals of *C. sativus*) and Podophyllotoxin (isolated from rhizome of *P. hexandrum*) was screened through chemical, biochemical and electrochemical techniques. The chemical assays allowed an evaluation of their reducing power and radical scavenging activity, while biochemical assays evaluated the lipid peroxidation inhibition capacity, using brain cells as models; the electrochemical evaluation was performed by cyclic voltammetry and differential pulse voltammetry. All the species proved to have antioxidant activity, and particularly, by the electrochemical techniques, it has been shown that these drugs showed scavenging ability on superoxide anion produced by electrochemical reduction of oxygen. The RSA values of crocin, kaempferol, podophyllotoxin and ascorbic acid and gallic acid used as standards were examined and compared against one another. Crocin was the most efficient species presenting the lowest EC_{50} values in the chemical and biochemical assays, and the highest ''antioxidant power'' in the electrochemical assays. The work described in this study showed that cyclic voltammetry and differential pulse voltammetry can be considered as important techniques for the evaluation of antioxidant properties of crocin, kaempferol and PTOX. The results also infer that these drugs can be recommended as good natural antioxidants as they possess a tendency to donate electrons and could react with free radicals to convert them into more stable products and to terminate radical chain reactions.

Acknowledgements

The authors are thankful to the Head, department of chemistry, Dr. Hari Singh Gour University Sagar (M.P.) India, for providing necessary laboratory facilities and the University Grants Commission New-Delhi for financial support under its special assistance programme.

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