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Original Article

POTENTIAL *IN VITRO* ANTIOXIDANT AND PROTECTIVE EFFECT OF *CASSIA FISTULA* LINN. FRUIT EXTRACTS AGAINST INDUCED OXIDATIVE DAMAGE IN HUMAN ERYTHROCYTES

RIZWANA ABID[a], RIAZ MAHMOOD[a,*], RAJESH K P[a], KUMARA SWAMY B E[b]

[a] Department of PG Studies and Research in Biotechnology and Bioinformatics, Jnanasahyadri, Kuvempu University, Shankaraghatta 577451, Shivamogga, Karnataka, India, [b] Department of PG Studies and Research in Industrial Chemistry, Jnanasahyadri, Kuvempu University, Shankaraghatta 577451, Shivamogga, Karnataka, India

Emil: rmahmood@kuvempu.ac.in, abid.rizwana@gmail.com

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ABSTRACT

Objective: To evaluate the antioxidant potential and protective effect of *Cassia fistula* Linn. on hydrogen peroxide induced oxidative damage in erythrocytes.

Methods: The *in vitro* antioxidant potency was screened by various established chemical, biochemical and electrochemical techniques. The Chemical assays allowed an evaluation of their total phenolic and flavonoid contents, total antioxidant capacity, reducing power, radical scavenging activities and metal chelating activity. Both, CFE (*C. fistula* ethanolic extract) and CFA (*C. fistula* aqueous extract) were analyzed for phenolic acids and flavonoids by HPLC. The biochemical assays were employed to evaluate the lipid peroxidation and protective effect on induced oxidative damage in erythrocytes. Electrochemical measurement of CFE and CFA was determined by cyclic voltammetry.

Results: CFE showed strong antioxidant activity as well as >90% protection of erythrocytes. This might be due to the presence of high 464.3 ± 0.02 µg EGA mg^{-1}total phenolics and 272.5 ± 0.5 µg EQ mg^{-1} total flavanoids while, CFA showed 75% antioxidant and protective activity possibly due to 250.84 ± 0.25 µg EGA mg^{-1} total phenolics and 195 ± 0.5 µg EQ mg^{-1} total flavonoid content. Moreover, HPLC of CFE and CFA exhibited widely known various good antioxidant molecules such as gallic acid, coumaric acid, ellagic acid, rutin, quercetin, myricetin and kaempferol. The cyclic voltammetric behavior shows the broad peak towards anodic potential represents the antioxidant property of CFE and CFA.

Conclusion: The present study clearly indicates that, *C. fistula* Linn. Fruit is an excellent source of natural antioxidant.

Keywords: *Cassia fistula* L, Antioxidant, Cyclic voltammetry, Oxidative damage inhibition, HPLC.

INTRODUCTION

Generation of free radicals and reactive oxygen species (ROS) during aerobic cellular metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress [1]. Free radicals and ROS are responsible for causing a large number of diseases including cardiovascular disease [2], cancer [3], neurodegenerative disorders [4-6], cirrhosis [7], ulcerative colitis [8], aging [9] and atherosclerosis [10]. Human body has multiple mechanisms especially enzymatic and non-enzymatic antioxidant systems to protect cells and their constituents against ROS and free radicals induced damage [11]. Antioxidants are the substances, when present at low concentrations compared to those of oxidizable substrates significantly delays or prevents oxidation [12].

In general, erythrocytes have been used as an *in vitro* model to study oxidative damage, because they are considered as prime targets for free radical attack owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the redox active hemoglobin molecules, which are potent promoters of ROS [13, 14]. The peroxidation of erythrocyte membranes is observed and undergoes changes in cell morphology when it gets exposed to harsh conditions of H_2O_2, which indicates the oxidative damage on the erythrocytes.

The development and utilization of more effective antioxidants of natural origin are desired. In this context, much attention has been paid to natural antioxidants of plant origin. The antioxidant activity of natural compounds is mainly due to their redox property, which allows them to act as proton donors, metal ion chelators and singlet oxygen quenchers [15]. *Cassia fistula* Linn. (Caesalpiniaceae), known as Indian Laburnum is a medicinal plant of immense importance and distributed in various regions including Asia, South Africa, China, the West Indies and Brazil. *C. fistula* L. is rich in polyphenols, tannin, anthraquinones, rehein I, emodin II, kaempferol, β-sitosterol, betulinic acid, flavonol glycosides and proanthocyanidin [16, 17].

The present study has been carried out to evaluate *in vitro* antioxidant property using different models and protective effect of *Cassia fistula* Linn. Fruit extracts against induced oxidative damage of normal erythrocytes. The extracts were analysed for phenolic acids and flavonoid by high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Chemicals

2,2-Diphenyl-2-picrylhydrazyl (DPPH), 2,2_-azino-bis(3-ethylbenzo thiazoline)-6-sulfonic acid (ABTS), nitroblue tetrazolium sodium salt (NBT), nicotinamide adenine dinucleotide phosphate reduced (NADH) was obtained from Sigma Chemical Co. (St. Louis, MO, USA), butylated hydroxyl anisole (BHA), trichloroacetic acid (TCA), Folin–Ciocalteu reagent were purchased from Merck (Germany). Ferrozine, gallic acid, ascorbic acid, ferrous chloride were procured from Himedia Pvt. Labs. (Mumbai, India). All other chemicals and solvents used were of analytical grade. Purified water from in a Mili-Q water purification system (Millipore, Bedford, MA, USA) was used in all the experiments.

Plant material collection and preparation of extracts

C. fistula L. fruits were collected from the orchard of Kuvempu University, Shankarghatta, Shimoga, Karnataka, India. The materials were identified and authenticated by the Department of Studies in Botany, Kuvempu University. Fruits were shade dried so as to bring down the initial large moisture content to enable its prolonged storage life, pulverized mechanically. The material was defatted using petroleum ether in the Soxhlet apparatus. Further, hot extraction was carried out with defatted material (500 g) successively with chloroform and ethanol. The extracts were concentrated to dryness under reduced pressure in a rotary evaporator (Buchi Flawil, Switzerland) to yield dried petroleum ether, chloroform and ethanol extracts. Distilled water was added to the dried plant material and brought to a boil for 30 min. This

decoction was filtered and dried for further use as aqueous extract. The highest yield was obtained using ethanol and water as extraction solvents. Ethanol (yield: 22.09%, w/w) and aqueous extracts (16.92%) of *C. fistula* were labeled as CFE and CFA respectively and stored in desiccators to avoid oxidation.

Qualitative phytochemical screening of CFE and CFA

The extracts were qualitatively analyzed to confirm the presence of possible active constituents by employing the standard tests [18].

Quantitative analysis of CFE and CFA

Determination of total phenolic content

The total phenolic content in the CFE and CFA was measured by Folin–Ciocalteu method [19]. Briefly, 1 ml of CFE and CFA (100 µg) was mixed with 2 ml of Folin-Ciocalteu reagent (diluted 1:10, v/v) followed by the addition of 2 ml of sodium carbonate (7.5%, w/v) and mixed thoroughly, allowed to stand for 90 min at room temperature and absorbance was measured against the blank at 750 nm using spectrophotometer (Systronics, PC based double beam spectrophotometer 2202). Total phenolic content of the extract was expressed in terms of equivalent to gallic acid (EGA, µg $mg^{-1}$of dry mass). All samples were analyzed in triplicate and the results were averaged.

Determination of total flavonoid content

Total flavonoid content of CFE and CFA was determined according to the modified method [20] using quercetin as standard. Briefly, 5 ml of extracts (200 µg) in millipore water was mixed with 300 µl of sodium nitrite (5%) and 300 µl of aluminum chloride (10%), this reaction mixture was incubated at room temperature for 6 min followed by the addition of 2 ml of sodium hydroxide (1 M). Later, the volume in each test tube was made up to 10 ml by adding 2.4 ml of millipore water. Absorbance was measured against the blank at 510 nm. Total flavonoid content of the extract was expressed in terms of equivalent to Quercetin (EQ, µg mg^{-1} of dry mass).

***In vitro* antioxidant activity**

Total antioxidant capacity

Total antioxidant capacity of CFE and CFA was performed according to the procedure of Prieto *et al*., [21]. 300 µl of CFE and CFA at different concentrations (0-160 µg) was combined with 3 ml of reagent mixture (4 mM ammonium molybdate, 0.6 M sulfuric acid and 28 mM of sodium phosphate). The tubes were capped and kept for incubation at 95°C for 90 min, after cooling to room temperature the absorbance of the content was measured at 695 nm against the blank. The total antioxidant capacity of each extract is expressed as equivalents of ascorbic acid.

Total reductive capability

Total reduction capability of CFE and CFA was estimated by using the method of Oyaizu [22]. The different concentration of CFE and CFA (0-300 µg) in 1 ml of millipore water was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.5) and 2.5 ml of potassium ferricyanide (1%). The mixtures were incubated at 50°C for 20 min and 2.5 ml of 10% trichloroacetic acid were added. Then the mixture was centrifuged for 10 min at 3000 rpm. 2.5 ml of millipore water and 0.5 ml of ferric chloride (0.1%) was added to 2.5 ml of supernatant. The absorbance was measured at 700 nm against blank. Higher absorbance of the reaction mixture indicated greater reducing power. Total reducing capacity of each extract is expressed as equivalents of quercetin.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The free radical scavenging activity of CFE and CFA was evaluated using DPPH method [23]. Each extract at different concentrations was added to 3 ml of 0.004% DPPH in methanol and the mixtures were incubated at room temperature in dark condition for 30 min. The scavenging activity of extracts against DPPH radical was determined by measuring the absorbance at 517 nm. DPPH radical scavenging activity of BHT was assayed for comparison. Radical scavenging activity was calculated using the following Equation (1):

$$\% \text{ inhibition} = \left[\frac{\text{Acontrol} - \text{Atest}}{\text{Acontrol}}\right] \times 100$$

Where $A_{control}$ is the absorbance of the control (without extract) and A_{test} is the absorbance of the extract reaction. IC_{50} value was calculated using the following Equation (2):

$$IC50 = \left[\left(\frac{\sum C}{\sum I}\right) \times 50\right]$$

Where, ∑C is the sum of extracts concentrations used to test and I is the sum of percentage of inhibition at different concentrations.

ABTS radical scavenging activity

CFE and CFA were screened for ABTS (2, 2'-azinobis-3-ethyl-benzothiozoline-6-sulphonic acid) radical scavenging activity according to the method of Arnao *et al.,* [24] with slight modification. ABTS radical scavenging activity of BHA was assayed for comparison. The stock solution containing equal volume of (v/v) 7.4 mM ABTS and 2.6 mM potassium persulfate was mixed and kept for overnight at room temperature under dark condition. 150 µl of CFE and CFA at different concentrations were allowed to react with 2.85 ml of ABTS solution for 2 h in dark condition. The scavenging activity of extracts against ABTS radical was determined by measuring the absorbance at 734 nm. Percentage of inhibition and IC_{50} value was calculated using equation (1) and Equation (2).

Superoxide radical scavenging activity

Superoxide anion radical scavenging activity of CFE and CFA was determined using the slight modified method of Nishimiki *et al.,* [25], all the reagents were prepared in phosphate buffer (pH 7.4). 1 ml of NBT (156 µM), 1 ml of NADH (468 µM) and 2 ml of extracts at different concentrations were added to each test tube. To this 100 µl of PMS (60 µM) was added to initiate the reaction and incubated at 25°C for 5 min followed by the measurement of absorbance at 560 nm against the blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion radical scavenging activity. Gallic acid was used as standard. Percentage of inhibition and IC_{50} value was calculated using Equation (1) and Equation (2).

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of CFE and CFA was measured by using deoxyribose method [26]. All the tubes containing the reaction mixture of 2.5 ml of 2-deoxyribose (3 mM), 0.2 ml of ferric chloride (0.1 mM), 0.2 ml of EDTA (0.1 mM),0.2 ml of ascorbic acid (0.1 mM) and 0.2 ml of H_2O_2 (2 mM) in phosphate buffer (pH 7.4, 20 mM). To this 0.2 ml of various concentrations of the extracts was added to give a total volume of 1.2 ml. The mixtures were kept in a water bath at 37 °C for 30 min. 0.2 ml of chilled 15% trichloroacetic acid was added followed by 0.2 ml of 1% thiobarbituric acid in 0.25 N HCl. Then the mixture was incubated at 100°C for 30 min and allowed to room temperature. The absorbance was measured at 532 nm against the blank. Percentage of inhibition and IC_{50} value was calculated using Equation (1) and Equation (2).

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity of CFE and CFA was measured according to Marcocci *et al.,* [27]. The reaction mixture containing 2 ml of sodium nitroprusside (10 mM in 0.5 M phosphate buffer, pH 7.4) and extracts at different concentrations were incubated for 150 min at 25°C. The reaction mixture (0.5 ml) was mixed with 1 ml of sulfanilamide (1% in 5% phosphoric acid) and allowed to stand for 5 min at 25°C. 1 ml of 0.1% (α-napthyl)-ethylenediamine was added to the resultant mixture and incubated for 30 min at 25°C. Absorbance of the pink coloured chromophore was measured at 546 nm. Percentage of inhibition and IC_{50} value was calculated using Equation (1) and Equation (2).

Lipid peroxidation inhibition assay

TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected according to the method of Halliwell and Guttridge [12]. 10% of chicken liver homogenate in 0.15 M potassium chloride was prepared. 0.5 ml of liver homogenate and 1 ml of CFE and CFA at different concentrations were taken in

test tubes. Lipid peroxidation was induced by adding 50 µl of ferrous sulfate (0.07 M) and incubated at room temperature for 30 min. The reaction was terminated by the addition of chilled 1.5 ml of acetic acid (20%, pH 3.5) containing 20% TCA followed by the addition of 50 µl of TBA (0.8% TBA in 1.1% SDS). The resultant mixture was incubated in boiling water bath for 60 min. After cooling, 5 ml of butanol was added and centrifuged at 3000 rpm for 10 min. Absorbance of the organic supernatant was measured at 532 nm. Percentage of inhibition and IC_{50} value was calculated using Equation (1) and Equation (2).

Metal chelating activity

The chelation of ferrous ions by CFE and CFA was determined according to the method of *Dinis et al.,* [28]. About 3 ml of extracts at different concentrations were taken in different test tubes followed by the addition of 50 µl of ferrous chloride (2 mM). The reaction was initiated by the addition of 20 µL ferrozine (5 mM), and then the mixture was shaken vigorously and allowed to stand for 10 min at room temperature. After equilibrium, absorbance of the solution was measured at 562 nm against the blank. EDTA was used as standard for comparison. Percentage of inhibition and IC_{50} value was calculated using Equation (1) and Equation (2).

***In vitro* inhibition of human erythrocyte hemolysis assay**

Preparation of erythrocytes

For erythrocyte isolation, 2 ml of blood was obtained from a healthy volunteer and collected in heparinized sterile tube. Erythrocytes were isolated and stored according to the method of Yang *et al.,* [29]. Briefly, blood samples collected were centrifuged (3000 rpm,10 min) at 4°C, erythrocytes were separated from the plasma and buffy coat and were washed three times by centrifugation (3000 rpm, 5 min) in 10 volumes of 10 mM phosphate buffered saline (PBS, pH 7.4). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes were stored at 4°C and used within 6 h for further studies.

Inhibition of human erythrocyte hemolysis

The inhibition of human erythrocyte hemolysis by CFE and CFA was estimated according to the method of Tedesco *et al.,* [30] with slight modifications. To 100 µl of 5% (v/v) erythrocytes suspension in PBS, 50 µl of CFE and CFA at different concentrations were added. To each tube, 100 µl of 100 µM H_2O_2 (in 0.1 M PBS pH 7.4) was added. A negative control was also maintained by adding 100 µl of 100 µM H_2O_2 to erythrocytes suspension. The reaction mixture was incubated at 37 ºC for 3 h and diluted with 8 ml of PBS and centrifuged at 3000 rpm for 10 min. Absorbance of the resulting supernatant was measured at 540 nm to determine the percentage of hemolysis. BHA was used as standard for comparison. To assess the possible hemolysis, percentage of hemolysis was calculated by taking hemolysis caused by 100 µM H_2O_2 as 100%. Percentage of inhibition and IC_{50} value was calculated using Equation (1) and Equation (2).

Protective effect on erythrocytes structural morphology

To evaluate protective effect of CFE and CFA, 50 µl of erythrocyte suspension in PBS was added to all test tubes followed by the addition of extracts (1 mg ml^{-1}) and 100 µl of 200 µM H_2O_2 in PBS (pH 7.4). Simultaneously, negative control (H_2O_2 alone) and positive control (erythrocyte suspension alone) were maintained for comparative evaluation. The mixture was incubated at 37°C for 60 min and centrifuged at 3500 rpm for 10 min and the cell pellet were inspected for morphological changes using Nikon Eclipse 80i microscope at 40x magnification.

Electrochemical measurements of antioxidant activity: Cyclic voltammetry

Apparatus and preparation of bare carbon electrode

Cyclic voltammetry study was performed with Model CHI-660c electrochemical work station. A conventional three electrode cell were employed throughout the experiments. The electrochemical cell contained the carbon paste electrode (CPE) as a working electrode, platinum counter electrode (PCE) and saturated calomel electrode (SCE) as reference [31].

The bare carbon electrode was prepared by mixing of graphite powder and silicon oil at a ratio of 70:30 (w/w) in an agate mortar until a homogenous paste was obtained. The paste was packed into the homemade cavity (3 mm in diameter) and then smoothened on a butter paper. Electrical contact was provided by a copper wire connected to the paste at the end of the tube and cyclic voltamogram was produced using 10 mm dopamine.

The experiment was repeated with a modified carbon paste electrode, in which the carbon paste was blended with extracts and then used in filling the electrode. The electrochemical response of dopamine (0.1 mM in phosphate buffer solution, pH 7.2) at modified CPE was observed at a scan rate 0.1 V s^{-1}.

HPLC–UV analysis

Phenolic acids and flavonoids from CFE and CFA were analyzed by HPLC (Model LC-10ATVP. Shimadzu Corp, Kyoto, Japan). Samples were separated on a reversed phase Shimpak C_{18} column (5 µm, 250 mm×4.6 mm) [32]. Phenolic content in both CFE and CFA were detected using octadecylsilyl silica gel as stationary phase. Solvent system consisting of [A] phosphoric acid: water (0.5: 99.5, v/v), [B] acetonitrile was used as mobile phase at a flow rate of 1 ml min^{-1}. Phenolic acid standards such as gallic acid, ellagic acid, ferulic acid, p-coumaric acid, mandelic acid and vanillic acids were employed for identification of phenolic acids present in CFE and CFA by comparing the retention time under similar experimental conditions. UV detector at 220 nm was used for analysis.

Flavonoid content in CFE and CFA was detected using octadecylsilyl silica gel as stationary phase. Solvent system consisting of methanol, water and phosphoric acid (50:49.6:0.4, v/v) was used as mobile phase at a flow rate of 0.5 ml min^{-1}. Rutin, quercetin, kaempferol, myricetin, luteolin were used as a reference standard to identify the flavonoids in CFE and CFA. UV detector at 350 nm was used for analysis.

Statistical analysis

All the experiments were performed in triplicate and results are reported as mean ± SEM. The Dunnet's test was used for pair wise comparison by using graph pad prism 5.

RESULTS

Qualitative analysis of CFE and CFA

The preliminary phytochemical investigation of CFE revealed the presence of various classes of compounds such as flavonoids, alkaloids, terpenoids, tannins, glycosides, carbohydrates, quinones, organic acid, fats and oils and the absence of sterols, saponins and proteins. On the other hand, CFA was found to be containing flavonoids, glycosides, carbohydrates, proteins, quinones, fats and oil.

Quantitative analysis of CFE and CFA

Total phenolic content in CFE and CFA was expressed as equivalent to gallic acid (EGA) and was found to be 464.3 ± 0.02 and 250.84 ± 0.25 µg mg^{-1} of dry CFE and CFA, respectively. The total flavonoid content of CFE and CFA was found to 272.5 ± 0.5 and 195 ± 0.5 µg mg^{-1} of dry extract as equivalent to quercetin in CFE and CFA respectively.

Evaluation of *in vitro* antioxidant properties

Total antioxidant and reductive capability

Total antioxidant capacity of both CFE and CFA has shown significant antioxidant capacity expressed as equivalents to ascorbic acid. Among them, CFE showed significant activity over CFA (**Fig.1**). However, CFE and CFA have shown significant reductive capability when compared to standard quercetin.

Like antioxidant activity, the reducing power of CFE and CFA increased with increasing concentration of extracts, hence both can serve as free radical inhibitors or scavengers.

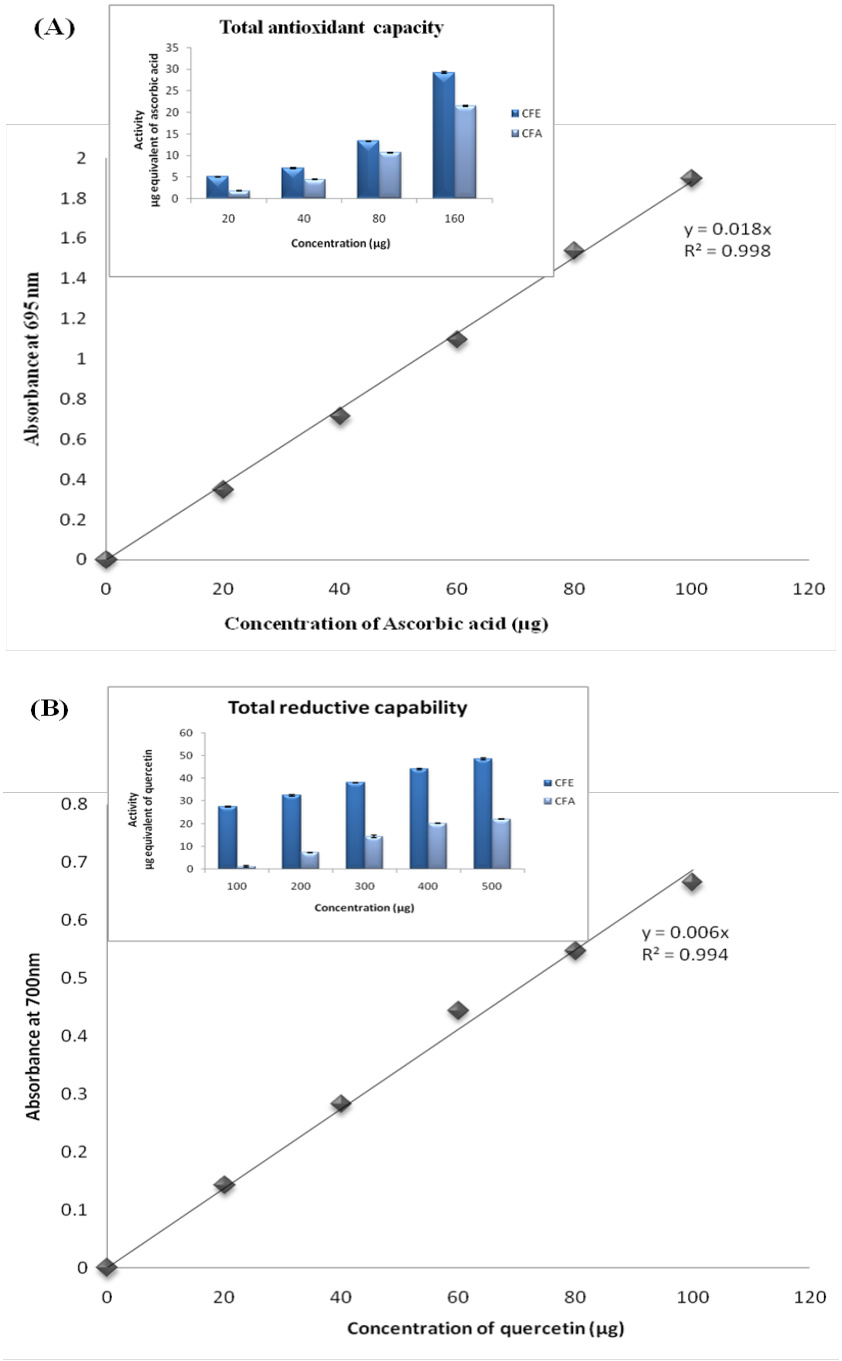

Fig.1: It shows plots (A) total antioxidant capacity and (B) total reductive capability of CFE and CFA.

DPPH, ABTS free radical scavenging and metal chelating activity of extracts

DPPH and ABTS free radical scavenging activity of CFE and CFA was expressed in terms of percentage of inhibition and IC_{50} values (µg ml^{-1}). Among CFE and CFA, CFE showed better radical scavenging activity (IC_{50}; DPPH: 722.15 ± 2.72 µg ml^{-1}, ABTS: 92.38 ± 3.67 µg ml^{-1}) whereas CFA showed moderate activity (IC_{50}; DPPH: 2503.34 ± 0.89µg ml^{-1}, ABTS: 108.36 ± 0.73µg ml^{-1}) compared to the standard BHA (IC_{50}; DPPH: 36.28 ± 0.267 µg ml^{-1}, ABTS: 7.44 ± 0.078 µg ml^{-1}). Metal chelating activity of extracts demonstrates that CFE has better Fe^{2+}chelating capacity than the CFA. The extracts act on the metal ion in a dose dependent manner. The results of above activity of CFE and CFA were illustrated in **Table 1**.

Superoxide, nitric oxide and hydroxyl radical scavenging activities of extracts

CFE and CFA were screened for superoxide radical scavenging activity by PMS–NADH–NBT system. The IC_{50} values of quenching the radicals were found to be 828.50 ±1.96 µg ml^{-1} and 1248.31±1.33 µg ml^{-1} for CFE and CFA respectively. In the present study, CFE showed a better NO radical scavenging activity (IC_{50}: 1232.64 ± 1.73µg ml^{-1}) and CFA showed a moderate activity with IC_{50}: 2940.13 ± 0.97µg ml^{-1}. Both CFE and CFA were examined for their ability to act as hydroxyl radical scavengers. CFE showed a better hydroxyl radical scavenging activity (IC_{50}: 609.03 ± 0.64 µg ml^{-1}) than CFA, which exhibited a moderate activity (IC_{50}: 1748.86 ± 0.65 µg ml^{-1}).

The results of above activities of CFE and CFA clearly indicate strong concentration dependent activity (**Table 2**).

Lipid peroxidation inhibition assay

The CFE showed a better LPO inhibition with IC_{50} of 596.70 ± 0.59 µg ml^{-1}whereas CFA showed less LPO inhibition activity (IC_{50}:1083.61± 0.51µg ml^{-1}) which is depicted in **Table 3**.

Protective effect of CFE and CFA on oxidative damage in erythrocytes

The study showed that the extracts did not pose any harmful effects or toxicity on the erythrocytes. Protective effect of different concentrations of CFE and CFA on H_2O_2 induced hemolysis of erythrocytes for 3 h are shown in **Table 4**. 100% hemolysis was observed when erythrocytes were incubated with H_2O_2 due to induced stress, but they were stable in the presence of both CFE and CFA. Both the extracts showed dose dependent protective activity.

CFE (IC_{50}: 607.87 ± 0.96 µg ml^{-1}) showed significant protective effect than the CFA (IC_{50}: 1606.21 ± 1.71 µg ml^{-1}). This may be attributed to the presence of high amount of polyphenols and flavanoids. The cellular morphology of human erythrocytes under H_2O_2 induced oxidative stress condition is illustrated in **Fig. 2.** Normal erythrocytes (**Fig. 2A**) appeared as typical discocytes and under induced oxidative stress by H_2O_2, change in the cell shape was observed (**Fig. 2B**). The morphological changes induced by H_2O_2 were almost recovered to the normal form when the cells were treated with CFE and CFA (**Fig. 2C and D**).

Evaluation of antioxidant activity by electrochemical method

The cyclic voltammograms obtained at a scan rate of 0.1 V s^{-1}showed cathodic peak potential E_{pc} shifting towards anodic direction with broad peak potential E_{pa} as the oxidized antioxidant molecules characteristically exhibit decreased cathodic current with reciprocal increased anodic current (**Fig.3A and B**). Interestingly, in the present study broad peak was observed towards anodic potential, which represents the antioxidant property of CFE and CFA.

HPLC–UV analysis

HPLC was used to determine the phenolic acids and flavonoids in CFE and CFA as shown in **Fig. 4** and **Table 5**. Phenolic acids detected in CFE were gallic acid, coumaric acid, ellagic acid and two unknown peaks at 220 nm (**Fig. 4A**) with retention times of 2.13, 3.89 and 5.27 min respectively, among them, gallic acid was the most abundant (130.29 mg g^{-1}). CFA showed the presence of gallic acid and two unknown peaks with retention time of 2.38 min, as compared to standard molecules (**Fig. 4B**). UV spectral peaks of CFE and CFA at 350 nm along with standard flavonoids confirmed the presence of rutin, quercetin, myricetin and kaempferol in CFE (**Fig. 4C**) with retention times of 2.01, 3.10, 3.82 and 5.74 min respectively. CFA showed the presence of rutin and quercetin with retention times 2.09 and 3.60 min respectively (**Fig. 4D**).

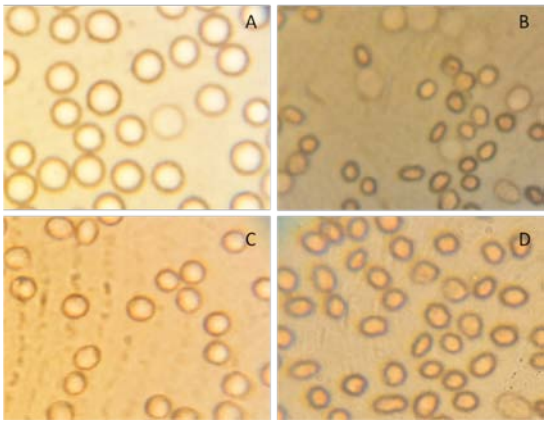

Fig.2: It shows morphology of (A) normal erythrocytes, (B) erythrocytes + H_2O_2, (C) erythrocytes + H_2O_2+ CFE and (D) erythrocytes + H_2O_2+ CFA (100x).

Table 1: It shows DPPH, ABTS radical scavenging and metal chelating activity of CFE and CFA

S. No.	Activity	Extracts	Concentration in µg ml^{-1}	% of inhibtion	IC_{50} (µg ml^{-1})	Standard (µg ml^{-1})
1.	DPPH radical scavenging activity	CFE	200	23.39 ± 0.15	722.15 ± 2.72	36.28 ± 1.45
			400	34.92 ± 0.29		
			600	50.19 ± 0.39		
			800	51.61± 1.12		
			1000	66.24 ± 0.19		
			1500	85.22 ± 0.59		
		CFA	1000	34.12 ± 0.12	2503.34 ± 0.89	
			2000	44.01 ± 0.01		
			3000	62.88 ± 0.52		
			4000	72.37 ± 0.10		
			5000	86.23 ± 0.15		
2.	ABTS radical scavenging activity	CFE	50	28.58 ± 1.72	92.38 ± 3.67	7.44 ± 0.07
			75	41.91 ± 0.95		
			100	54.35 ± 0.15		
			125	63.85 ± 0.45		
			150	81.90 ± 0.40		
		CFA	50	18.75 ± 2.05	92.38 ± 3.67	
			75	33.85 ± 0.55		
			100	47.50 ± 0.20		
			125	57.20 ± 2.90		
			150	73.40 ± 1.60		
3.	Metal chelating activity	CFE	1000	41.28 ± 0.28	1706.42 ± 0.98	28.92±0.10
			2000	70.30 ± 0.20		
			3000	83.37 ± 0.36		
			4000	98.06 ± 0.13		
		CFA	1000	33.48 ± 0.25	2298.63 ± 1.41	
			2000	47.72 ± 0.47		
			3000	75.45 ± 0.45		
			4000	80.55 ± 0.17		
			5000	89.08 ± 0.07		

Values are expressed as mean ± SEM

Table 2: It shows superoxide, nitric oxide and hydroxyl radical scavenging activity of CFE and CFA

S. No.	Activity	Extracts	Concentration in µg ml^{-1}	% of inhibtion	IC_{50} (µg ml^{-1})
1.	Superoxide radical scavenging activity	CFE	200	24.44 ± 0.45	828.50±1.96
			400	46.09 ± 0.32	
			800	68.94 ± 0.08	
			1200	75.19 ± 0.25	
			1600	77.41 ± 0.40	
			2000	82.10 ± 0.44	
		CFA	1000	75.20 ± 0.33	1248.31±1.33
			1500	78.00 ± 0.49	
			2000	80.47 ± 0.10	
			2500	82.60 ± 0.15	
			3000	84.27 ± 0.25	
2.	Nitric oxide radical scavenging activity	CFE	400	17.94 ± 0.12	1232.64 ± 1.73
			800	37.07 ± 0.55	
			1200	47.95 ± 0.55	
			1600	65.91 ± 0.10	
			2000	74.51 ± 0.40	
		CFA	1000	12.45 ± 0.25	2940.13 ± 0.97
			2000	23.50 ± 0.08	
			3000	53.43 ± 0.51	
			4000	80.68 ± 0.13	
3.	Hydroxyl radical scavenging activity	CFE	200	19.01 ± 0.01	609.03 ± 0.64
			400	27.40 ± 0.20	
			600	50.07 ± 0.06	
			800	65.30 ± 0.30	
			1000	84.27 ± 0.06	
		CFA	500	14.10 ± 0.10	1748.86 ± 0.65
			1000	28.25 ± 0.15	
			1500	42.05 ± 0.05	
			2000	58.55 ± 0.35	

Values are expressed as mean ± SEM.

Table 3: It shows lipid peroxidation inhibition activity of CFE and CFA

S. No.	Activity	Extracts	Concentration in µg ml^{-1}	% of inhibtion	IC_{50} (µg ml^{-1})
1.	Lipid peroxidation inhibition assay	CFE	100	57.14 ± 0.14	596.70 ± 0.59
			500	59.32 ± 0.11	
			1000	64.54 ± 0.22	
			1500	78.76 ± 0.12	
		CFA	500	48.11 ± 0.11	1083.61± 0.51
			1000	57.16 ± 0.05	
			1500	60.81 ± 0.04	
			2000	64.63 ± 0.31	

Values are expressed as mean ± SEM

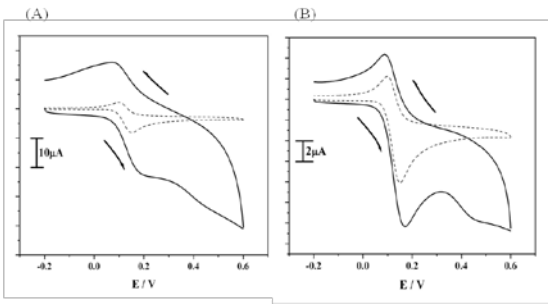

Fig. 3: It shows the voltamograms of CFE (A) and CFA (B) at a scan rate 0.1 V s^{-1}

DISCUSSION

Many botanicals possess antioxidant properties and these herbal antioxidants protect against a plethora of diseases by contributing to the total antioxidant defense system of the human body [33]. Several studies have shown possible benefits of antioxidants like flavonoids, polyphenolics and vitamins from plant sources in altering, reversing or preventing the negative effects of oxidative stress [30]. The present study is to evaluate *in vitro* antioxidant property and protective effect of *Cassia fistula* Linn. fruit extracts on oxidative stress induced by H_2O_2 in normal erythrocytes.

The total phenolic content in CFE and CFA was estimated using Folin-Ciocalteu reagent that produce blue colour by reducing yellow hetero polyphosphomolybdate-tungstate anions [34] and the total flavonoid content of CFE and CFA was determined using spectrophotometric method with aluminum chloride. The phenolic content of plants may contribute directly to their antioxidant action [35]. Earlier reports clearly reveal that various classes of compounds like β-sitosterol, betulinic acid, anthraquinone glycosides, flavonol glycosides, kaempferol, kaempferol-3-rhamnoside, α-amyrin, β-amyrin, pectins, tannins are present in species of *Cassia* such as *C. tora, C. javanica, C. didymobotrya, C. nodosa, C. uniflora, C. acutifolia* [16].

Total antioxidant capacity of both CFE and CFA was evaluated by phosphomolybdenum method according to the procedure described by Prieto *et al.,* [21]. Previous report of Sirappuselvi and Chitra [36]

showed polyphenolic compounds present in *C. tora* contributes significantly to the total antioxidant capacity of the fruits. Studies suggested that *C. fistula* has good reducing power. Irshad *et al.,* [37] mentions the reducing activity of methanolic extracts of plant over power the hexane extracts. Bhalodia *et al.,* [38] reported that flower extract of *C. fistula* showed significant ferric reducing power. Thus, the present result with fruit extracts also corroborates the earlier results with reference to the reductive abilities.

DPPH and ABTS are the stable free radicals and the measurement is based on hydrogen donating ability of antioxidant molecules present in CFE and CFA to reduce purple colored DPPH and blue colored ABTS radical to colorless. The results are in agreement with previous report of Siddhuraju *et al.,* [39] where pulp extract of *C. fistula* at 50 µg showed 15.7% of DPPH radical scavenging activity. Metal chelating activity of extracts was estimated using the method of Dinis *et al.,* [28]. Ferrozine can quantitatively form complexes with Fe^{2+}resulting in the red color of the solution, this colour diminishes in the presence of chelating agents. Phenols possess a hydroxyl group which helps it to act as a strong antioxidant agent. They can stabilize metal ion or chelate them and protect the cells that can be damaged from the action of transition metal ions.

Similar results have been reported by Sumathy *et al.,* [40] where the flavonoid rich fraction of *C. auriculata* petals could bleach ABTS radicals and chelate Fe^{2+}ions efficiently at the concentration 20 µl ml^{-1}.

Table 4: It shows protective effect of CFE and CFA on oxidative damage in erythrocytes

S. No.	Activity	Extracts	Concentration in µg ml^{-1}	% of inhibtion	IC_{50} (µg ml^{-1})	Standard (µg ml^{-1})
1.	Erythrocyte haemolysis inhibition assay	CFE	200	32.98 ± 0.39	607.87 ± 0.96	72.49± 1.70
			400	47.50 ± 0.25		
			600	62.29 ± 0.08		
			800	84.24 ± 0.20		
			1000	95.75 ± 0.04		
		CFA	1100	12.53 ± 0.40	1606.21 ± 1.71	
			1200	27.57 ± 0.44		
			1300	32.52 ± 0.20		
			1400	54.45 ± 0.42		
			1500	75.27 ± 0.24		

Values are expressed as mean ± SEM.

Table 5: It shows quantitative RP-HPLC analysis of CFE and CFA

S. No.	Phenolic acids			Flavonoids		
	Standard	CFE (mg g^{-1})	CFA (mg g^{-1})	Standard	CFE (mg g^{-1})	CFA (mg g^{-1})
1.	Gallic acid	130.29	96.95	Rutin	18.53	136.84
2.	Coumaric acid	4.31	--	Quercitin	91.18	22.07
3.	Ellagic acid	9.72	--	Myricetin	4.45	--
4.	Hydroxyl benzoic acid	--	--	Kaempferol	8.42	--
5.	Vanillic acid	--	--	Luteolin	--	--

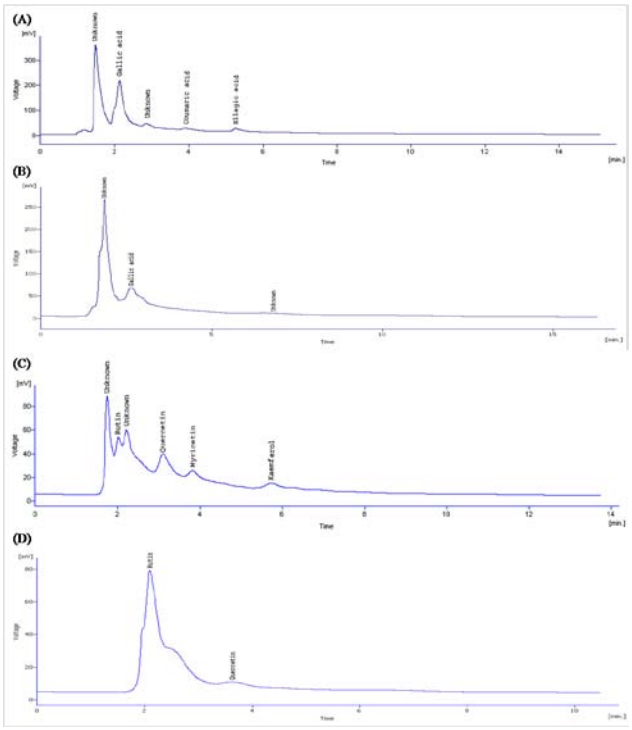

Fig. 4: RP-HPLC chromatograms of phenolic acids of (A) CFE (B) CFA, RP-HPLC chromatograms of flavonoids (C) CFE (D) CFA.

CFE and CFA were screened for superoxide radical scavenging activity by PMS–NADH–NBT system [41] in which superoxide anions were generated by PMS–NADH coupling reaction and decreased absorbance with antioxidants indicates consumption of the superoxide anion in the reaction mixture. Nitric oxide (NO) is a labile gaseous free radical with important roles in physiological and pathological conditions. Lui *et al.,* [42] found that ischemia-reperfusion injury increased the production of NO and induced the expression of iNOS mRNA in the kidney. NO is generated when sodium nitroprusside reacts with oxygen to form nitrite, incubation with antioxidants inhibits nitrite formation by competing with oxygen to react with nitric oxide directly. Due to this ability, there was a decrease in pink colored chromophore. $OH^{\bullet}$ radical is an extremely reactive free radical formed in biological systems, it was measured by the deoxyribose method [26].

Fenton reaction (Ferric-ascorbate–EDTA–H_2O_2) generates hydroxyl radicals, which reacts with deoxyribose to produce thiobarbituric acid reactive substances (TBARS). The hydroxyl quencher reduces TBARS production and formation of pink chromogen by competing with deoxyribose for hydroxyl radicals. Similarly, in a related species of *C. tora* Sirappuselvi and Chitra [36] have reported that, methanolic extract scavenged superoxide anion radicals markedly and inhibited nitric oxide moderately in a dose dependent manner with IC_{50} values of 38.19 µg ml^{-1} and 49.36 µg ml^{-1} respectively. Further, EC_{50} values of hydroxyl radical scavenging was reported by Irshad *et al.,* [37]. The methanolic and hexane extracts of pulp and seeds could scavenge 50% of the radicals at 0.889, 1.058, 2.075, 1.723 mg ml^{-1} respectively. These results also clearly indicate the presence of hydroxyl radical scavenging abilities.

Lipid peroxidation (LPO) may cause peroxidative tissue damage during inflammation, cancer, toxicity of xenobiotics and aging [43]. LPO was an oxidative deterioration process of polyunsaturated fatty acids (PUFA) which is induced by radical and the extent of LPO was assayed by detecting the amount of MDA. TBA reacts with MDA to form a pink complex.

The erythrocyte is considered as an excellent *in vitro* model to study the antioxidant activity since its membrane contains abundant polyunsaturated fatty acids, extremely susceptible to free radical mediated peroxidation [44, 45]. It is quite possible that, the location of the extract components into the membrane bilayer and the resulting restriction on its fluidity might hinder the diffusion of H_2O_2 and its consequent damaging effects [46]. The oxidative stress leads to echinocyte formation because of membrane damage, which in turns leads to change in whole red blood cell structural conformation and its functioning [47]. Protective property of red wine and mango peel extracts against erythrocyte hemolysis through restoration of erythrocytes cell shape and integrity has been reported by Tedesco et al. [30] and Ajila and Prasada Rao [48].

To substantiate the *in vitro* antioxidant activity by CFE and CFA, a new advanced electrochemical method of cyclic voltammetry was conducted. Generally, electrolysis occurs at the surface of electrode in response to a change in potential in order to maintain the surface concentration of redox species at the values required by the Nernst equation. Therefore, the rate of change of potential is directly proportional to the rate of electrolysis and the current. Antioxidants are oxidized at an electrode and enhance anodic potential [49]. In the present study broad peak was observed towards anodic potential, which represents the antioxidant property of CFE and CFA and electrochemical behavior is attributed to the presence of antioxidant molecules such as flavonoids and polyphenols. It is desirable to carry out a thorough study of phenolic profile in order to identify and quantify the active substances. The analysis of phenolic acids and flavonoids using HPLC has been considered for the medicinal plant to evaluate its antioxidant property [32]. The presence of well-known compounds such as gallic acid, quercetin, myricetin and kaempferol in the extracts of this plant clearly indicates the medicinal value of this plant. As we are aware that these compounds are scientifically used as reference standards in the evaluation of antioxidant potentials [50]. Thus, indicates that the polyphenols present in CFE and CFA could be responsible for the beneficial effects. The profound antioxidant effect of CFE over CFA might be due to the presence of the higher amount of bioactive compounds in CFE than CFA.

CONCLUSION

The present study shows that the *C. fistula* L. fruit extracts has remarkable antioxidant and protective activity against induced oxidative damage in erythrocytes. These results confirm the popular use of the plant as therapeutic agent to alleviate disturbances relevant to the oxidative stresses and related diseases. Thus, *C. fistula* L. could be exploited as a valuable source of antioxidant agent for pharmaceutical industry.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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