

Caesalpinia Crista Linn. Induces Protection against DNA and Membrane Damage

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ABSTRACT

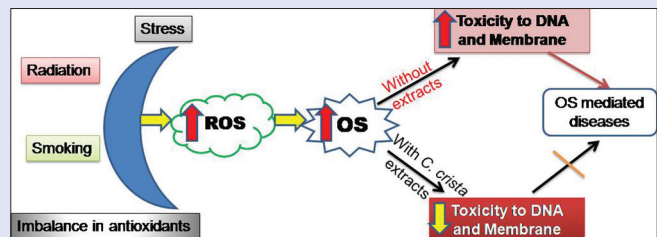
Background: *Caesalpinia crista* is a medicinal herb used to cure various ailments in subtropical and tropical regions of Southeast Asia. **Objective:** The objective of this evaluation of *C. crista* against free radical induced DNA and erythrocyte damage. **Materials and Methods:** The profiles of polyphenol and flavonoid were quantified through reversed-phase high-performance liquid chromatography. Free radical induced DNA and membrane damage were performed using H₂O₂ as oxidative agent. **Results:** The total polyphenol content of *C. crista* leaf ethyl acetate extract (CcEA) was 94.5 ± 3.8 mg/gGAE, CcME (*C. crista* leaf methanol extract) was 52.7 ± 2.8 mg/gGAE, and CcWE (*C. crista* leaf Water extract) was 31.84 ± 1.8 mg/gGAE. Total flavonoid content of CcEA was 60.46 ± 2.3 mg/gQE, CcME was 46.26 ± 1.8 mg/gQE, and CcWE was 20.47 ± 1.1 mg/gQE. The extracts also exhibited good antioxidant activity as confirmed by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), hydroxyl scavenging, reducing power, and total antioxidant assays. Among the three extracts, CcEA and CcME showed better protection against red blood cell (RBC) hemolysis and DNA damage as confirmed by electrophoretic study. Further, Scanning electron micrograph data showed that CcEA revealed the free radical induced structural alterations in RBC. **Conclusion:** These findings suggest that *C. crista* contains bioactive molecules and can inhibit oxidative stress and can be source of further study to use this in herbal medicine.

Key words: Antioxidants, *Caesalpinia crista*, DNA protection, free radicals, membrane damage, oxidative stress

SUMMARY

- ROS are generated under normal biological systems. These ROS generated can be scavenged by endogenous and exogenous cellular mechanisms. Environmental stress, radiation, smoke etc. elevates ROS dramatically. This leads to significant damage to cellular biomolecules like DNA and cell structures. Plants as a large reservoir of drugs for protecting DNA and cell structures from oxidative stress. Polyphenols present in the *C. crista* extracts

acts through several mechanisms to quench free radicals. Extracts exhibited potent antioxidant properties and also protected DNA and cell membrane from oxidative damage. Hence this can be used in herbal medicine for treating oxidative stress mediated diseases.



Abbreviations used: ABTS: 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); CcEA: *C. crista* leaf ethyl acetate extract; CcME: *C. crista* leaf methanol extract; CcWE: *C. crista* leaf Water extract; DPPH: 2,2-diphenyl-1-picrylhydrazyl; GAE: Gallic acid Equivalent; H₂O₂: Hydrogen Peroxide; QE: Quercetin Equivalent; RNS: Reactive Nitrogen Species; ROS: Reactive Oxygen Species; SEM: Scanning Electron Microscope.

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INTRODUCTION

The oxidative stress is the part of normal cellular activities in the metabolic system.^[1] When the oxidative stress is not managed properly, it may result in several disease conditions, namely, neurodegenerative disorders,^[2] metabolic disorders,^[3] and cardiovascular diseases.^[4] The imbalance between antioxidants and free radicals leads to oxidative stress.^[5] Free radicals can be derived from exogenous sources such as radiations, environmental contaminants, stress, and cigarette smoke which cause disturbance in homeostasis between free radicals and antioxidants in our body.^[6] Therefore, excessive production of reactive oxygen species (ROS) can damage cellular biomolecules such as nucleic acids, proteins, lipids, and resulting in pathological conditions such as cancer, neurodegenerative diseases, and other stress-related diseases.^[7-9] DNA and erythrocytes have been extensively used as simple model to study the effect of bioactive molecules on induced DNA and erythrocyte membrane damage.^[10,11] DNA damage is mainly caused by ROS/RNS. ROS/RNS-induced DNA damage results in single- and double-stranded DNA breaks and formation of DNA adduct. DNA damage accumulates in body cells and which further leads to loss of gene expression and loss of functions.^[12]

The present study is planned to use natural product, plant as a source of novel drug candidate for protecting DNA and erythrocyte from oxidative damage. Natural bioactives acts through several mechanisms to quench free radicals, reducing peroxides, chelating transition metals, and also antioxidative defense enzyme system.^[13] The plant derived botanicals, extracts, and their bioactive constituents have spectrum of biological activities. The interest in secondary metabolites such as polyphenols, flavonoids, alkaloids, and terpenoids have considerably

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increased in recent times because of their potential role in preventing oxidative stress-induced diseases.^[14] Different medicinal plants have been used in the preparation of drugs for treating various ailments. Many research findings have shown that plants can play effective role against DNA damage.^[15] Nowadays, there is an intensive research on identifying novel antioxidants from naturally available sources. These antioxidants counteract free radical ions and inhibit oxidative damage caused by free radicals.^[16] Further, it is essential to identify and isolate bioactive compounds which are responsible for therapeutic use.

Caesalpinia crista Linn. belongs to the family *Caesalpinaceae*. It is widely distributed throughout India, subtropical and tropical regions of Southeast Asia.^[17] Traditionally, extracts of *C. crista* are used as stress relaxation health drink by forest dwellers and also used as health tonic for rheumatism and backache.^[18,19] The most of the parts of *C. crista* such as stem, root, leaf, and seed contains cassane and norcassane such as diterpenoids.^[20] The extracts of *C. crista* have varied bioactivities, namely, anti-inflammatory,^[21] antihelmintic activity,^[22] memory enhancer,^[23] free radical scavenging activity,^[24] anticancer,^[25] and hepatoprotective.^[26] The previous experimental study provides the evidence that *C. crista* could able to inhibit the amyloid β (42) aggregation from monomers and oligomers and also able to disaggregate the preformed fibrils.^[27] Furthermore, aqueous extract of *C. crista* could act as anti-inflammatory agent by inhibiting 5-lipoxygenase *in vitro*.^[21] Earlier findings suggest that botanical preparations are nontoxic or very low toxicity toward human cells.^[28] In the current study aimed at elucidating the profiles of polyphenols and flavonoids and determining their role in combating oxidative stress-induced DNA and erythrocyte membrane damage.

MATERIALS AND METHODS

Chemicals

Agarose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Folin-Ciocalteu reagent, and trichloroacetic acid were purchased from Sisco Research Laboratories. λ -DNA was purchased from Bengaluru Genei, India. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, H_2O_2 , 2,2-diphenyl-1-picrylhydrazyl radical, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2-deoxy-D-ribose, TRIS base, ascorbic acid (AA), Na_2EDTA , ethidium bromide (EtBr), caffeic acid, p-coumaric acid, cinnamic acid, ferulic acid, gallic acid, gentisic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, myricetin, daidzein, quercetin, kaempferol and ethylenediaminetetraacetic acid, thiobarbituric acid (TBA) were purchased from Sigma Chemicals Co., (St. Louis, MO, USA). All other chemicals used in the experiment were of analytical grade.

Collection of plant material

C. crista plant was collected in Pachapur Hobli, Belgaum district, Karnataka, India. Lying between 15° 52' North latitudes and 74° 34' East longitude. Collected plant was identified from the botanist (taxonomic deposit number: 417358). The collected plant was washed with running tap water to remove soil and other dust particles followed by washing with distilled water. The leaf sample was separated and shade dried at room temperature (25°C) for 1 week. The dried leaf was finely powdered using domestic mixer and sieved using 18 mm mesh size sieve.

Preparation of solvent extract

Fifteen grams of finely powdered leaf was extracted with 150 mL of Hexane for 6 h, followed by extraction with 150 mL of ethyl acetate for 12 h using Soxhlet apparatus. The extract is filtered using 0.45 mm filter paper; the extract was then concentrated at 40°C. Further, the extract was freeze dried to achieve complete removal of solvent and stored at 4°C for further use. The residue obtained from ethyl acetate was extracted with 150 mL of methanol for 12 h using Soxhlet apparatus and it is filtered using 0.45 mm

filter paper. The extract was then concentrated at 40°C followed by freeze drying for complete removal of solvent and stored at 4°C for further use. The residue obtained from methanol extraction was further extracted with 10 g of dried residue were added into 100 mL of sterile double-distilled water, followed by boiling for 20 min, and the infusion obtained was filtered with sterile filter paper Whatman no 1 under sterile conditions. The extract was then concentrated by freeze drier (DELVAC company, model no 00-12). *C. crista* leaves were extracted in ethyl acetate (CcEA), methanol (CcME), and water (CcWE) these extracts were used for further studies. The extracts obtained were dissolved in dimethyl sulfoxide and water with known concentration for further studies.

Estimation of total polyphenols

The total polyphenols of *C. crista* extracts were determined by Folin-Ciocalteu method.^[29] Extracts of different volumes were mixed with aqueous Na_2CO_3 (1.5 mL) and vortexed followed by adding 500 μL of Folin-Ciocalteu reagent and incubated for 5 min at room temperature. The solution was incubated in the dark for 2 h. The absorbance was measured at 734 nm using ultraviolet (UV)/visible light spectrophotometer. The result was expressed in gallic acid equivalent (GAE) using (0–100 $\mu\text{g}/\text{mL}$) standard curve.

Identification of polyphenols in *Caesalpinia crista* extracts by reversed-phase high-performance liquid chromatography

Detection and quantification of polyphenols in the *C. crista* extracts were done according to the previously described method with slight modifications using reversed-phase performance liquid chromatography (HPLC) system (Agilent Model 1200 series) with diode array detector (operating at 254 nm and 320 nm).^[30] A gradient solvent system was used which consists of solvent A; water: acetic acid (98:2) and solvent B; acetonitrile was used as mobile phase at a flow rate of 1 mL/min for a total run time of 60 min. The gradient elution was done as follows: 100% A to 85% A in 30 min, 85% A to 50% A in 20 min, 50% A to 0% A in 5 min, and 0% A to 100% A in 5 min followed by 4 min post run for column reconditioning. Known concentration of polyphenol standards such as caffeic acid, p-coumaric acid, ferulic acid, gallic acid, gentisic acid, protocatechuic acid, chlorogenic acid was used for identification and quantification of phenolic acids present in the extracts.

Analysis of total flavonoids

The total flavonoid content of *C. crista* extracts was determined by adopting earlier described method.^[31] Quercetin (0–100 $\mu\text{g}/\text{mL}$ in methanol) was used as a standard reference. The standards and the extract solutions (500 μL) were mixed with 0.1 mL of 10% (W/V) aluminum chloride, 0.1 mL of potassium acetate, 1.5 mL of methanol, and 2.8 mL water. For the blank, both potassium acetate and aluminum chloride were added, and their volume was replaced by water. The reaction mixture was incubated for 30 min at room temperature, and the absorbance was taken at 415 nm. The result was expressed as quercetin equivalent using (0–100 $\mu\text{g}/\text{mL}$) standard curve.

Identification of flavonoids in *Caesalpinia crista* extracts by reversed-phase high-performance liquid chromatography

Detection and quantification of flavonoids in the *C. crista* extracts were done following earlier described method with slight modifications.^[32] The standards used were catechin, myricetin, daidzein, quercetin, and kaempferol. The column used was reversed-phase C18 column (4.6 mm \times 250 mm) HPLC system (Agilent-Model 1200 series),

and detector is diode array detector (operating at 260 nm). A gradient solvent system is used which consists of solvent A; water: acetic acid (98:2) and solvent B; methanol: acetic acid (99:1). Gradient elution was linear to 10% B in 5 min, 23% B in 31 min, and 35% B in 43 min, followed by 6 min washing with 100% B, and equilibrated for 6 min at 100%. A with total run time of 55 min. The detection and quantification of flavonoid were done at 260 nm.

Measurement of antioxidant activity

2,2-diphenyl-1-picrylhydrazyl scavenging assay

The effect of *C. crista* extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined according to the method described earlier with minor modification.^[33] A 100 µM solution of DPPH in methanol was prepared. The plant extracts containing different concentrations in terms of GAE (0–25 µgGAE) were mixed with 1 mL of DPPH solution. The mixture was shaken vigorously and left in the dark at room temperature for 20 min. The absorbance of the resulting solution was measured at 517 nm. The control contained all the reagents except extracts. The capacity to scavenge DPPH radical was calculated using the following equation.

The activity is represented as % radical scavenging that is calculated by:

$$\% \text{ DPPH radical scavenging} = (A_c - A) \times 100 / A_c$$

Where "A_c" is the absorbance of the control tube and "A" is the absorbance of the tube with "c" concentration of sample.

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) Radical cation decolorization assay

The antioxidant activity was analyzed by ABTS method described earlier.^[34] 7 mM concentration of ABTS was diluted in water. ABTS radical cations were generated by mixing 7 mM ABTS and 2.45 mM potassium persulfate. The reaction mixture was incubated at room temperature for 12–16 h in dark. After 12–16 h, the reaction mixture was diluted in methanol to obtain an absorbance of 0.70 ± 0.020 at 734 nm. In this study, (0–25 µg/mL) of different concentrations of *C. crista* extracts was mixed with 1000 µl of ABTS solution, and the absorbance was measured using a UV/visible spectrophotometer (Shimadzu) exactly after 5 min of initial mixing. AA was used as positive control. A dose-dependent curve was plotted to calculate the IC₅₀ value.

Ferric reducing antioxidant power assay

The reductive potential of the *C. crista* extracts was determined using standard method.^[35] Different concentrations of *C. crista* extracts (0–200 µg) in 0.5 mL of water was mixed with equal volumes of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide (K₃Fe (CN)₆). The mixture was incubated for 20 min at 50°C. At the end of incubation, an equal volume of 10% trichloroacetic acid was added to the mixture and centrifuged at 3200 ×g for 10 min. The supernatant was mixed with distilled water and 0.1% ferric chloride at 1:1:0.2 (v/v/v), and the absorbance were measured at 700 nm. An increase in the absorbance of the reaction mixture indicates the potential reducing power of the sample. AA was used as a standard for comparison.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was analyzed by deoxyribose method.^[36] Hydroxyl radicals were generated using Fenton reagent (Ascorbate-EDTA-H₂O₂-Fe³⁺ method). The total reaction mixture contains 2-deoxy-2-ribose (2.6 mM), ferric chloride (20 µM), H₂O₂ (500 µM), AA (100 µM), and plant extracts with various concentrations. The total reaction volume made up to 1 mL with phosphate buffer (100 µM, pH 7.4). The reaction mixture was incubated in 37°C for 1 h to initiate the reaction. After incubation period over 0.8 mL of the reaction mixture was added to the 2.8% trichloroacetic acid (1.5 mL), followed by 1% TBA (1 mL), and 0.1% sodium dodecyl sulfate (0.2 mL).

The reaction mixture was then heated at 90°C for 20 min to obtain color, later cooled and 1 mL of double distilled water was added. After the absorbance was read at 532 nm with respective blank sample. The percentage of inhibition was calculated by following equation:

$$(\% \text{ Inhibition}) = (A_0 - [A_1 - A_2]) / A_0 \times 100$$

Where: A₀ is the absorbance of the control without a sample,

A₁ is the absorbance in the presence of the sample and deoxyribose and

A₂ is the absorbance of the sample without deoxyribose.

Total antioxidant capacity

The total antioxidant capacity of extracts was evaluated by the phosphomolybdenum method.^[37] The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acid pH. In this assay, 0.1 mL of extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes containing reaction mixture were incubated at 95°C for 90 min. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol in the place of extract was used as the blank. The antioxidant activity was expressed as the number of gram equivalent of AA. The calibration curve was prepared by mixing AA (10–100 µg/mL) with methanol.

Preparation of erythrocytes

Rat erythrocytes were isolated and stored as per previously described method.^[38] Briefly, blood samples collected were centrifuged (1500 ×g, 5 min) at 4°C, erythrocytes were separated from the plasma and buffy coat and were washed 3 times using 10 volumes of 20 mM phosphate-buffered saline (pH 7.4 PBS). Each time the cell suspension centrifuged at 1500 ×g for 5 min. The supernatant and buffy coats of white cells were carefully removed with each wash. Erythrocytes thus obtained were stored at 4°C and used within 6 h for further studies.

Protective effect on erythrocytes structural morphology

Rat erythrocytes (50 µL) were incubated with and without 50 µg *C. crista* extracts and treated with 100 µL of 200 µM H₂O₂ for 30 min at 37°C. After incubation, the incubate was centrifuged at 1500 ×g for 10 min, and the cell pellets were processed and were fixed in 3% glutaraldehyde on a coverslip.^[39,40] After fixing on the cover slip, the cells were dehydrated in an ascending series of acetone (30%–100%). The dried samples were mounted on a aluminum stub (100–200 Å) using double-sided tape and coated with gold film with a thickness of 10–20 nm using sputter coater (Polaron, E 5000, SEM coating system). The cells were examined under a scanning electron microscope (SEM) (Model No, LEO 425 VP, Electron microscopy LTD, Cambridge, UK).

In vitro assay of inhibition of rat erythrocyte hemolysis

The inhibition of rat erythrocyte hemolysis by the *C. crista* extracts was determined as per previously described method with slight modifications.^[41] The rat erythrocyte hemolysis was performed with hydrogen peroxide as free radical initiator. To 200 µL of 10% (v/v) suspension of erythrocytes in PBS, *C. crista* extracts with different concentrations (0–200 µg) were added. To this, 100 µL of 500 µM H₂O₂ (in PBS pH 7.4) was added. The reaction mixture was incubated at 37°C for 30 min and was centrifuged at 2000 ×g for 10 min. The absorbance of the resulting supernatant was measured at 410 nm by taking 200 µL of reaction mixture with 800 µL PBS to determine the hemolysis. Likewise, the erythrocytes were treated with hydrogen peroxide and without inhibitors (extracts) to obtain a complete hemolysis. The absorbance of the supernatant was measured at the same condition. Percentage of hemolysis was calculated by taking hemolysis caused by 200 µM H₂O₂ as 100%. The IC₅₀ values were calculated, and the concentration required for the inhibition of 50% hemolysis.

Prevention of λ -DNA damage by *Caesalpinia crista* extracts

Oxidative λ -DNA damage was prevented by *C. crista* leaf extract and it was assayed.^[42] λ -DNA (0.5 μ g) with and without extracts (50 μ g) was incubated with Fenton reagent (1 mM FeSO₄, 25 mM H₂O₂ in tris buffer 10 mM, pH 7.4) in a final reaction volume of 30 μ L for 1 h at 37°C. Relative difference between oxidized and native DNA were analyzed on 1% agarose gel prepared in tris-acetate-EDTA buffer (pH 8.5) at 50 V for 3 h at room temperature. The gel was documented (Uvitec Company, software platinum 1D, UK), and the band intensity was determined.

Ethidium bromide binding to DNA by fluorescence analysis

Protective effect of *C. crista* extracts against oxidative λ -DNA damage was analyzed by adopting previously described method.^[10] The change in fluorescence of EtBr bound to DNA was measured. The assay was carried out by incubating λ -DNA (1 μ g) with 1 mM FeSO₄, 25 mM H₂O₂ in 10 mM Tris-HCl (pH 7.4) for 1 h. The protection of oxidative λ -DNA damage was analyzed in the presence of 50 μ g of *C. crista* extracts for 1 h. The samples thus prepared were mixed with 5 μ g of EtBr, and the fluorescence was recorded at excitation at 535 nm and emission at 600 nm.

Melting temperature studies

The effect of *C. crista* extracts on the λ -DNA integrity was measured by thermal denaturation studies using Spectrophotometer (Ultrospec, 4300 probe) equipped with thermo-programmer and data processor (Amersham Pharmacia Biotech, Hong Kong). λ -DNA (1 μ g) was incubated with 1 mM FeSO₄, 25 mM H₂O₂ in 10 mM Tris-HCl (pH 7.4) for 1 h for monitoring the oxidative λ -DNA damage in the presence of extract (50 μ g) for 1 h. The melting profiles (melting temperature [T_m]) of λ -DNA were recorded at different temperatures ranging from 25°C to 95°C. The T_m value was determined graphically from the absorbance versus temperature plots.

Statistical analysis

Three independent experiments were conducted in triplicate, and the data were reported as mean \pm standard deviation. Determined using the statistical program SPSS (Armonk, NY: IBM Corp) for Windows, version 10.0, with level of significance of $P < 0.05$.

RESULTS

Polyphenol content in *Caesalpinia crista* extracts and identification of phenolic acids by reversed-phase high-performance liquid chromatography

The amount of total polyphenols present in various extracts was measured by Folin-Ciocalteu method. Quantification was done using standard graph, we found that CcEA 94.5 \pm 3.8 mg/gGAE, CcME 52.7 \pm 2.8 mg/gGAE, and CcWE 31.84 \pm 1.1 mg/gGAE [Figure 1]. The major phenolic acids present in the extracts were identified and quantified using reversed-phase HPLC (RP-HPLC) with standards. The different phenolics in *C. crista* in the following decreasing order: CcEA; gentisic acid > ferulic acid > p coumaric acid whereas in CcME; gallic acid > p coumaric acid > ferulic acid, and CcWE; gallic acid > caffeic acid > ferulic acid, respectively [Table 1].

Flavonoids content in *Caesalpinia crista* extracts and identification of flavonoids by reversed-phase high-performance liquid chromatography

Total flavonoid content in the extracts expressed as quercetin equivalent [Figure 1]. The results showed that CcEA in terms of quercetin equivalent content was 60.46 \pm 2.3 mg/g, CcME was 46.26 \pm 1.8 mg/g,

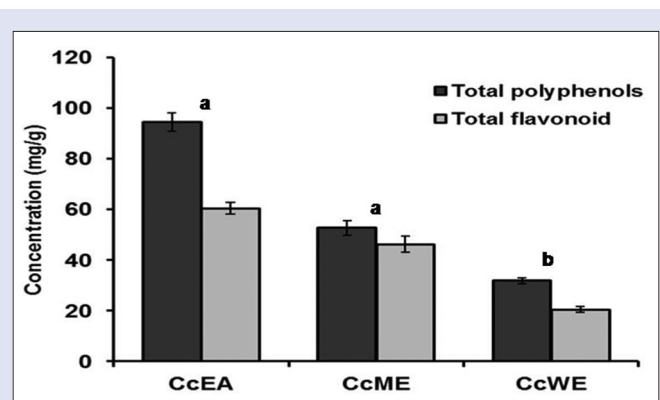


Figure 1: Total polyphenol and flavonoid content of leaf extracts of *Caesalpinia crista*. Extracts of *Caesalpinia crista* were quantitated for total polyphenol and flavonoid content. The values were expressed mg/g as gallic acid equivalent and mg/g as QE, respectively. The values are mean of three replicates \pm standard deviation. Means followed by letters (a and b) are significantly different ($P < 0.05$)

Table 1: Phenolics and flavonoid profiles of *Caesalpinia crista* extracts

Constituents	CcEA (mg/g)	CcME (mg/g)	CcWE (mg/g)
Phenolics			
Gallic acid	-	0.66 \pm 0.004	2.31 \pm 0.012
Gentisic acid	2.52 \pm 0.34	-	-
Chlorogenic acid	-	-	-
Caffeic acid	0.137 \pm 0.062	-	0.666 \pm 0.008
p-coumaric acid	0.357 \pm 0.11	0.423 \pm 0.06	-
Ferulic acid	0.511 \pm 0.13	0.398 \pm 0.005	0.558 \pm 0.04
Cinnamic acid	0.282 \pm 0.012	-	-
Flavonoids			
Catechin	-	-	5.49 \pm 0.8
Rutin	1.63 \pm 0.05	0.42 \pm 0.05	0.80 \pm 0.009
Quercetin	-	-	5.84 \pm 0.09
Myricetin	0.76 \pm 0.005	-	-
Genistin	1.62 \pm 0.11	0.32 \pm 0.06	-
Kaempferol	0.58 \pm 0.06	-	-

Different extracts of *C. crista* were subjected to HPLC analysis along with standards and the peak area, concentration of polyphenols and flavonoids were calculated, and expressed as mg/g. Values provided are mean of three replicates \pm SD. CcEA: *C. crista* leaf ethyl acetate extract; CcME: *C. crista* leaf methanol extract; CcWE: *C. crista* leaf water extract; *C. crista*: *Caesalpinia crista*; SD: Standard deviation; HPLC: High-performance liquid chromatography

and CcWE was 20.47 \pm 1.1 mg/g. Among the three extracts analyzed, CcEA, CcME has high flavonoid content when compared to CcWE. The different flavonoids in *C. crista* in the following decreasing order CcEA; rutin > genistin > myricetin in CcME; rutin > genistin whereas, in CcWE; quercetin > catechin > rutin were major flavonoids present in the extracts, respectively [Table 1].

2,2-diphenyl-1-picrylhydrazyl free radical scavenging effect of *Caesalpinia crista* extracts

The leaf extracts were able to reduce the stable DPPH radical into yellow-colored product. Extracts showed dose-dependent scavenging activity of DPPH radicals. The IC₅₀ values CcEA was 23.9 \pm 1.8 μ gGAE, CcME was 30.3 \pm 2.2 μ gGAE, and CcWE was 55.9 \pm 3.1 μ gGAE and standard AA was 8.36 \pm 0.9 μ g. The activity of the extracts was compared with the standard AA [Figure 2a].

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation decolorization assay

The antioxidant activity of the phenolic compounds was analyzed by ABTS radical method. The radical ABTS by reacting with oxidizing agent potassium persulphate it becomes dark blue color. The antioxidants present in the extracts as strong hydrogen donor, in which the decrease in the absorbance of the final solution was measured at 734 nm. The antioxidant activity of extracts in terms of IC_{50} Values CcEA $38.9 \pm 1.8 \mu\text{g}$, CcME was $54.4 \pm 2.1 \mu\text{g}$, and CcWE was $98.4 \pm 3.8 \mu\text{g}$ and in standard AA was $16.1 \pm 0.8 \mu\text{g}$. Average IC_{50} values determined by ABTS assay were almost lower as compared to the values determined by DPPH assay [Figure 2b].

Hydroxyl radical scavenging activity of *Caesalpinia crista* extracts

A hydroxyl radical is one of the major found reactive species in biological systems. The antioxidant effects of *C. crista* extracts was examined by their ability to counteract free radicals formed. Hydroxyl radical scavenging ability of extracts was analyzed using 2-deoxy-2-ribose method. The results indicate that IC_{50} values CcEA was $89.7 \pm 3.1 \mu\text{g}$; CcME was $76.8 \pm 2.4 \mu\text{g/mL}$ and CcWE was $220 \pm 5.8 \mu\text{g/mL}$ and standard AA was $72.17 \pm 2.4 \mu\text{g/mL}$ [Figure 2c]. This indicates that ability of the extracts to participate with deoxyribose and to scavenge hydroxyl radicals. This decreases chromogen product formation which reveals hydroxyl radical scavenging ability.

Ferric reducing antioxidant power assay

Reducing capacity of the extracts indicates the potential of antioxidants present. The results of this assay indicate that there is a gradual increase in absorbance at 700 nm in a concentration-dependent manner. The trend of different extracts was in the decreasing order CcEA > CcME > CcWE [Figure 2d]. This result gives an insight that the extracts have the ability to scavenge various free radicals.

Total antioxidant content

Phosphomolybdenum method involves the reduction of Mo (VI) to Mo (V), and this principle is used to estimate total antioxidant content

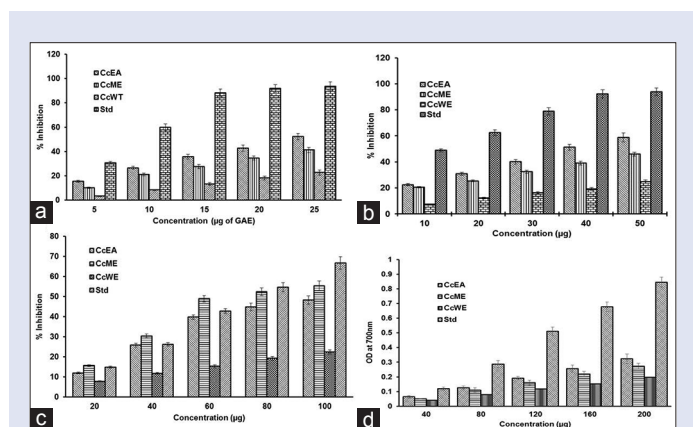


Figure 2: Antioxidant activities of *Caesalpinia crista* extracts. (a) Free radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl, (b) 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), (c) hydroxyl radical scavenging activity, (d) ferric reducing antioxidant power of *C. crista* leaf ethyl acetate extract, *C. crista* leaf methanol extract, and CcWE of *Caesalpinia crista*. Ascorbic acid was used as positive control. The data points represent means \pm standard deviation of three independent determinations

spectrophotometrically. The formation of green-colored Mo (V) end product after prolonged incubation, and the reducing capacity of the antioxidants in the extract were measured at 695 nm. The total antioxidant activity of the extracts found to be decrease in the order of CcEA > CcME > CcWE [Figure 3]. AA was used as a reference standard. The antioxidant activity of extracts is attributed to polyphenols and other antioxidant molecules present in the extracts.

Protective effect *Caesalpinia crista* extracts on red blood cell structural morphology

Protective effects of *C. crista* extracts on oxidative damage were analyzed in the SEM *in vitro* SEM of erythrocytes incubated with H_2O_2 alone and extracts [Figure 4]. Control erythrocytes appeared as classic discocytes while exposure to H_2O_2 resulted in a significant change in the cell shape and distinct echinocyte formation. The changes in the morphology induced by oxidative system were prevented when the cells were treated with extracts. When compounds inserts into the inner layer, stomatocytes are formed. However, speculated-shaped echinocytes are formed when it locates into the outer layer. Thus, result in the present study indicates that extracts prevent the morphological changes induced by oxidants in red blood cell.

Inhibition of rat erythrocytes hemolysis

Oxidants lyse the erythrocyte membrane and leaches out the hemoglobin pigment into the medium. In the current study, membrane damage or hemolysis was induced using H_2O_2 . The protective effect against oxidative damage was studied using *C. crista* extracts. Oxidants lyse the erythrocytes membrane and lead to release of hemoglobin and found in the medium. The medium turns into red, and it was measured at 410 nm. The different extracts inhibited hemolysis in a dose-dependent manner. All the extracts showed corresponding inhibitory activities against membrane damage. The IC_{50} values for the corresponding extracts CcEA, CcME, and CcWE, respectively are $88.56 \pm 1.88 \mu\text{g}$, $112.4 \pm 3.88 \mu\text{g}$, and $164.28 \pm 3.24 \mu\text{g}$ [Figure 5].

Inhibition of Fe^{2+} induced λ -DNA damage by *Caesalpinia crista* extract

In the current study, the *C. crista* extracts showed DNA protection caused by the radicals generated by Fenton reaction. Figure 6 results

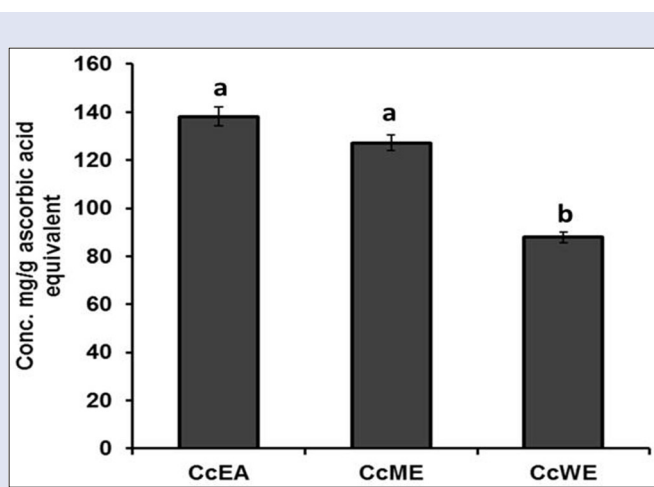


Figure 3: Total antioxidant content of *Caesalpinia crista* extracts. Data represent mean \pm standard deviation, letter a, b indicates statistical difference between the extracts at $P < 0.05$

indicate that λ -DNA in the presence of radicals and with extracts showed increase in the band intensity. Where λ -DNA with radicals and without extracts showed no band in 1% agarose gel. Further, treating λ -DNA with extract alone did not alter DNA integrity which suggests the extract itself is not toxic to λ -DNA. The DNA damage protection activity of *C. crista* is ascribed to polyphenols and antioxidant molecules that are present in the extract.

Ethidium bromide Binding studies

Free radical causes damage to DNA and base modifications. The measurement of λ -DNA damage by $\text{FeSO}_4/\text{H}_2\text{O}_2$ is done by noting the

changes in fluorescence of EtBr on binding to λ -DNA in the presence and absence of extracts. EtBr fluorescence for intact λ -DNA was 51.51, whereas 27.65 for λ -DNA treated with FeSO_4 in the presence of H_2O_2 . The significant decrease in the fluorescence intensity in the case of λ -DNA treated with FeSO_4 and H_2O_2 was due to the damage of DNA by hydroxyl radicals generated. However, the fluorescence intensity for DNA in the presence of $\text{FeSO}_4/\text{H}_2\text{O}_2$, and *C. crista* extracts was 47.7, 41.98 and 45.96 for CcEA, CcME, and CcWE, respectively [Figure 7]. The higher fluorescence intensity observed may be attributed to the free radical scavenging activity of polyphenols and antioxidant molecules in extracts.

Melting temperature studies

The T_m study provides a structure and dynamics of DNA. The T_m of λ -DNA was 70.5°C , whereas it was 54.8°C , when it was treated with $\text{FeSO}_4/\text{H}_2\text{O}_2$ [Table 2]. Low T_m in the case of λ -DNA treated with $\text{FeSO}_4/\text{H}_2\text{O}_2$ was due to the damage of DNA in the presence of hydroxyl radicals. Whereas, T_m value for DNA in the presence of CcEA with

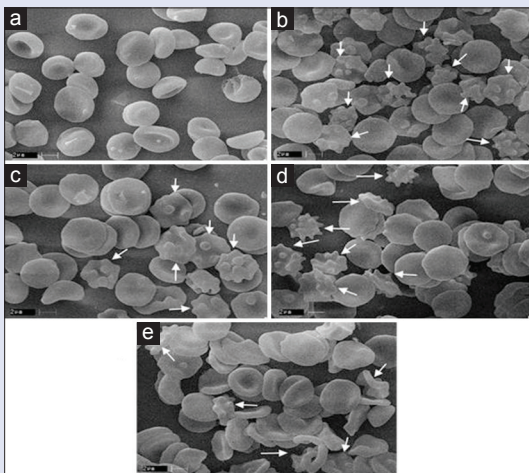


Figure 4: Scanning electron micrograph of normal erythrocytes and protective effect *Caesalpinia crista* against H_2O_2 induced oxidative damage on red blood cell. (a) Red blood cell + H_2O_2 ; (b) red blood cell + H_2O_2 ; (c) red blood cell + H_2O_2 + *C. crista* leaf ethyl acetate extract; (d) red blood cell + H_2O_2 + *C. crista* leaf methanol extract; (e) red blood cell + H_2O_2 + CcWE. $\times 10000$

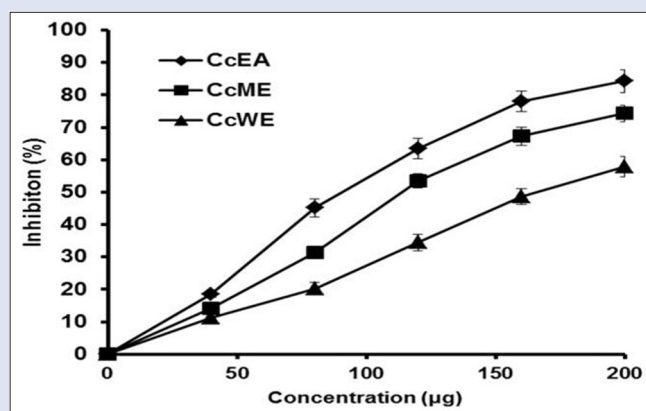


Figure 5: *In vitro* protective effects of *Caesalpinia crista* extracts against H_2O_2 induced hemolysis of rat erythrocytes. Protective effects of *Caesalpinia crista* extracts against H_2O_2 induced hemolysis of rat erythrocytes. The data points represent means \pm standard deviation of three independent determinations

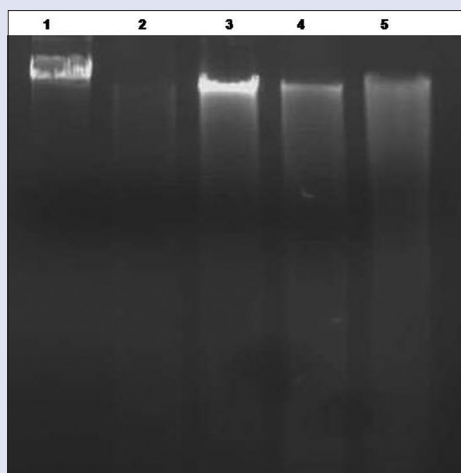


Figure 6: Gel electrophoresis image of λ -DNA damage inhibition of *Caesalpinia crista* extracts. Gel electrophoresis image of λ -DNA damage inhibition by *Caesalpinia crista* extracts after 1 h of incubation of reaction mixture. Lane 1: 0.5 μg DNA alone; Lane 2: 0.5 μg DNA + 1 mM FeSO_4 + 25 mM H_2O_2 ; Lane 3: 0.5 μg DNA + 1 mM FeSO_4 + 25 mM H_2O_2 + 50 μg *C. crista* leaf ethyl acetate extract; Lane 4: 0.5 μg DNA + 1 mM FeSO_4 + 25 mM H_2O_2 + 50 μg CcME; Lane 5: 0.5 μg DNA + 1 mM FeSO_4 + 25 mM H_2O_2 + 50 μg CcWE

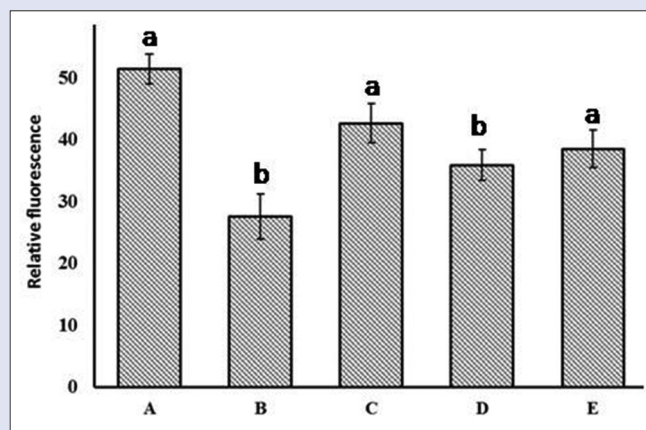


Figure 7: Ethidium bromide binding to DNA. (A) λ -DNA; (B) λ -DNA + FeSO_4 + H_2O_2 ; (C) λ -DNA + *C. crista* leaf ethyl acetate extract + FeSO_4 + H_2O_2 ; (D) λ -DNA + *C. crista* leaf methanol extract + FeSO_4 + H_2O_2 ; (E) λ -DNA + CcWE + FeSO_4 + H_2O_2 . Values are mean \pm standard deviation ($n = 3$), means with different letters are significantly different at $P < 0.05$

Table 2: Melting temperature of DNA treated with *Caesalpinia crista* extracts

Sample	T _m value
DNA	70.5°C±1.3 ^a
DNA + FeSO ₄ + H ₂ O ₂	54.8°C±2.1 ^a
DNA + CcEA + FeSO ₄ + H ₂ O ₂	71.6°C±1.9 ^c
DNA + CcME + FeSO ₄ + H ₂ O ₂	68.3°C±1.1 ^c
DNA + CcWE + FeSO ₄ + H ₂ O ₂	67.3°C±1.4 ^b

Values are mean±SD ($n=3$), means with different letters within a column are significantly different at $P<0.05$. CcEA: *C. crista* leaf ethyl acetate extract; CcME: *C. crista* leaf methanol extract; CcWE: *C. crista* leaf water extract; T_m: Melting temperature; *C. crista*: *Caesalpinia crista*; SD: Standard deviation

FeSO₄/H₂O₂ was 71.6°C, CcME with FeSO₄/H₂O₂ was 68.3°C, and CcWE with FeSO₄/H₂O₂ was 67.3°C. The higher T_m of λ-DNA in the presence of extracts indicates that extracts prevented free radical-induced DNA damage. The mechanism of prevention may be due to the scavenging of free radicals by the polyphenols and antioxidants present in the extracts.

DISCUSSION

The botanicals may be derived from various plant parts such as roots, stem, leaves, bark, and seed. The bioactive molecules have ability to target many pathological conditions.^[43] The free radicals are the cause of many disease conditions. The previous studies suggests that free radical scavenging capability of bioactive molecules play an important role in the prevention of chronic diseases such as cardiovascular disorders, metabolic disorders, neurodegenerative diseases.^[13,44] The free radicals causes DNA and membrane damage. Earlier research findings indicate that the electron donating capacity of bioactive molecules coupled with antioxidant activity helps to prevent oxidative stress.^[45,46] Among the free radical species, hydroxyl radical is found in higher concentration in the body. The antioxidants present in the extracts have the ability to quench hydroxyl radicals and prevent lipid peroxide formation.^[47] Hence, the bioactives present in the extracts have predominant role in minimizing oxidative stress and its toxicity effect. Earlier findings from the researchers found that hydroalcoholic and methanolic extracts of *C. crista* have possess rich antioxidant activity.^[26,48] In our current study, the free radical scavenging capability of *C. crista* extracts was evaluated using various chemical assays, and the results found that CcEA and CcME showed to have greater antioxidant activity.

Oxidant damages the cell integrity and leads to disruption in cell rigidity, shape, and leads to the formation of echinocytes. Thus, formed cells eventually affect the functioning of erythrocytes.^[49] When oxidant or foreign molecules enter into the erythrocytes leads to changes in the shape and integrity of the membrane due to discrepancy of two monolayers of the red cell membrane.^[50,51] Oxidative stress generates free radicals, causes DNA damage and it implicates for various generative diseases. The presence of antioxidant molecule in the medicinal plants is reported to have protective function against DNA damage.^[52]

Oxidants produced may lead to changes in the erythrocyte membrane structure as exhibited by decreased cytoskeletal protein content and also changes in the morphology and function.^[53] However, ample number of research findings indicates that many medicinal plants can play cytoprotective role.^[10,51,54] Sarkar *et al.* showed that methanolic extract of *C. crista* showed protection against Fe(2+)-mediated oxidative DNA damage.^[26] From our results, it is evident that *C. crista* extracts such as CcEA and CcME were effective in reducing the oxidative stress-induced membrane damage and it was confirmed by SEM [Figure 4].

The drugs from botanicals have increased potency and lesser adverse effects and have become the potential therapeutic interventions for prevention and treatment of many ailments.^[55] The findings of this indicate that CcEA and CcME have a significant effect on preventing

DNA and membrane damage. The correlation between antioxidant property and protective role of *C. crista* against free radical-induced DNA and membrane damage suggests the contribution of polyphenols and other antioxidants. Hence, the *C. crista* extracts may be used as safe antioxidant source and can be further developed into nutraceutical product for many health benefits.

CONCLUSION

The present study shows that *C. crista* extracts rich in polyphenols. The extracts CcEA and CcME exhibited rich antioxidant properties. It also exhibited protective role against oxidative stress-induced DNA and erythrocyte membrane damage. As the *C. crista* extracts showed various antioxidant properties in many different systems. It could be used as a source of therapeutic or nutraceutical product to be incorporated in herbal medicine.

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Conflicts of interest

There are no conflicts of interest.

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