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ORIGINAL ARTICLE

LIBS-Based Detection of Antioxidant Elements in Seeds of *Emblica officinalis*

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Abstract The aim of the study was to determine the effect of the elements of the extract of seed from *Emblica officinalis* on antioxidant enzymes and osmotic fragility of erythrocytes membrane in normal as well as streptozotocin-induced severely diabetic albino Wister rats. The results revealed that the untreated diabetic rats exhibited increase in oxidative stress as indicated by significantly diminished activities of free radical scavenging enzymes such as catalase (CAT) and superoxide dismutase (SOD) by 37.5% ($p<0.001$) and 18.6% ($p<0.01$), respectively. However, the *E. officinalis* seed extract treatment showed marked improvements in CAT and SOD activities by 47.09% ($p<0.001$) and 21.61% ($p<0.001$), respectively. The enhanced lipid peroxidation by 30.87% ($p<0.001$) in erythrocytes of untreated diabetic rats was significantly accentuated in the extract treated animals by 23.72% ($p<0.001$). The erythrocytes showed increased osmotic fragility due to diabetes in terms of hemolysis. It attained the normal level in diabetic treated group. The findings thus suggest that *E. officinalis* seed extract has the potential to be exploited as an agent to boost the antioxidant system in the diabetic animal model. Laser-induced breakdown spectroscopy has been used as an analytical tool to detect major and minor elements like Mg, Fe, Na, K, Zn, Ca, H, O, C, and N present in the extract. The higher concentration of Ca (II), Mg (II) and Fe (II) as reflected by their intensities are responsible for the antioxidant potential of *E. officinalis*.

Keywords *Emblica officinalis* · Erythrocytes · Diabetes · Oxidative stress · Osmotic fragility

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Introduction

Increasing evidences from experimental and clinical studies suggest that the oxidative stress plays a major role in the pathogenesis of diabetes mellitus. Abnormally high levels of free radicals along with simultaneous decline of antioxidant defense can lead to a damage of cellular organelles and enzymes, increased lipid peroxidation, and development of complication of diabetes mellitus. Free radicals are formed disproportionately in diabetes by glucose oxidation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins.[1] The long-term hyperglycemic conditions lead to numerous alterations in cell membrane properties such as enhanced rigidity, permeability for cations, and transmembrane potential in its absolute magnitude.[2] The rigidity, biochemical organizations, as well as dynamic properties of erythrocyte membrane are considerably altered in the diabetic state, resulting in impaired cell function. Generally, the life spans of erythrocytes are shown to be decreased in

diabetic patients. Erythrocyte membrane hyperpolarization has been shown in diabetic cells due to constant oxidative stress that can be responsible for long-term complications in diabetes.[3] High hemoglobin (Hb) contents of erythrocytes make them vulnerable to oxidative damage by reactive oxygen species (ROS) as Hb and its oxidative breakdown products stimulate lipid peroxidation (LP). High ferrous ion concentration aggravates the problem mainly through the formation of ferryl hemoglobin[4] and, in part, through the Fenton reaction of hydrogen peroxide (H_2O_2) with ferrous ion (Fe^{2+}) of Hb that generates the powerful hydroxyl radicals. Red blood corpuscles (RBC) have a competent antioxidant system, and any ROS formed are effectively scavenged. Erythrocyte defense mechanism against oxidative damage is very efficient and is located in both the cytosol and membrane domains. The cytosolic antioxidant system is relatively complex and includes enzymes such as superoxide dismutase (SOD) and catalase (CAT). RBC membrane contains vitamin E as a major chain-breaking antioxidant and membrane stabilizer that avoids LP through its action on a variety of free radicals. In order to expand treatment options, there is a need to continue to explore the relationship between free radicals, diabetes, and its complications and to elucidate the mechanisms by which increased oxidative stress accelerates the development of diabetic complications.

In recent years, a number of plants commonly used to treat diabetes in traditional system of medicine have been explored scientifically by our research group for analysis of their chemical constituents, pharmacological activities, and their role in diabetes management.[5–8] The application of some of these plant products as complementary and alternative medicine in treatment of diabetes has been found to be of high significance.[9–11] To understand the biological activities of the extracts of medicinal plants, it is important to know their elemental constituents present as trace minerals. Laser-induced breakdown spectroscopy (LIBS) technique has been successfully applied for the analysis of trace elements and radioactive elements in solid, liquid, and gas. Recently, Rai et al. has also postulated the utility of LIBS technique on the plant product.[8,12–15]

Emblica officinalis (Family Euphorbiaceae) has been extensively used in traditional Indian system of medicine as an important constituent for scavenging reactive oxygen species.[16] The available reports indicated that different parts of *E. officinalis* have been used as anti-cancer,[17] antimutagenic,[18] antidiabetic,[19] hepatoprotective,[18] and also as antiulcer agents.[20] The aqueous extract of its seeds was found to have high anti-diabetic potential.[21] However, a close review of the available literature indicates that no considerable attention has been paid to explore the impact of extract of *E. officinalis* seeds on to the status of RBC membrane and levels of antioxidant enzymes. The present study was therefore designed to study the impact of seed extract of *E. officinalis* on the integrity of erythrocyte membrane of severely diabetic animals. We have monitored the activities of antioxidative enzymes such as CAT and SOD, lipid peroxidation, and osmotic fragility of RBC membrane. The results indicated that the extract of seeds from *E. officinalis* has the potential to normalize the oxidative stress in severely diabetic subjects.

Materials and Method

Materials

Fresh fruits of *E. officinalis* were purchased from the local market of Allahabad (India) as authenticated by Prof. Satya Narayan, taxonomist, Department of Botany, University of Allahabad, Allahabad, India. A voucher specimen has been submitted. The seeds were collected from the fruits cut into pieces and shade-dried. The dried seeds (4 kg) were mechanically crushed and extracted with distilled water using Soxhlet up to 48 h. The extract was filtered and concentrated in rotatory evaporator at 35±5°C under reduced pressure to obtain a semisolid material, which was then lyophilized to get a powder (yield about 10.5%, *w*/*w*).

Animal Care and Maintenance

Studies were conducted on 3-month-old male albino Wistar rats of 150–200 g body weight. Animals obtained from Central Drug Research Institute, Lucknow, India were housed in polypropylene cages at an ambient temperature of 27±3°C and 45±5% relative humidity with 12 h each of dark and light cycles. Animals were fed pellet diet (Golden feed, New Delhi, India) and water ad libitum. We had obtained the necessary approval from Institutional Ethical Committee to carry out the study.

Induction of Diabetes

Diabetes was induced by a single intraperitoneal injection of freshly prepared streptozotocin (STZ) 50 mg kg^{-1} body weight in 0.1 M citrate buffer (pH 4.5) to overnight-fasted rats. After 3 days of STZ administration, rats with marked hyperglycemia (FBG >250 mg/dl and PPG >350 mg/dl)[22] were selected for the study.

Blood Sampling

Animals were given mild anesthesia with chloroform. In order to draw blood, syringe needle was inserted just below the xyphoid cartilage and slightly to the left of midline. Blood was carefully aspirated from the heart into EDTA-coated tubes.

Erythrocytes were isolated by centrifugation for 20 min at 10,000×*g*. The plasma and buffy coat were removed by aspiration. The cells were washed three times with 0.9% NaCl (physiological saline); packed cell volume was recorded and finally suspended in an equal volume of the phosphate buffer (50 mM, pH 7.4) to get an erythrocyte suspension.

Experimental Design

The most effective dose of the seed extract (300 mg kg^{-1}) identified in previous findings[21] was used for the treatment of severely diabetic rats. The experiment was carried out into three groups (I, II, and III) containing six rats in each: group I—normal control; group II—diabetic control; and group III—diabetic treated with the seed extract. Control rats (groups I and II) received orally vehicle (distilled water only), while group III received orally the seed extract (300 mg kg^{-1}, body weight) suspended in distilled water up to 30 days once a day.

Assay of Activities of Antioxidant Enzymes in Erythrocyte Membrane

Superoxide Dismutase

The activity of SOD in the RBCs hemolysate was assayed spectrophotometrically according to Marklund and Marklund,[23] and the auto-oxidation of pyrogallol was measured at 412 nm for 3 min with or without the enzyme protein. One unit of the enzyme activity was expressed as 50% inhibition of auto-oxidation of pyrogallol in 1 min.

Catalase

The activity of CAT was determined by the method of Aebi.[24] Briefly, 100 μl of the hemolysate added with 10 μl absolute alcohol was incubated for 30 min at 0°C followed by re-addition of 10 μl absolute alcohol. The mixture was incubated for 30 min at 0°C followed by the addition of 10 μl Triton X-100. An aliquot of 50 μl was taken up in 1.25 ml of H_2O_2 (0.066 M) in phosphate buffer (50 mM, pH 7), and decrease in absorbance was measured at 240 nm for 60 s in a spectrophotometer. An extinction coefficient of 43.6 $M^{-1}cm^{-1}$ was used to determine the enzyme activity. Here, 1 U of CAT activity equals the moles of H_2O_2 degraded per minute per milligram Hb.

Estimation of LP in Erythrocytes Membrane

Malondialdehyde (MDA), a product of lipid peroxidation (LP), was determined according to the method of Ohkawa et al.[25] In brief, the sample was added to 8.1% SDS, vortexed, and incubated for 10 min at room temperature. This was followed by the addition of 375 μl of 20% acetic acid and 0.6% thiobarbutoric acid and placed in boiling water bath for 60 min. The mixture was allowed to cool. To it, 1.25 ml of butanol–pyridine (15:1) was added and the mixture was centrifuged at 640×*g* for 5 min with 1,1,3,3-tetramethoxy propane as the standard. MDA concentration was expressed as nanomoles per milligram prrotein in the membranes and as nanomoles per gram Hb in whole erythrocytes.

Determination of Osmotic Fragility of Erythrocyte Membrane

Osmotic fragility of the erythrocyte membrane was monitored using the method described by O'Dell et al.[26] with slight modification. An aliquot (100 μl) of washed erythrocyte suspension was added to the tubes containing 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, and 0.9% buffered salt solution (pH 7.4). Tubes were allowed to stand at room temperature for 30 min, centrifuged at 1270×*g* for 10 min to pellet the cells, and the absorbance of the supernatant was measured at 540 nm.

Detection of Trace Elements

A schematic experimental setup[13] was used for recording the LIBS spectra. The LIBS spectra of *E. officinalis* fruits extract powder, dissolved in distilled water, was recorded for identifying the presence of the best set of elements responsible for its antioxidant potential. The four-channel spectrometer equipped with CCD (Ocean optics LIBS 2000+) consisting four-grating was used to get the dispersed light from the plasma. A pulsed laser beam from a Q-switched Nd:YAG laser (Continuum Surellite III-10) was focused on the sample using a converging lens (Quartz) of 30-cm focal length; the temperature of the locally heated region rose rapidly and resulted in plasma formation on sample surface. The emitted light from micro-plasma was collected using an optical fiber tip placed in the vertical plane at 450 with respect to the laser beam and finally fed into an entrance slit of the multichannel spectrometer (Ocean Optics LIBS2000+) equipped with CCD and four gratings. The spectra presented in each Figures. 5, 6, and 7 are the average of 100 scans (100 shots). The initial three gratings had a resolution of 0.1 nm covering the wavelength range 200–310, 310–400, and 400–510 nm, respectively, while the fourth grating, called broadband grating, covered the wavelength range from 200 to 1100 nm and had a resolution of 0.75 nm. All the four gratings were used simultaneously to record the LIBS spectra. LIBS spectra

were recorded for aqueous extract of *E. officinalis* at 2-Hz repetition rate and 175-mJ laser energy.

Preliminary Phytochemical Screening of the Seed Extract

Qualitative phytochemical analysis of the crude seed extract for flavonoids was determined according to Kokate[27] and Harborne.[28] The seed extract (200 mg) was dissolved in 100 ml ethanol and filtered. Two milliliters of this filtrate was mixed with equal volume of concentrated HCl followed by the addition of the magnesium ribbon. The appearance of tomato red color indicated the presence of flavonoids in the extract of *E. officinalis*.

Statistical Analysis

Data were statistically evaluated using one-way ANOVA, followed by a post hoc Newman–Kuel's test using the Graphpad Prism 3 computer software. The values were considered significant at $p<0.05$.

Results

Osmotic Fragility

The osmotic fragility was measured in terms of percent hemolysis of erythrocytes in normal, severely diabetic treated and untreated rats. The results are shown in Figure 1. It was found to be significantly increased in the diabetic rat's RBC membrane. However, the osmotic fragility of the diabetic rats was recovered significantly ($p<0.001$) near normal in the RBCs upon the treatment with the seed extract of *E. officinalis* (Figure 1).

Activity of Antioxidant Enzymes

The effect of *E. officinalis* seed extract treatment on STZ-induced diabetic rats has been shown in Figure 2. The result indicated an 18.6% decrease in SOD activity when compared to the normal control group. The activity of SOD, however, got improved by 21.61% due to the treatment of diabetic rats with the seed extract of *E. officinalis*. The activity of CAT activity in diabetic control group registered a significant decrease (37.5%) in comparison to the normal control group. The activity of CAT got increased by 47.09% in diabetic treated group when compared with untreated diabetic group (Figure 3).

Lipid Peroxidation

Erythrocyte lipid peroxidation was measured in terms of MDA concentration. The result demonstrated in Figure 2 showed an increase of 30.87% in diabetic control group with respect to normal control rats. Erythrocytes from the diabetic rats treated with seed extract exhibited a recovery of 23.72% in lipid peroxidation (Figure 4).

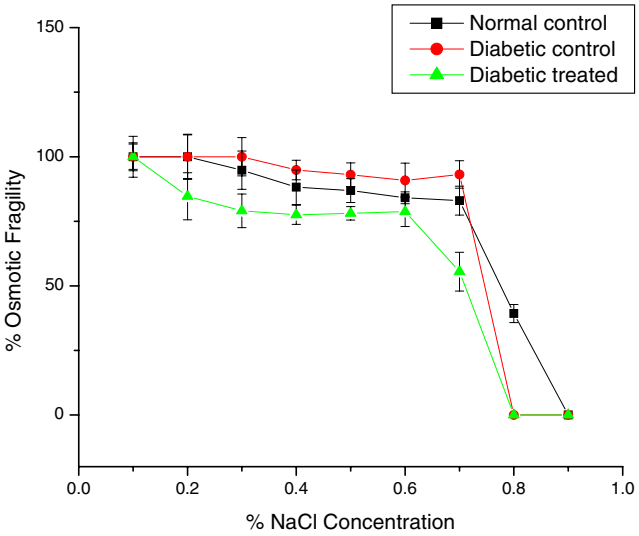

Fig. 1 Effect of the *E. officinalis* seed extract on osmotic fragility (*OF*) of erythrocytes of rats. The OF of erythrocytes of normal (*N*), diabetic control (*DC*), and diabetic treated (*DT*) with *E. officinalis* seed extract as described in "Materials and Method." OF has been defined as percent hemolysis of erythrocytes

Analysis of Antioxidative Elements

The LIBS spectra of *E. officinalis* fruit extract, in different spectral range shown in Figures 5, 6, and 7, were taken at

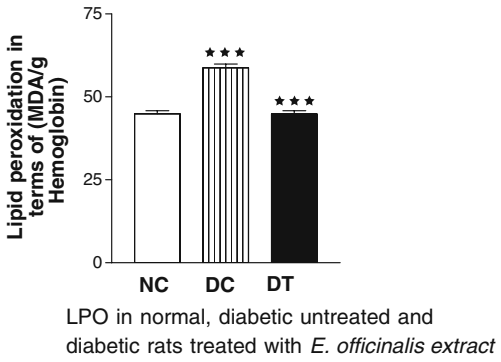

Fig. 2 Effect of the *E. officinalis* seed extract on superoxide dismutase (*SOD*) activity from erythrocytes of rats. The activity of SOD was determined in the hemolysates prepared from erythrocytes of normal (*N*), diabetic control (*DC*), and diabetic treated (*DT*) rats as described in "Materials and Method." One International unit (*IU*) of enzyme activity has been defined as 50% inhibition of pyrogallol auto-oxidation per minute. *Specific activity of enzyme is expressed as activity (IU)/gram hemoglobin $\times 10^3$. Values are expressed as mean +SD; $n=6$, where n is the number of determinations. [a]Values significantly different from DC group at $p<0.001$. [b]Values are significantly different from *E. officinalis*-treated group at $p<0.001$

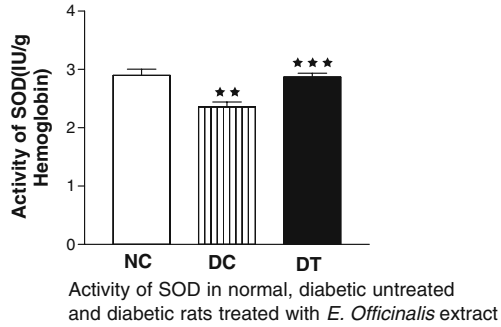

Fig. 3 Effect of the *E. officinalis* seed extract on catalase (*CAT*) activity from erythrocytes of rats. The activity of CAT was determined in the hemolysates prepared from erythrocytes of normal (*N*), diabetic control (*DC*), and diabetic treated (*DT*) rats as described in "Materials and Method." One international unit (*IU*) of catalase activity was defined as micromoles of H_2O_2 decomposed per minute. *Specific activity of enzyme is expressed as activity (IU)/gram hemoglobin. Values are expressed as mean + SD; n=6, where n is the number of determinations. [a]Values significantly different from control at $p<0.001$. [b]Values are significantly different from DC group at $p<0.001$.[c]Value significantly different from *E. officinalis*-treated group at $p<0.05$

optimized experimental conditions. It clearly revealed the presence of Mg, Na, Cl, Ca, H, O, C, and N elements in the spectral range λ 200–900 nm. According to the Boltzmann distribution law, intensity is directly related to concentration[29]; therefore, the intensity of observed spectral lines corresponding to the major and minor elements present in the extract speaks about their concentrations and helped in defining their role in diabetes-induced oxidative stress management.

Discussion

The previous finding of our research group has suggested that the aqueous seed extract of *E. officinalis* reduces the

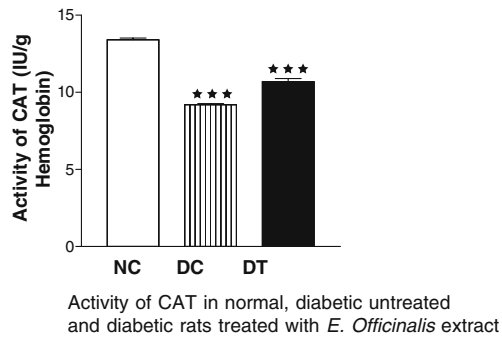

Fig. 4 Effect of the aqueous extract of *E. officinalis* on lipid peroxidation (*LPO*) has been expressed in terms of nanomolar of malondialdehyde (*MDA*) produced/gram hemoglobin. Values are expressed as mean + SD; n=6, where n is the number of determinations. [a]Values significantly different from control at $p<0.001$.[b]Values are significantly different from DC group at $p<0.001$. [c]Value significantly different from *E. officinalis*-treated group at $p<0.05$

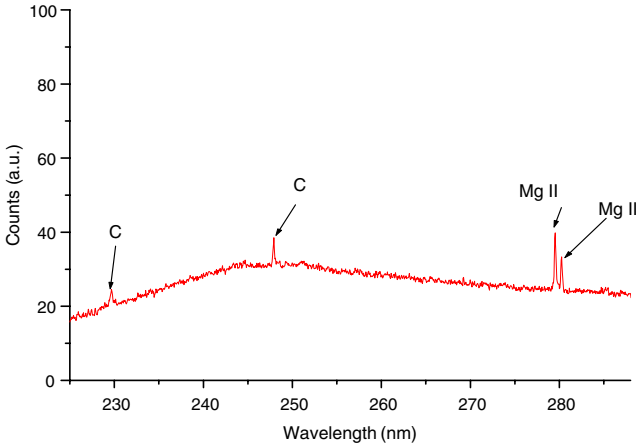

Fig. 5 Laser-induced breakdown spectra of *E. officinalis* in the spectral range 225–285 nm

high blood glucose level in normal as well as diabetic models.[21] This extract has been shown to be nontoxic to humans and experimental animals. Many polyphenols such as ellagitannin phyllemblin, ellagic acid, trigalloylglucose, phyllantidin, mucic acid, emblicannin, and furosin have been reported to be present in this extract.[30] Antioxidant activities of many of these poly phenols are reported.[31] Antioxidant defenses in the red cell can temper the negative influence of free radicals and related reactions and keep them in check.[32] Many enzymatic and non-enzymatic factors constitute antioxidant protection of the body and maintain the physiological level of ROS.[33] Superoxide dismutase and catalase are among the enzymes that quench the ROS generated. Decrease in the activity of CAT in erythrocytes observed in diabetic rats improved

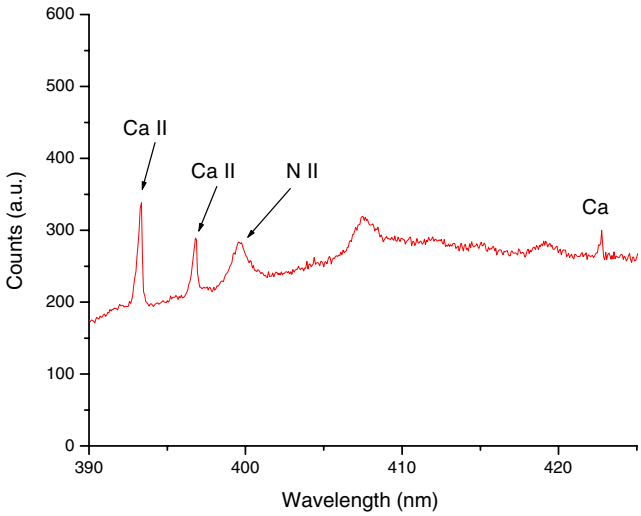

Fig. 6 Laser-induced breakdown spectra of *E. officinalis* in the spectral range 390–425 nm

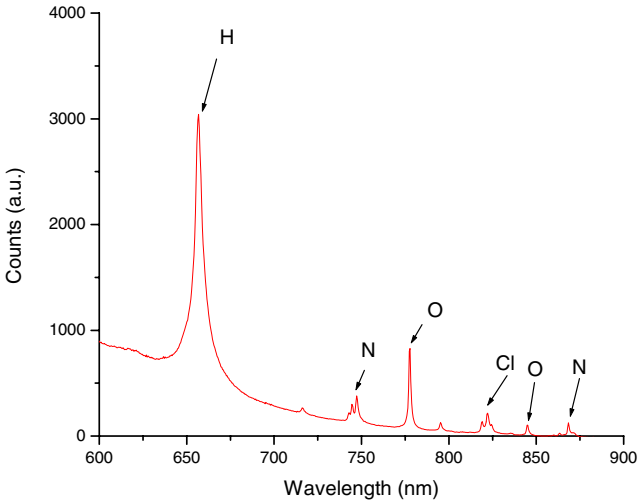

Fig. 7 Laser-induced breakdown spectra of *E. officinalis* in the spectral range 600–900 nm

significantly upon the treatment with the seed extract of *E. officinalis*. The activity of SOD which is responsible for the reduction of superoxide radicals into water and oxygen was observed to be sufficiently diminished in diabetic rats in the present investigation. It was found that the oral administration of seed extract increases the SOD activity in STZ-induced diabetic rats. Similar results have also been reported by other workers in different types of herbal extracts in diabetic models,[34,35] which substantiate the efficacy of *E. officinalis* seed extract in alleviating diabetic oxidative stress.

Extensive lipid peroxidation in biological membranes causes alteration in fluidity such as a decrease in its membrane potential and an increase in its permeability to different ions followed by an eventual rupture.[36] The determination of MDA level in erythrocytes gives an estimate of the rate of lipid peroxidation in cell membrane. Peroxidation of lipids occurs when pro-oxidant substances react with unsaturated fatty acids of biological membranes. The significantly elevated lipid peroxidation in diabetic rat erythrocytes, which was brought to normal with the treatment of the extract of seeds from *E. officinalis*, exhibits the antioxidative effect of this plant.

Table 1 Intensity ratio of elements w.r.t. C (247.8 nm)

Element/C (247.8nm)	Intensity ratio
C (247.8)/C (247.8)	1
Mg II (279.553)/C (247.8)	3.06977
Mg II (280.271)/C (247.8)	2.04378
Ca II (393.366)/C (247.8)	39.2093
Ca II (396.847)/C (247.8)	18.31737
Ca (422.673)/C (247.8)	5.41176

Table 2 Intensity ratio of elements w.r.t. O (844.62 nm)

Element//O (844.62nm)	Intensity ratio
H (656.27)/O (844.62)	134.07815
N (746.83)/O (844.62)	1.72071
N (868.02)/O (844.62)	0.68573
O (777.41)/O (844.62)	9.95708
O (844.62)/O (844.62)	1
Cl (822.17)/O (844.62)	1.21311
Na (819.47)/O (844.62)	2.47649

Osmotic fragility has been studied as a marker of hemolysis in erythrocytes at different salt concentrations.[37] It was observed that erythrocytes from the diabetic rats were more fragile in comparison to the normal ones. The erythrocyte osmotic fragility of diabetic rats was accentuated by the seed extract treatment. It might be possible that oxidative stress-dependent impairment in erythrocytes stability leads to osmotic stress-imposed hemolysis in animals. The ratio of intensities of detected elements (Mg, Na, Ca, C, H, O, and N) to the intensity of reference lines C and O, which were the essential constituents of plant materials, was estimated to evaluate their proportional concentration. Since gratings of different resolutions were used, the whole spectra was divided into two parts: the first covered the wavelength range from 200 to 510 nm with 0.1-nm resolution, and the second lies in the wavelength range 510–1100 nm with 0.75-nm resolution. To find the intensity ratios of spectral lines, the C line (247.88 nm) as reference line for the spectral range of λ 200–510 nm and O line (844.62 nm) as the reference line for spectral wavelength range of 500–1100 nm had been selected. Thus, the intensity ratios of Mg/C, Ca/C, Na/O, H/O, and N/O were calculated and are given in Tables 1 and 2. Mg and Ca are essential for the activity of antioxidant enzymes, including superoxide dismutase and catalase.[38] The higher concentration of Ca (II) and Mg (II) as reflected by their intensities are responsible for the antioxidant potential of *E. officinalis*.

From this study, we can conclusively infer that the oral administration of aqueous extract of *E. officinalis* seeds exhibits antioxidant potential and also involves improvement in osmotic stability of RBC.

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