



Research article

The protective and antioxidant effects of *Hygrophila schulli* seeds on oxidative damage of DNA and RBC cellular membraneMd. Shoriful Islam^a, Mst. Shahnaj Parvin^b, Md. Ekramul Islam^{b,*}^a Dept. of Pharmacy, Islamic University, Kushtia, 7003, Bangladesh^b Dept. of Pharmacy, University of Rajshahi, Rajshahi, 6205, Bangladesh

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ABSTRACT

Medicinal plants are sources of antioxidant which may protect the body against oxidative stress related diseases and can be used as human food supplements. In this investigation, seeds of *Hygrophila schulli* (M. R. Almeida & S. M. Almeida) (Fam.-Acanthaceae), a herbaceous plant well known for its medicinal properties, has been examined for antioxidant activity of crude methanolic extract (CME) and its fraction using *in vitro* and *in vivo* assay as well as their protective activity against oxidative damage of DNA and RBC. Total phenolic and flavonoid content have also been estimated using the aluminum chloride colorimetric and Folin-Ciocalteu method. Among the different fractions of CME, Ethyl acetate fraction (EAF) had higher antioxidant activity *in vitro* assay and was selected for *in vivo* antioxidant activity in cadmium intoxicated mice. The EAF showed a significant ($p < 0.05$) increase in serum catalase and SOD activity compared to the control group. TBARS levels were restored to 17.42 and 19.19 nmol/mg protein, respectively, after treatment with EAF and standard ascorbic acid (AA); compared to the normal group (14.96 nmol/mg protein). Similarly, levels of albumin, bilirubin, uric acid, and alkaline phosphatase were also brought back to normal levels. EAF's protective role against oxidative damage of DNA has shown a significant reduction in destroying of nicked DNA. RBC as a target of oxidation by H₂O₂ and HOCl, EAF showed inhibition of oxidation in a concentration-dependent manner, compared to standard gallic acid. In this study, we confirmed that EAF could scavenge reactive oxygen species (ROS) thus preventing DNA strand scission and the extract can be used as a functional food or nutraceutical product for health benefits.

1. Introduction

Medicinal plants are a source of a wide variety of bioactive compounds which act as antioxidants and protect the human body against different oxidative stress related diseases, and are being used as food supplements. Among various medicinal plants, *Hygrophila schulli* (M. R. Almeida & S. M. Almeida) (English- Marsh Barbel; Local Bangla- Shulamardan; family-Acanthaceae), has gained much importance in the recent past [1, 2]. The species, a thorny sub-herb among which many grow as aquatic plants [3], is distributed throughout the Indian subcontinent, Myanmar and tropical Africa. In traditional medicine of Ayurveda, the extracts of the plant are used for treatment of various ailments like diabetes and dysentery [4]. Moreover, it has been reported that since ancient times, along with the whole plant, roots, seeds and ashes are commonly used in traditional medicine as antibacterial, diuretic, demulcent, stomachic and for the treatment of jaundice, rheumatism, tonic, hypnotic, diarrhea, dysentery, urinary calculi, urinary discharge,

anti-inflammatory, joint pain, biliousness, eye problems, abdominal troubles, anemia, cough, arthritis and gastric disorders [5]. The species has been placed under 'least concern' category (IUCN 2011).

Various types of chemical compounds are confirmed and isolated by scientific reports from this plant like saponins, alkaloids, steroids, tannins, flavonoids, phenols, and triterpenoids [6]. Similarly, the literature review revealed some notable pharmacological effects like anti-nociceptive, anti-tumor, antioxidant, hepatoprotective, hypoglycemic, haematinic, diuretic, free radical scavenging, anthelmintic, anti-inflammatory, antipyretic, anabolic, and androgenic activities [7]. It has also been reported that the free radical scavenging activity of *H. schulli* seed extract is comparable to that of reference standard antioxidants. In addition, it could prevent oxidative damage of various biomolecules, and provide notable protection against oxidative damage [8].

Plants are the vital sources of various bioactive substances [9] and medicinal plants still now to be the main resource against many diseases [10]. In the literature review, there are some scientific reports about

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seeds but no reports on protection against oxidative damage to DNA and RBC. Regular cellular functions, like oxidative phosphorylation and lipid peroxidation, generate ROS that can induce oxidative damage to DNA. It is known that about ten thousand oxidation reactions every day affect the DNA in each of our cells [11]. In the literature review, there are some scientific report on *H. schulli* seeds but no report on the protection of oxidative damage of DNA and Erythrocytes. *H. schulli* seeds are believed to be effective and have been utilized in many systems of traditional medicine's formula by various ethnic communities [12]. Therefore, the goal of this project was to explore the protective activity against oxidative damage on DNA and erythrocytes with antioxidant activities for future investigation toward the finding a new, potent and safe antioxidant compound as well as the possibility of using extracts of this species as potential food supplements.

2. Materials and methods

2.1. Plant material

The seeds of *H. schulli* from matured fallen were collected from the ground of Chapainawabganj, Bangladesh in December 2016. It was taxonomically identified and preserved by the Dept. of Botany, University of Rajshahi, Bangladesh with specification number 102019-37.

2.2. Preparation of extract

Collected air-dried seeds without removing the seeds coat were ground into a coarse powder using a grinder machine and the coarse powder (400 g) was soaked in chilled methanol (1:4; w/v) and kept in a cold room (4 °C) for 10 days with occasional stirring. The mixture was then filtered through cotton and Whatman No. 1 filter paper and concentrated in a rotary evaporator (model C-303; Sibata, Japan) at 150 mbar pressure and 40 °C temperature. The process was repeated two times. The concentrated crude methanolic extract (CME) was fractionated with the organic solvent of different potentials into petroleum ether fraction (PEF), chloroform fraction (CHF), ethyl acetate fraction (EAF), and the rest of the residue left was designated as aqueous fraction (AQF).

2.3. Experimental animal model

Healthy albino male mice (24–31 g) were procured from the branch of animal research, Dept. of Pharmacy, Jahangirnagar University, Dhaka, Bangladesh. The animals were kept under 12 h light/dark cycles at 25 °C in conventional plastic cages and nourished with regular pellet diet and tap water. All the mice were allowed 1 week to acclimate to the housing conditions before starting the experiment. The experiment on mice was conducted under the rules of the Institute of Biological Sciences (IBS), University of Rajshahi. The experimental procedure was authorized by the Institutional Animal, Medical Ethics, Biosafety, and Biosecurity Committee (IAMEBBC) at the IBS, University of Rajshahi. (Approval memo no-82/320/IAMEBBC/IBSc).

2.4. Phytochemical analysis

2.4.1. Total phenolic content (TPC)

Folin-Ciocalteu colorimetric method described by Meda et al. [13] was used to determine the phenolic compounds in CME and fractions. Briefly, an aliquot of 1 ml of CME/fractions solution (50 µg/ml) was mixed with 2.5 ml of Folin-Ciocalteu reagent. Then, 14% of sodium carbonate solution (0.3 ml) was added to the above mixture and incubated at room temperature for 20 min. Afterwards, the absorbance was taken at 760 nm in a spectrophotometer (Shimadzu UV Mini-1240, Japan). A calibration curve for gallic acid in the range of 10–200 µg/ml was prepared in the same manner and results were expressed as milligrams of gallic acid equivalent per gram (mg of GAE/g) of extract.

2.4.2. Total flavonoid content (TFC)

The total flavonoid was determined according to the aluminum chloride colorimetric method [14]. Briefly, 0.5 ml CME/fractions solution (200 µg/ml) was mixed with 5% NaNO₂ (0.15 ml) solution in a test tube. After 5 min of incubation, 10% AlCl₃ (0.3 ml), 1 ml NaOH (1 mM), and 550 µl distilled water were added. The same procedure was followed for the standard solution of quercetin and calibration curve was constructed. The absorbance was read at 510 nm and the results expressed as milligram of quercetin equivalent per gram (mg of QE/g) of extract.

2.5. In vitro antioxidant activity

2.5.1. DPPH radicals scavenging activity

The radical scavenging activity of the CME/fractions was determined as described by N. Jahan et al [15]. CME and various fractions at 5–100 µg/ml were mixed separately with 1 ml of methanol solution of DPPH (0.008% w/v) The mixture was homogenized and incubated in dark for 20 min, and absorbance was measured at 517 nm. Ascorbic acid was used as the antioxidant standards. The percentage of free radical scavenging was estimated using the following equation-

$$\% \text{ Scavenging} = \frac{\text{The absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Concentration providing 50% inhibition (IC₅₀) is calculated from the graph plotted for scavenging percentage against the sample concentration.

2.5.2. Hydrogen peroxide scavenging activity

The ability of the CME/fractions to scavenge hydrogen peroxide was assessed by the replacement titration method [16]. Reaction medium containing 0.1 mM H₂O₂, 3% ammonium molybdate, 2M H₂SO₄, 1.8M KI and CME/fractions (200 µg/ml) were mixed and the solution was titrated with Na₂S₂O₃ (5.09 mM) until the yellow color vanished. Scavenging activity was calculated by the following equation.

$$\% \text{ Scavenging} = \frac{\text{The volume of Na}_2\text{S}_2\text{O}_3 \text{ for control} - \text{Volume of Na}_2\text{S}_2\text{O}_3 \text{ for a sample}}{\text{The volume of Na}_2\text{S}_2\text{O}_3 \text{ for control}} \times 100$$

2.5.3. Reducing power activity

The assay was based on the reduction of colorless potassium ferricyanide [K₃Fe (CN)₆] to its blue ferrous-colored form due to electrons donation by antioxidants. The ferric reducing power was expressed in terms of absorbance of the sample [17].

2.5.4. Total antioxidant capacity

The total antioxidant capacity of CME/fractions was determined by the spectrophotometric phosphomolybdenum method [18]. Precisely, solutions of the extracts (2 ml, 5–100 µg/ml) in methanol were incubated with 3 ml of the reaction composition (containing 0.6M sulfuric acid, 28 mM sodium phosphate, and 1% w/v ammonium molybdate) and incubated at 95 °C for 90 min. Then the absorbance of the cooled aqueous solution of each was measured at 695 nm against a blank.

2.6. In vivo antioxidant activity

2.6.1. Animal grouping

In vivo antioxidant activity was determined by using the cadmium (Cd)-induced hepatotoxicity of the mice model. After seven days of acclimatization, mice were randomly divided into seven groups of five mice each. Group-I served as normal control and group-II received 8 mg/kg body weight (b.w.) of Cd, Group-III–V were simultaneously treated with 8 mg/kg body weight (b.w.) of each and 100, 200, and 400 mg/kg of

b.w of the EAF, respectively. Mice in group-VI treated with CME (400 mg/kg) and groups-VII treated with ascorbic acid (100 mg/kg) of b.w along with Cd 8 mg/kg b.w.

2.6.2. Preparation of serum and tissue homogenates

At the end of 7 days of treatment, all the animals were sacrificed and blood serum was collected by centrifugation (Biobase BKC-TL4IV, China) at 3000 rpm for 10 min at 4 °C temperature. The serum was used immediately for the biochemical analysis. The animals were quickly dissected and the liver and brain removed. Then these organs were homogenized and supernatants obtained after centrifugation were stored frozen overnight and used for analysis of antioxidant enzyme activities (SOD and CAT), and concentration of thiobarbituric acid reactive substances (TBARS).

2.6.3. Estimation of biochemical parameters

The concentration of albumin, total bilirubin, uric acid and alkaline phosphatase were determined in accordance with the principles described by Doumas et al., [19], Weigl et al., [20] and Fossati et al., [21], Akpınar [22], respectively.

2.6.4. Antioxidant enzymatic activities

Superoxide dismutase (SOD) activity was determined at 560 nm as described by Marklund et al., [23] and the enzyme activity was expressed as unit (U)/mg Tissue.

Another antioxidant enzyme, catalase (CAT) activity was measured at 240 nm by the Aebi method [24], and the values were expressed as $\mu\text{mol H}_2\text{O}_2$ decomposed/min/mg of tissue.

2.6.5. Thiobarbituric acid reactive substances (TBARS) assay

The mixture of tissue homogenate (10%, w/v) with SDS (8.1%, w/v) was vortexed. After 5 min, acetic acid (20%) and TBA (0.8, w/v) was added to the mixture and heated at 95 °C for 60 min. Then the mixture was centrifuged at 4000 rpm for 15 min at room temperature. Finally, the colored layer was measured at 532 nm and the concentration of MDA was calculated using the following equation [25].

$$\text{Concentration of MDA (mM)} = \frac{\text{Absorbance}(532) - \text{Absorbance}(600)}{155}$$

2.7. Protection of human erythrocytes against oxidative damage

2.7.1. Protection of erythrocytes

Heparinized blood samples were obtained from healthy volunteers via venapuncture and erythrocytes were isolated by centrifugation at 2500 rpm for 10 min, washed two times with PBS and finally re-suspended using the same buffer to a hematocrit level of 5%. The erythrocytes suspension was then incubated at 37 °C in the presence or absence of CME/fractions (CHF, EAF) at different concentration with 100 $\mu\text{M H}_2\text{O}_2$ or 125 $\mu\text{M HOCl}$ (final concentrations). CME/fractions were added to the erythrocytes suspension 10 min prior to addition of the oxidizing agents. The erythrocyte suspension incubated with PBS served as control. After 15 min of incubation the absorbance of the supernatant was taken at 540 nm and hemolysis percentage was calculated using the formula [26] –

$$\% \text{ Inhibition of hemolysis} = \frac{\text{The absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.7.2. Lipid peroxidation

Erythrocyte suspension (25%) was incubated 30 min at 37 °C with CME/fractions and gallic acid as standard, separately. Then, H_2O_2 (20 mM), PBS, SDS, trichloroacetic acid (0.75 ml of 28%), and TBA (0.5 ml, 1%) was added to the reaction mixtures were kept at 90 °C for 30 min. The absorbance of the resulting pink-colored layer was taken at 532 nm

with centrifugation [25]. The percent inhibition was calculated using the following formula:

$$\% \text{ Inhibition of lipid peroxidation} = \frac{\text{The absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.8. Protection of DNA against oxidative damage

Protection of plasmid DNA from harmful effects of Fenton's reagent was evaluated by DNA nicking assay [27] with minor modifications. Different concentrations of the sample (250 $\mu\text{g/ml}$ to 50 $\mu\text{g/ml}$) and plasmid DNA (5 μg) were mixed with Fenton's reagent (0.5 μl) and incubated for 30 min at 37 °C. After incubation the reaction mixture (10 μl) was taken and electrophoresed using 1 % agarose in TBE buffer at 90 V for 1 h. The gel was stained with EtBr, viewed utilizing trans-illuminating UV light, and finally photographed were taken by gel documentation system, (Acinterlab, USA).

2.9. Statistical analysis

All the experiments are expressed as means \pm SD of the responses of triplet per sample and the results of *in vivo* tests are expressed as means \pm SD of the number of experimental animals of each group. One-way ANOVA coupled with Tukey's *post hoc* test at $p < 0.05$ was used to determine the statistical significance between groups. Microsoft Excel 2013 and Graph Pad Prism 8 software were used for the statistical analysis.

3. Results

3.1. Phytochemical analysis

The antioxidant constituents like total phenolic content were 270.63 ± 3.64 mg of GAE/gm. extract for EAF followed by CME, AQF, CHF, PEF, and 234.70 ± 5.46 mg of QE/gm. of dry extract for the total flavonoid content of EAF followed by AQF, CME, PEF, and CHF (Table 1).

3.2. In vitro antioxidant activity

The DPPH scavenging activity of the sample measured by the spectrophotometric method in terms of IC_{50} values were 3.7, 14, 19, 40.5, and 58.3 $\mu\text{g/ml}$ for EAF, CME, AQF, CHF, and PEF respectively; compared with standard BHT (3.1 $\mu\text{g/ml}$). Further, at a concentration of 200 $\mu\text{g/ml}$, the % of scavenging exhibited by EAF (57.82 %) was most comparable with a reference standard (BHT) exhibited 86.04% in the hydrogen peroxide scavenging assay model (Table 2).

For reducing power, the highest absorbance activity was found in EAF (3.26) with moderate AQF (2.58) compared with standard GA (4.66) at the concentration of 50 ($\mu\text{g/ml}$). Similarly for the total antioxidant assay, at the concentration of 200 ($\mu\text{g/ml}$) the highest absorbance activity were

Table 1. Total phenolic and flavonoid content of CME and fractions of *H. schulli* seeds.

Fractions	TPC (mg of GAE/g)	TFC (mg of QE/g)
CME	226.06 \pm 4.35	154.45 \pm 2.79
PEF	27.67 \pm 4.04	104.53 \pm 3.02
CHF	87.03 \pm 1.80	55.75 \pm 2.53
EAF	270.63 \pm 3.64	479.70 \pm 5.46
AQF	173.20 \pm 5.82	223.61 \pm 5.77

All values are expressed as mean \pm SD (n = 3), SD = Standard deviation, CME = Crude methanol extract, CHF = Chloroform fraction, EAF = Ethyl acetate fraction, AQF = Aqueous fraction, GA = Gallic acid, TPC = Total phenolic content. TFC = Total flavonoid content.

Table 2. *In vitro* antioxidant activity of CME, fractions, and standard.

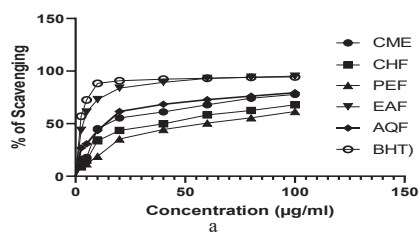
Name of Experiment/ Fractions	DPPH radical scavenging assay (IC ₅₀)	Hydrogen peroxide scavenging assay (% SCV for 200 µg/ml)	Reducing power capacity (Absorbance for 50 µg/ml)	Total Antioxidant Assay (Absorbance for 200 µg/ml)
CME	14	35.88	2.171 ± 0.002	0.811 ± 0.013
PEF	58	24.96	0.722 ± 0.005	0.322 ± 0.007
CHF	40	28.45	0.716 ± 0.003	0.410 ± 0.013
EAF	3.1	57.82	2.861 ± 0.040	1.096 ± 0.059
AQF	19	43.35	2.383 ± 0.002	0.504 ± 0.022
Standard	2 (BHT)	86.04 (BHT)	3.565 ± 0.050 (GA)	1.608 ± 0.059 (GA)

All values are expressed as mean ± SD, (n = 3). Here, SD = Standard deviation, CME = Crude methanol extract, CHF = Chloroform fraction, EAF = Ethyl acetate fraction, AQF = Aqueous fraction, GA = Gallic acid, BHT = Butylated hydroxyl toluene.

found in EAF (1.296 ± 0.059) and CME (0.811 ± 0.013) showed moderate activity as compared to reference standard GA (1.608 ± 0.059). Therefore, the EAF demonstrated greater scavenging compared with others (Table 2; Figure 1).

3.3. *In vivo* antioxidant activity

Antioxidant activity (*In vivo*) was performed in cadmium (Cd) intoxicated mice, and administration of Cd significantly decreased the



activity of SOD (0.408 U/mg of tissue) as compared with normal mice (0.792 U/mg of tissue). Treatment with CME and EAF at 400 mg/kg in Cd intoxicated mice improved the level of SOD (0.621 and 0.774 U/mg of tissue, respectively). Also, a significant ($p < 0.05$) reduction in catalase activity was noticed in liver homogenate of cadmium-induced mice (2.411) compared to the control grouped mice (4.312) as well. TBARS level showed a significant increase in Cd intoxicated mice (35.48 ± 2.214 nmol/mg tissue) comparing to the normal value (17.192 ± 0.689 nmol/mg protein). After treatment with EAF, a significant ($p < 0.05$) decline in TBARS levels was observed as standard ascorbic acid-treated mice (Table 3).

Similarly, in EAF₄₀₀ mg/kg b.w. and CME₄₀₀ mg/kg b.w. treated mice albumin (18.05 and 13.03 gm/dl) and total bilirubin (2.15 and 1.8 mg/dl) levels were also restored towards normal value (17.19 gm/dl) and (2.26 mg/dl), respectively. Likewise, uric acid and ALP levels were decreased in the Cd-treated animals and EAF (400 mg/kg b.w.), as well as CME (400 mg/kg b.w.) of the extract, produced uric acid and ALP levels significantly ($P < 0.05$) Table 3.

3.4. Protection of human erythrocytes against oxidative damage

RBC as the target of oxidation by H₂O₂, EAF showed 60.81% inhibition of oxidation followed by CME (48.26%), and CHF (44.77%) in the decreasing order, comparing with the standard gallic acid (63.14%). EAF various fractions also suppressed HOCl-induced oxidation of erythrocytes moderately. Similarly, a significant decrease in lipid peroxidation in erythrocytes was observed treated with EAF, CME, and CHF (Table 4 & Figure 2).

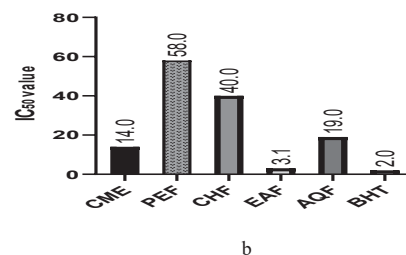


Figure 1. DPPH radical scavenging activity of CME and its fractions of *H. schulli* seeds. a: % of Scavenging activity on DPPH radical; b: IC₅₀ value. Each value is expressed as mean ± SD (n = 3), SD = Standard deviation.

Table 3. *In vivo* antioxidant assay of CME, EAF, and standard AA.

Name of Experiment	SOD (Enzyme unit (U)/mg Tissue)	Catalase (H ₂ O ₂ decomposed /min./mg of tissue)	LPO (TBARS level nmol/mg wet tissue)	Albumin (gm/dl)	Bilirubin (mg/dl)	Uric acid (mg/dl)	Alkaline phosphatase (U/l)
Positive control/ Normal group	0.792	4.312 ± 0.644	17.192 ± 0.689	17.52 ± 0.526	2.36 ± 0.343	104.1 ± 3.978	20.6 ± 2.408
Negative Control (Cd 8 mg/kg)	0.408*	2.411 ± 0.557*	35.48 ± 2.215*	10.37 ± 0.873*	1.57 ± 0.306*	59.97 ± 4.725*	04.0 ± 2.646*
Cd with EAF (100 mg/kg)	0.516**	2.860 ± 0.771	29.30 ± 1.976**	11.88 ± 1.271**	1.675 ± 0.340	66.6 ± 6.195	11.75 ± 2.217**
Cd with EAF (200 mg/kg)	0.594**	3.245 ± 0.595	23.63 ± 1.186**	13.64 ± 2.230	1.84 ± 0.230**	73.40 ± 3.140**	14.60 ± 2.074**
Cd with EAF (400 mg/kg)	0.676**	3.937 ± 0.628	21.17 ± 1.018**	16.05 ± 0.354**	2.05 ± 0.212	90.8 ± 3.394**	17.00 ± 2.828**
Cd with CME (400 mg/kg)	0.568**	3.042 ± 0.543	24.65 ± 1.270**	13.03 ± 2.346	1.76 ± 0.251	65.4 ± 7.077	16.67 ± 1.528**
Standard AA (100 mg/kg)	0.686**	4.056 ± 0.339**	19.19 ± 0.901**	15.70 ± 1.098**	2.02 ± 0.330	87.42.42 ± 3.129**	18.50 ± 2.646**

All values are expressed as mean ± SD (n = 2–5); analysis of variance followed by unpaired t-test. SD = Standard deviation, CME = Crude methanol extract, CHF = Chloroform fraction, EAF = Ethyl acetate fraction, AQF = Aqueous fraction, Cd = Cadmium, AA = Ascorbic acid, SOD = Superoxide dismutase, LPO = Lipid peroxide.

* $p < 0.05$, significant compared with the normal group.

** $p < 0.05$, significant compared with Cd (negative control) group.

Table 4. Effect of CME, CHF, and EAF against oxidative damage of human erythrocytes.

Sample with dose	H ₂ O ₂ induced assay (% of inhibition)	HOCl induced assay (% of inhibition)	Lipid peroxidation (% of inhibition)
CME (500 µg/ml)	48.26	41.19	49.61
CHF (500 µg/ml)	41.77	30.11	35.05
EAF (500 µg/ml)	62.81	57.13	70.50
GA (500 µg/ml)	86.14	84.37	91.68

All values are expressed as mean \pm SD (n = 3). Here, SD = Standard deviation, CME = Crude methanol extract, CHF = Chloroform fraction, EAF = Ethyl acetate fraction, AQP = Aqueous fraction, GA = Gallic acid.

3.5. Protection of DNA against oxidative damage

The protective effect of samples by Fenton's reagent-induced oxidative DNA damage was determined in the plasmid DNA of *E. coli* cells based on the DNA Nicking assay. The qualitative visualization of DNA by gel electrophoresis showed that EAF at the various concentration (L3 to L7, 300 to 100 µg/ml, respectively) and GA as standard (L8, 100 µg/ml) protect DNA from destruction as compared to Fenton's reagent treated DNA (L2). Here, supercoiled DNA was broken into an open circular form compared to the untreated DNA (Figure 3).

4. Discussion

As far as our concern, this study is the first report on the oxidative induced DNA, RBC cellular membrane protective effect, and *in vivo* antioxidant activities of *H. schulli* seeds along with repeated *in vitro* antioxidant activity.

Phenolic compounds or polyphenols are thought to be strong chain-breaking antioxidants [28]. Phytochemical analysis in the EAF of seeds has shown that the amount of total flavonoids and flavonols is low but the amount of total phenolic component is relatively high.

For *in vitro* antioxidant activity, four methods were used including the most widely used DPPH method [29] as well as hydrogen peroxide scavenging for direct measurement of radical scavenging activity. *H. schulli* has shown good radical quenching activity against both DPPH and hydrogen peroxide radicals. In the present study, CME and its fraction showed moderate to potent DPPH free radicals scavenging activity. As the concentration of extracts was increased the DPPH scavenging activity also increased, as indicated by percentage inhibition. EAF had the lowest IC₅₀ value when compared with other fractions or CME and very near to standard BHT (Table 2). In H₂O₂ scavenging assay, all the extracts showed different levels of scavenging activity but EAF again exhibited strongest H₂O₂ scavenging activity compared to other fractions or CME. Although H₂O₂ is not very reactive, high penetrability of the cellular membrane leads to OH formation when it reacts with Fe²⁺ or the superoxide anion radical in the cell which can initiate lipid peroxidation and cause DNA damage in the body [30].

So, the extract of *H. schulli* seeds specially EAF may serve as an important source of free radical scavengers. Moreover, our findings observed for radical scavenging support the findings of Vijaya [31], which stated that the ethanolic extract of *H. schulli* seeds display potent DPPH antioxidant activities and high total phenolic and flavonoid contents.

On the other hand, reducing power and total antioxidant capacity assay methods indirectly evaluates antioxidant activity. The reducing properties of antioxidants are generally related to the presence of reductants and based on the reduction of Fe³⁺ to Fe²⁺, which are shown to break down radical chains by donating an atom [32] to act as antioxidants. It was observed that CME/fractions were able to donate electrons to ferric ions which converted to ferrous ions to form a blue complex and measured at 532 nm. The total antioxidant capacity was indicated by the reduction of molybdenum (VI) to molybdenum (V), with the formation of the green complex by the CME and its fractions. In both cases the activity was expressed in terms of absorbance. Reducing power and total antioxidant capacity were increased with increase in concentration of CME/fractions. Table 2 shows the absorbance value of 50µg/ml for reducing power and 200µg/ml for total antioxidant capacity assay. Among the fractions and CME, EAF showed the highest activity in both assay as it was observed for the phenolic and flavonoid contents and DPPH activity. So the results revealed that the EAF is rich in phenolic compounds, well-known reducing agents responsible for the antioxidant capacity of this fraction.

For the function of the enzymatic antioxidants, some exogenous antioxidants like vitamin A, C, E, and some metals are essential which are of dietary origin [33]. Also, dietary sources enhanced the level of non-enzymatic antioxidants such as ALP, albumin, bilirubin, uric acid at the extracellular fluid of the body that binds with extracellular free radical generating metals (iron and copper) and ensures the limited survival of reactive oxygen species such as superoxide and H₂O₂ in the extracellular fluid. Enzymatic antioxidants also provide natural protection against oxidative stress of the cell [34]. Therefore, simultaneous use of Cd and extractives on the animal model showed an intention of balancing the amounts of these enzymes in animal cells. In Cd-treated mice the level of SOD and catalase was decreased whereas administration of EAF at different doses significantly improves to the level of normal mice in a dose dependent manner.

Another marker of *in vivo* antioxidant assay is lipid peroxidation (LPO) which is thought of as the primary mechanism for Cd toxicity despite its inability to directly generate free radicals [35]. Cd raises the LPO and changes the antioxidant defense mechanism, creating oxidative damage, and treatment with EAF along with Cd showed protective effects against LPO [36]. In this study, EAF treated groups showed a dose-dependent rise of TBARS level which indicates the lipid peroxidation inhibition ability of the extractives. This evidence is in agreement with scientific reports describing the inhibitory potential of different plant extracts against lipid peroxidation [37, 38]. Thus, the potential of anti-lipid peroxidation also signs of their polyphenol content including flavonoids.

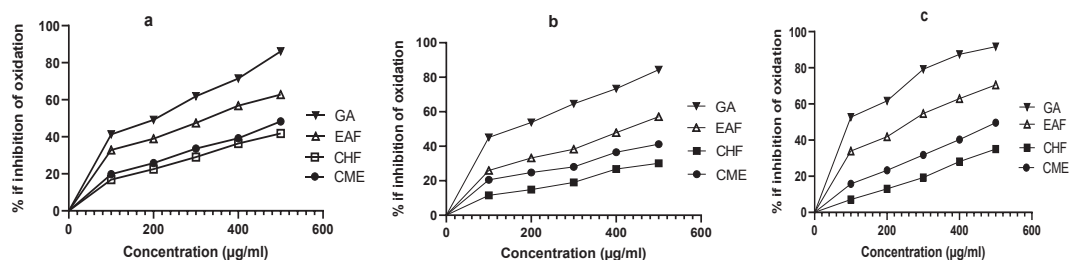


Figure 2. Protection of Human Erythrocyte against H₂O₂ (a) and HOCl (b) induced oxidative damage and lipid peroxidation (c). All values are expressed as mean \pm SD (n = 3) SD = Standard deviation.

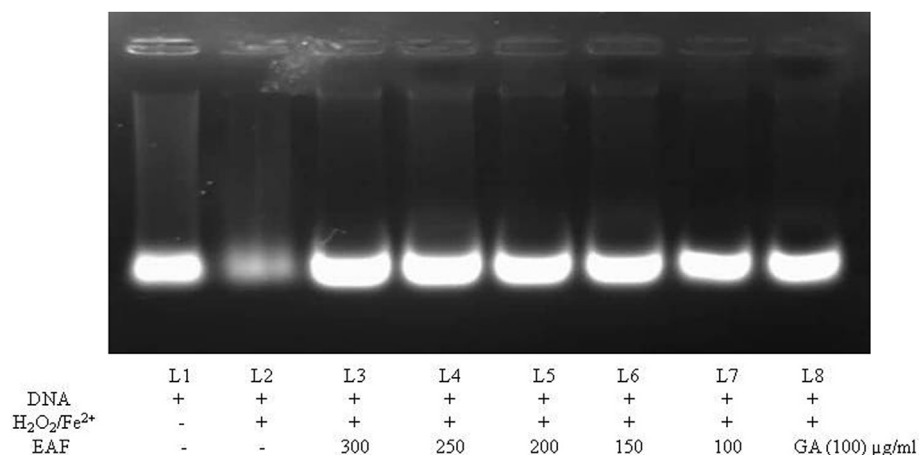


Figure 3. DNA nicking assay. Lane-1: Untreated DNA; Lane-2: DNA + Fenton's reagent; Lane-3: DNA + Fenton's reagent + EAF (300 mg/ml); Lane-4: DNA + Fenton's reagent + EAF (250 mg/ml); Lane-5: DNA + Fenton's reagent + EAF (200 mg/ml); Lane-6: DNA + Fenton's reagent + EAF (150 mg/ml); Lane-7: DNA + Fenton's reagent + EAF (100 mg/ml); Lane-8: DNA + Fenton's reagent + Gallic Acid (100 mg/ml).

Due to the presence of a high concentration of polyunsaturated fatty acids and oxygen transport in cell membranes, erythrocytes are the main targets for ROS attack [39]. Thus, erythrocytes are very convenient for the assessment of the intracellular antioxidant activities of various compounds. Many reagents can be used to induce oxidative stress in erythrocyte test models like hydrogen peroxide, hypochlorous acid which can easily penetrate through the cell membrane. Erythrocytes were affected both from inside and outside [40, 41]. The plant extracts are capable of protecting erythrocytes against oxidative damage, thereby maintaining the basal levels of GSH and MDA concentration [42]. EAF of *H. schulli* showed concentration-dependent protection against evaluated hydrogen peroxide, hypochlorous acid as well as lipid peroxidation. So far no reports are available on protecting erythrocytes cell membrane damage by *H. schulli* seeds.

Another noble experiment of this study was the evaluation of antioxidant activity using DNA models like DNA Nicking assay in which plasmid DNA was exposed to the Fenton's reaction. Fenton's reaction produced hydroxyl radicals that induced oxidative DNA strands breaking and yield fragmented forms of DNA [43]. The addition of EAF resulted in a moderate decrease in the formation of nicked DNA and it increased the supercoiled form of DNA as well as protected from its oxidative destruction by Fenton's reagent (Figure 3). The protection of DNA indicated the presence of flavonoids and phenolic compounds like trycin and quercetin in *H. schulli* seeds which can prevent the production of ROS by complexing cations [44]. Extracts prepared from black raspberry [45] seed and various natural resources [46] could either stop or slow down the process of DNA damage. So these results indicate that *H. schulli* seeds is a potential source of antioxidants that prevent DNA damage. To the simplest of our knowledge, no previous literature has reported the DNA protecting activity by *H. schulli* seeds. Therefore, the results reported here would appear to be the first report.

5. Conclusion

Taken together our findings indicate that the methanolic extract and its fraction EAF of *H. schulli* (Buch.-Ham.) could have strong antioxidant activity both *in vitro* and *in vivo*. Our results also proved that the EAF was able to protect erythrocytes and DNA against oxidative damage. Therefore these results showed that the EAF has a potential antioxidant activity that would be used for the human being. Besides, it would be a potential food supplement as it is used in various systems of traditional medicine for centuries. However, further studies are still required to isolate the novel compounds and to elucidate the molecular mechanisms underlying the effects.

Declarations

Author contribution statement

Md. Shoriful Islam: Performed the experiments; Wrote the paper.
Mst. Shahnaj Parvin: Analyzed and interpreted the data.
Md. Ekramul Islam: Conceived and designed the experiments.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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References

- [1] B. Ghule, P. Agrawal, P. Lal, D. Kothari, N. Kotagale, Separation and quantification of lupeol in *Hygrophila schulli* by high-performance thin-layer chromatography, *JPC-J. Planar Chromatogr. Mod. TLC* 34 (1) (2021) 79–87.
- [2] G.H. Tekulu, A. Desta, M.G. Hiben, E.M. Araya, Anti-nociceptive and anti-inflammatory activity of *Hygrophila schulli* leaves, *J. Inflamm. Res.* 13 (2020) 497.
- [3] D.D. Huyen, New record of species *Hygrophila episcopalis* R. Ben.(R. Ben.)(Acanthaceae) for the flora of Vietnam, *Acad. J. Biol.* 34 (2) (2012) 187–189.
- [4] A.K. Nadkarni, *Indian Materia Medica*, vol. 1, Popular Prakashan, Mumbai, 2007, pp. 668–669.
- [5] A. Patra, S. Jha, P.N. Murthy, S. Satpathy, Antibacterial activity of *Hygrophila spinosa* T. Anders leaves a comparative study, *Int. J. Pharm. Tech. Res* 1 (2009) 837–839.
- [6] A.Z. Hussain, S. Kumaresan, GC-MS analysis and antimicrobial activity of *Hygrophila auriculata*, *Arch. Appl. Sci. Res.* 5 (5) (2013) 163–168.

- [7] M.S. Hussain, S. Fareed, M. Ali, *Hygrophila auriculata* (K. Schum) Heine: ethnobotany, phytochemistry and pharmacology, *Asian J. Tradit. Med.* 5 (4) (2010) 121–131.
- [8] V.C. Lobo, A. Phatak, N. Chandra, Antioxidant and free radical scavenging activity of *Hygrophila schulli* (Buch.-Ham.) Almeida and Almeida. Seeds, *Adv. Bio. Res.* 1 (2010).
- [9] A.A. Tayel, W.F. El-Tras, S.H. Moussa, S.M. El-Sabbagh, Surface decontamination and quality enhancement in meat steaks using plant extracts as natural biopreservatives, *Foodborne Pathog. Dis.* 9 (8) (2012) 755–761.
- [10] J.M. Alves-Silva, A. Romane, T. Efferth, L. Salgueiro, North African medicinal plants traditionally used in cancer therapy, *Front. Pharmacol.* 8 (2017) 383.
- [11] A. Ciccia, S.J. Elledge, The DNA damage response: making it safe to play with knives, *Mol. Cell* 40 (2) (2010) 179–204.
- [12] N.K. Sethiya, N.M. Ahmed, R.M. Shekh, V. Kumar, P.K. Singh, V. Kumar, Ethnomedicinal, phytochemical and pharmacological updates on *Hygrophila auriculata* (Schum.) Hiene: an overview, *J. Integrat. Med.* 16 (5) (2018) 299–311.
- [13] A. Meda, C.E. Lamien, M. Romito, J. Millogo, O.G. Nacoulma, Determination of the total phenolic, flavonoid and proline contents in Burkina Faso honey, as well as their radical scavenging activity, *Food Chem.* 91 (3) (2005) 571–577.
- [14] T. Mabry, K.R. Markham, M.B. Thomas, *The Systematic Identification of Flavonoids*, Springer Science & Business Media, 2012.
- [15] N. Jahan, M.S. Parvin, N. Das, M.S. Islam, M.E. Islam, Studies on the antioxidant activity of ethanolic extract and its fractions from *Pterygota alata* leaves, *J. Acute Med.* 4 (3) (2014) 103–108.
- [16] X.Y. Zhang, *Principles of Chemical Analysis*, vol. 275, China Science Press, Beijing, 2000, p. 276.
- [17] L. Barros, P. Sabel Baptista, C.F.R. Ferreira, Effect of *Lactarius piperatus* fruiting body maturity stage on antioxidant activity measured by several biochemical assays, *Food Chem. Toxicol.* 45 (2007) 1731–1737.
- [18] M. Aguilar Urbano, M. Pineda Priego, P. Prieto, Spectrophotometric Quantitation of Antioxidant Capacity through the Formation of a Phosphomolybdenum Complex: Specific Application to the Determination of Vitamin E1, 2013.
- [19] B.T. Doumas, W.A. Watson, H.G. Biggs, Albumin standards and the measurement of serum albumin with bromocresol green, *Clin. Chim. Acta* 31 (1) (1971) 87–96.
- [20] E. Weigl, H. Bach, D. Krieg, Serum bilirubin in an obvious healthy population (author's transl), *Med. Klin.* 70 (15) (1975) 664–669.
- [21] P. Fossati, L. Prencipe, G. Berti, Use of 3, 5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymic assay of uric acid in serum and urine, *Clin. Chem.* 26 (2) (1980) 227–231.
- [22] D. Akpınar, P. Yarıçoğlu, N. Derin, Y. Alicigüzel, A. Açar, The effect of lipoic acid on antioxidant status and lipid peroxidation in rats exposed to chronic restraint stress, *Physiol. Res.* 57 (6) (2008).
- [23] S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, *Eur. J. Biochem.* 47 (3) (1974) 469–474.
- [24] H. Aebi, Catalase in vitro, *Methods Enzymol.* 105 (1984) 121–126. Academic Press.
- [25] S.Y. Park, S.H. Bok, S.M. Jeon, Y.B. Park, S.J. Lee, T.S. Jeong, M.S. Choi, Effect of rutin and tannic acid supplements on cholesterol metabolism in rats, *Nutr. Res.* 22 (3) (2002) 283–295.
- [26] U.P. Santos, J.F. Campos, H.F.V. Torquato, E.J. Paredes-Gamero, C.A. Carollo, L.M. Estevinho, E.L. Dos Santos, Antioxidant, antimicrobial and cytotoxic properties as well as the phenolic content of the extract from *Hancornia speciosa* Gomes, *PLoS One* 11 (12) (2016), e0167531.
- [27] U. Golla, S.S.R. Bhimathati, Evaluation of antioxidant and DNA damage protection activity of the hydroalcoholic extract of *Desmostachya bipinnata* L. Stapf, *Sci. World J.* (2014).
- [28] C.O. Chen, J.B. Blumberg, Are there age-related changes in flavonoid bioavailability, *Phytochem. Aging Health* (2008) 19–37. MS Meskin, WR Bidlack, RK Randolph, editores. CRC Press, Boca Raton, Florida.
- [29] S.M.B. Hashemi, M.S. Brewer, J. Safari, M. Nowroozi, M.H. Abadi Sherahi, B. Sadeghi, M. Ghafoori, Antioxidant activity, reaction mechanisms, and kinetics of *Matricaria recutita* extract in commercial blended oil oxidation, *Int. J. Food Prop.* 19 (2) (2016) 257–271.
- [30] M.N. Alam, N.J. Bristi, M. Rafiqzaman, Review on in vivo and in vitro methods evaluation of antioxidant activity, *Saudi Pharmaceut. J.* 21 (2) (2013) 143–152.
- [31] P. Singh, S.P. Vishwakarma, R.L. Singh, Antioxidant, oxidative DNA damage protective and antimicrobial activities of the plant *Trigonella foenum-graecum*, *J. Sci. Food Agric.* 94 (2014) 2497–2504.
- [32] F. Shahidi, P.K. Janitha, P.D. Wanasundara, Phenolic antioxidants, *Crit. Rev. Food Sci. Nutr.* 32 (1) (1992) 67–103.
- [33] B. Halliwell, J.M. Gutteridge, *Free Radicals in Biology and Medicine*, Oxford University Press, USA, 2015.
- [34] H.N. Abed, M.H. Hadwan, Clinical and thermodynamical properties of catalase enzyme in sera of Iraqi diabetic patients, *Bio Technol.: An Indian J.* 12 (1) (2016).
- [35] J.D. Eneman, R.J. Potts, M. Osier, G.S. Shukla, C.H. Lee, J.F. Chiu, B.A. Hart, Suppressed oxidant-induced apoptosis in cadmium adapted alveolar epithelial cells and its potential involvement in cadmium carcinogenesis, *Toxicology* 147 (3) (2000) 215–228.
- [36] B.I. Ognjanović, S.D. Marković, N.Z. Đorđević, I.S. Trbojević, A.Š. Štajin, Z.S. Saičić, Cadmium-induced lipid peroxidation and changes in antioxidant defense system in the rat testes: protective role of coenzyme Q10 and Vitamin E, *Reprod. Toxicol.* 29 (2) (2010) 191–197.
- [37] E.A. Irondi, G. Oboh, S.O. Agboola, A.A. Boligon, M.L. Athayde, Phenolics extract of *Tetrapleura tetrapleura* fruit inhibits xanthine oxidase and Fe²⁺-induced lipid peroxidation in the kidney, liver, and lungs tissues of rats in vitro, *Food Sci. Human Wellness* 5 (1) (2016) 17–23.
- [38] A.H. Thayyil, A.K. Muthu, M. Ibrahim, In vivo antioxidant and lipid peroxidation effect of various extracts from aerial parts of *Chomelia asiatica* (Linn) in rat fed with high fat diet, *Afr. J. Pharm. Pharmacol.* 10 (38) (2016) 810–816.
- [39] S. Sundaram, S. Anjum, P. Dwivedi, G.K. Rai, Antioxidant activity and protective effect of banana peel against oxidative hemolysis of human erythrocyte at different stages of ripening, *Appl. Biochem. Biotechnol.* 164 (7) (2011) 1192–1206.
- [40] C. Manna, P. Galletti, V. Cucciolla, G. Montedoro, V. Zappia, Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages, *J. Nutr. Biochem.* 10 (3) (1999) 159–165.
- [41] B.R. Van Dyke, P. Saltman, Hemoglobin: a mechanism for the generation of hydroxyl radicals, *Free Radical Biol. Med.* 20 (7) (1996) 985–989.
- [42] S. Luqman, S. Kaushik, S. Srivastava, R. Kumar, D.U. Bawankule, A. Pal, M.P. Darokar, S.P.S. Khanuja, Protective effect of medicinal plant extracts on biomarkers of oxidative stress in erythrocytes, *Pharmaceut. Biol.* 47 (2009) 483–490.
- [43] J. Cadet, J.R. Wagner, V. Shafirovich, N.E. Geacintov, One-electron oxidation reactions of purine and pyrimidine bases in cellular DNA, *Int. J. Radiat. Biol.* 90 (6) (2014) 423–432.
- [44] T. Jun, Z. Liancai, W. Bochu, Effects of quercetin on DNA damage induced by copper ion, *Int. J. Pharmacol.* 3 (1) (2007) 19–26.
- [45] M.H. Choi, S.M. Shim, G.H. Kim, Protective effect of black raspberry seed containing anthocyanins against oxidative damage to DNA, protein, and lipid, *J. Food Sci. Technol.* 53 (2) (2016) 1214–1221.
- [46] P. Kaur, S.S. Purewal, K.S. Sandhu, M. Kaur, DNA damage protection: an excellent application of bioactive compounds, *Bioresour. Bioproc.* 6 (1) (2019) 1–11.