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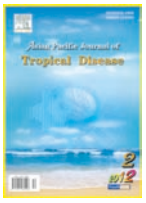

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Amelioration of oxidative stress by anthraquinones in various *in vitro* assays

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ABSTRACT

Objective: The use of natural phytoconstituents for food and as nutritional supplements is an easiest way to be healthier. Anthraquinone pigments have been traditionally used for various purposes viz. food colorants, textile staining, color paints and medicines. *Rubia cordifolia* L. is a perennial, herbaceous climbing plant belonging to family *Rubia*ceae. This plant contain substantial amounts of anthraquinones, especially in the roots. The present study deals with the bioactivity evaluation of phytoconstituents viz. alizarin and purpurin from *Rubia cordifolia*. **Methods:** The DNA protective and antioxidant potential of alizarin and purpurin was evaluated using different *in vitro* assays viz. DNA protection assay, ABTS assay, DPPH assay, Ferric ion reduction potential and Phosphomolybdenum assay. **Results:** Alizarin and purpurin exhibited good free radical scavenging activity in various assays. In DNA protection assay, alizarin showed more DNA protection against hydroxyl radicals generated by Fenton's reagent in comparison to purpurin. **Conclusions:** Being potent antioxidants, these natural coloring compounds can be boon to the food industry as nutraceuticals. Further, these phytochemicals can be explored for their anticancer activity and may serve as potent cancer chemopreventive molecules.

1. Introduction

Functional foods have one or more beneficial functions in the body, in addition to adequate nutritional effects, to either improve stage of health and well-being and/or reduce the risk of disease [1]. Natural plant products and their use in various food preparations have gained increased attention in recent years. Many phytoconstituents are investigated for potential use as novel nutraceuticals [2]. The antiradical activity of these natural plant products is of particular interest both because of their beneficial physiological activity on human cells and the potential they have to replace synthetic antioxidants used in foodstuffs [3]. Plants are the vast source of natural dyes/colourants. The northeastern sub-Himalayan region of India has plenty of plant species with dye-yielding properties. Villagers of these regions extract these dyes from different parts of plant mostly by boiling, scrapping, powdering to get the desired colour [4]. For centuries, natural dyes have been used for various purposes throughout the world, but use of synthetic dyes has lessened the value of natural colorants. In nineteen eighties, synthetic dyes have been reported to have carcinogenic effects, since then, dyes from natural sources are regarded as significant alternative [5,6,7]. During the process of food production and preservation, quality is always affected due to oxidation of food components. Numerous synthetic antioxidants have been used in last few decades for preservation of food quality but many synthetic antioxidants are suspected to be toxic, unsafe and number of possible health risks associated with them [8,9]. This insight has encouraged an upsurge in research into naturally-occurring products, in search of functional foods and alternative food additives. Antioxidants from natural sources have been getting more attention due to their safe nature and low toxicity. Number of reports have been available in the last decades regarding edible medicinal plants as source of antioxidants that can be utilised for the prevention of oxidative stress-related diseases[10-13]. Thus, search for phytochemicals with antioxidant and low cytotoxicity has become very important [14].

The Genus *Rubia* (Madder) belongs to family *Rubia*ceae,

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is found in different parts of the world. Numerous plants belonging to this family are reported to contain anthraquinones especially in their roots [15]. Anthraquinones have been known to exhibit a wide range of bioactivities such as antimicrobial, antifungal, hypotensive, analgesic, antimalarial [16]. Anthraquinones have extensive applications such as in food coloring, textile dyeing, paints and medicines [17,18]. Anthraquinones constitute the major group of naturally occurring quinones. Numerous anthraquinones have been isolated from roots of madder, among them alizarin (1,2-dihydroxy anthraquinone) and purpurin (1,2,4-trihydroxy anthraquinone) which gives the reddish orange or yellow dye are important one [19,20].

Keeping in mind, the importance of these colouring compounds, the present study was planned to evaluate genoprotective/antiradical efficacy of anthraquiones, purpurin and alizarin phytoconstituents from *R. cordifolia* L roots.

2. Materials and methods

2.1. Chemicals

2, 2-Diphenyl-1-picrylhydrazyl (DPPH), Ferric chloride, 2, 2-Azinobis (3- ethylbenzothiazoline-6-sulfonate) (ABTS), Ammonium molybdate were obtained from HiMedia Pvt. Limited. Mumbai, India. Ascorbic acid and Rutin were obtained from Sigma (St. Louis, MO, USA). pBR322 Plasmid DNA was purchased from Genei Pvt. Ltd., Bangalore (India). All other reagents were of analytical grade (AR).

2.2. Plant material and isolation

The roots of *Rubia cordifolia* were purchased from local market at Amritsar, Punjab, India. A voucher specimen no. 0342-B-03/2006, has been submitted to the Herbarium of the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab. The roots were washed with running water to remove any dust impurities and dried at 40℃. They were finely powdered and percolated with 80% hexane to obtained hexane extract. The hexane extract was subjected to column chromatography which yielded the "RCA fraction" on elution with hexane: ethyl acetate (94:6) (Fig. 1). Purpurin (CAS 81-54-9) was a kind gift from S. Arimoto-Kobayashi, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama, Japan.

2.3. Phytochemical analysis

The thin layer chromatography (TLC) of the isolated fraction "RCA" was performed on precoated Kieselgel 60F254 plates (Merck, Germany) which revealed it to be a single spot having same Rf value to standard alizarin, which was characterized structurally using mass spectroscopic technique. For this, 1 mg of compound was sonicated for 15 minutes with a mixture of acetonitrile and water of HPLC grade (50:50) at room temperature. Twenty microlitre of this solution was injected into the capillary tube of the mass spectrophotometer and the spectrum (fragmentation pattern) was recorded.

2. 4. Testing of Purpurin and Alizarin for antioxidant and genoprotective activities

2.4.1. Antioxidant activity

DPPH scavenging activity was carried out by the method of Blois [21] with slight modifications. Different concentrations (300 μl each) of test samples were dissolved in methanol and taken in test tubes in triplicates. Then 2 ml of 0.1 mM methanol solution of DPPH (2, 2-Diphenyl-1-picrylhydrazyl) was added to each of the test tubes and were shaken vigorously. After 30 minutes, absorbance was taken at 517 nm using UV-VIS spectrophotometer (Systronics 2202 UV-Vis Spectrophotometer, India). The control was prepared by adding vehicle solvent instead of test samples. Rutin was used as antioxidant standard.

$$\text{Radical scavenging activity\%} = A0 - A1/A0 \times 100$$

where A0 is the absorbance of DPPH solution. A1 is the absorbance of reaction mixture (containing test sample & DPPH solution).

$ABTS^{\cdot+}$ scavenging assay was carried out by the method given by Re et al. [22]. ABTS cations were generated by reacting 7 mM ABTS stock solution and 2.45 mM Potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS cation solution was diluted with ethanol to an absorbance of 0.70 ($\pm$0.02) at 734 nm. About 300 μl of test solution was added to the diluted ABTS cation solution (2 ml) and absorbance reading was taken up to 5 min (Systronics 2202 UV-Vis Spectrophotometer, India). Rutin was used as antioxidant standard.

$$\text{Radical scavenging activity\%} = A0 - A1/A0 \times 100$$

where A0 is the absorbance of ABTS solution. A1 is the absorbance of reaction mixture (containing test sample & ABTS solution).

Reducing potential of test samples was determined using the method of Oyaizu [23]. About 1 ml of different concentrations (40-200 μg/mL) of test samples were dissolved in methanol and taken in the test tubes in triplicates. To the test tubes, 2.5 ml of phosphate buffer (pH 6.6, 0.2M) and 2.5 ml of 1% Potassium ferricyanide solution was added. These contents were mixed well and were incubated at 50℃ for 20 minutes. After incubation, 2.5 ml of 10% trichloroacetic acid (TCA) was added and kept for centrifugation at 3000 rpm for 10 minutes. After centrifugation, 2.5 ml of supernatant was taken and to this, 2.5 ml of double distilled water was added, followed by addition of 0.5 ml of 0.1% ferric chloride. The absorbance was measured spectrophotometrically at 700 nm using UV-VIS spectrophotometer (Systronics 2202

UV-Vis Spectrophotometer, India). Increase in absorbance of reaction mixture was interpreted as increase in reducing ability of the test samples and the results were compared with ascorbic which was used as reference compound. The percentage of reduction of the sample as compared to the standard (ascorbic acid) was calculated using the formula:

% Reducing power = [1-(1-As/Ac) ×100]

where Ac = absorbance of standard compound at maximum concentration tested, and As = absorbance of sample.

The total antioxidant activity of the test sample was evaluated by the phosphomolybdenum method [24]. An aliquot of 0.3 ml of sample solution was mixed with 3 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped with silver foil and incubated at 95℃ for 90 min. The tubes were cooled to room temperature and the absorbance of aqueous solution was measured at 695 nm against a blank. Total antioxidant activity was expressed in relation of ascorbic acid and calculated by following formula:

% TAC = (As − Ac) /(Aaa-Ac)×100

where Ac was the absorbance of the control (blank, without extract), As was the absorbance in the presence of the extract and Aaa was absorbance of ascorbic acid.

2.4.2. Genoprotective activity

A DNA protection assay was performed using supercoiled pBR322 plasmid DNA with slight modifications according to our previous report [25]. Plasmid DNA was incubated with Fenton's reagents (30 mM H_2O_2, 50 mM ascorbic acid and 80 mM $FeCl_3$) containing test sample and the finally volume of the mixture was raised up to 20 μl. The mixture was then incubated for 30 min at 37℃ followed by addition of loading dye and electrophoresis was carried out in TAE buffer (40mM Tris base, 16mM acetic acid 1mM EDTA, pH 8.0) at 60 V for 2.5 h. DNA was analyzed followed by ethidium bromide staining using Gel Doc XR system (Bio-Rad, USA).

2.6. Statistical analysis

The results were presented as the average and standard error of three experiments. Regression analysis was carried out by best fit method and IC_{50} values were calculated using regression equation. The data were also analyzed for statistical significance using analysis of variance (one-way ANOVA) and the difference among means was compared by high-range statistical domain (HSD) using Tukey's test. The significance of results was checked at $*P \leqslant 0.05$.

3. Results

3.1. Structural elucidation of 'RCA' fraction

A red coloured fraction isolated from the hexane extract, namely 'RCA' fraction was characterized using mass spectroscopic technique. The mass spectrum of 'RCA' fraction gives parent peak at 241.16 and points to the molecular formula $C_{14}H_8O_4$. The structure of alizarin and purpurin is given in Fig. 2.

3.2. Antioxidant studies

In DPPH assay, alizarin and purpurin showed 71.77% inhibition (IC_{50} 20.55 μg/mL) and 57.58% (IC_{50} 72.96 μg/mL)

Table 1.
DPPH radical scavenging activity (%) of alizarin, purpurin and standard rutin (mean ± SE; n = 3). Means within the column followed by different superscript letters were significantly different at $*P \leqslant 0.05$.

S.No.	Concentration(μg/mL)	Alizarin	Purpurin	Rutin
1	40	55.08±0.417a	36.23±0.913a	28.48±0.260a
2	80	66.58± 1.192b	57.12±1.902b	53.15±0.770b
3	120	69.14± 0.253c	63.16±1.426c	73.33±0.220c
4	160	71.13±0.664cd	59.93±1.076d	74.61±0.655c
5	200	71.77±0.340d	57.58±0.565db	76.78±0.310d

Regression analysis: Alizarin (*r = 0.9628); Purpurin (*r = 0.9715); Rutin (*r = 0.9731). One way anova: Alizarin (F ratio =49.9657*: HSD =2.5452); Purpurin (F ratio =76.589*:HSD = 5.6189); Rutin (F ratio = 2836.862*: HSD = 1.7842).

Table 2.
ABTS cation radical scavenging activity (%) of alizarin, purpurin and standard rutin (mean ± SE; n = 3). Means within the column followed by different superscript letters were significantly different at $*P \leqslant 0.05$

S.No.	Concentration(μg/mL)	Alizarin	Purpurin	Rutin
1	1	28.00±0.449a	15.31±0.156a	22.44±1.140a
2	5	44.00±0.000b	54.59±0.255b	23.99±0.830b
3	10	60.96±0.285c	69.26±0.321c	28.20±0.790c
4	20	65.90±0.941d	91.20±0.201d	41.87±0.830d
5	40	96.45±0.000e	99.19±0.000e	56.04±0.910e
6	80	99.00±0.000f	99.19±0.000e	99.82±0.720f
7	120	99.00±0.000f	99.19±0.000e	99.82±0.230f

Regression analysis: Alizarin (*r = 0.9715); Purpurin (*r = 0.9591); Rutin (*r = 0.9934). One way anova: Alizarin (F ratio = 3875.401*: HSD = 2.2669); Purpurin (F ratio = 10427.14*: HSD = 1.4144); Rutin (F ratio = 38552.92*: HSD = 0.8363).

Table 3.
Reducing potential (%) of alizarin, purpurin in comparison to standard ascorbic acid (mean ± SE; n = 3). Means within the column followed by different superscript letters were significantly different at *$P \leq 0.05$

S.No.	Concentration(μg/mL)	Alizarin	Purpurin	Ascorbic acid
1	40	26.85±0.574[a]	28.45±0.195[a]	15.77±0.817[a]
2	80	64.97±2.489[b]	50.79±0.412[b]	42.77±1.550[b]
3	120	72.52±0.496[c]	72.29±0.770[c]	54.50±1.000[c]
4	160	80.52±0.401[d]	83.85±2.250[d]	78.07±1.070[d]
5	200	83.87±0.409e	94.95±0.137e	100.00±0.060e

Regression analysis: Alizarin (*r = 0.9695); Purpurin (*r = 0.9853); Ascorbic acid (*r = 0.9944). One way anova: Alizarin (F ratio = 1738.543*: HSD = 2.5656); Purpurin (F ratio = 1374.472*: HSD = 3.3221); ascorbic acid (F ratio = 7638.287*: HSD = 1.7349).

inhibition at highest concentration of 200 μg/mL respectively in comparison to rutin (IC_{50} 54.54 μg/mL) (Table 1). In ABTS assay, alizarin showed radical scavenging activity of 99% while purpurin showed 99.19% of inhibition at the highest tested concentration of 120 μg/mL (Table 2). Rutin showed IC_{50} of 24.78 μg/mL (Table 2)

Table 4.
IC50 values for alizarin, purpurin and standard compounds in different *in vitro* antioxidant assays.

	IC50 values (μg/mL)			
Assay	Alizarin	Purpurin	Rutin	Ascorbic acid
DPPH assay	20.55	72.96	54.05	–
ABTS.+ assay	4.91	4.60	24.78	–
Reducing power assay	62.17	71.27	–	76.71

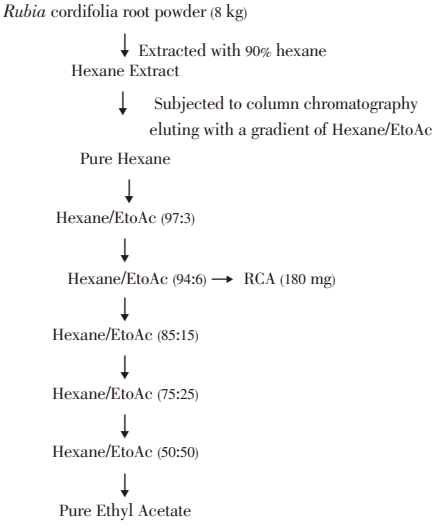

Figure 1. Isolation protocol of alizarin from *R. cordifolia* L. roots.

The reducing capacity of a compound Fe3+/ferricyanide complex to ferrous form may serve as indicator of its antioxidant potential. At the concentration of 200 μg/mL, the alizarin and purpurin exhibited 83.87% (IC_{50} 62.17 μg/mL) and 94.95% (IC_{50} 71.27 μg/mL) of inhibition in comparison to standard ascorbic acid (Table 3).The phosphomolybdenum or total antioxidant capacity assay is based on the reduction of Mo(VI) to Mo(V) on the addition of antioxidant sample and resulting in the formation of a greenish phosphate/Mo(V) complex (acidic pH & higher temperature). In present study, both anthraquinones viz. alizarin and purpurin reduced Mo(VI) to Mo(V) and the total antioxidant capacity of test samples is expressed in comparison to ascorbic acid and found to be 8.28% and 10.12% in comparison to ascorbic acid.

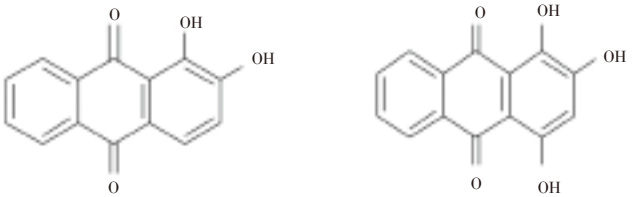

Figure 2. Structure of alizarin and purpurin.

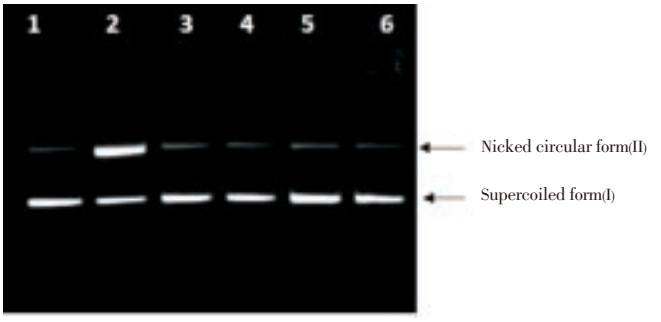

Figure 3. Effect of alizarin on the protection of supercoiled pBR322 DNA against hydroxyl radical generated by the fenton's reaction.
Lane 1: pBR322 DNA
Lane 2: pBR322 DNA + Fenton's reagent (DNA damage control),
Lane 3: pBR322DNA + Fenton's reagent + Alizarin (50 μg/mL),
Lane 4: pBR322 DNA + Fenton's reagent + Alizarin (100 μg/mL),
Lane 5: pBR322 DNA + Fenton's reagent + Alizarin (150 μg/mL),
Lane 6: pBR322 DNA + Fenton's reagent + Alizarin (200 μg/mL)

3.3. Genoprotective activity

When pBR322 plasmid DNA was exposed to Fenton's reagent, it caused conversion of DNA band from Form I (Native plasmid DNA) to Form II (single-stranded, nicked circular plasmid DNA) and Form III (Linear form). Alizarin was found to be more effective than purpurin. Alizarin showed its protective effect against hydroxyl radical generated by Fenton's reagent even at low concentration of 50 μg/mL. Purpurin showed protection at lower concentration only. Above 100 μg/mL, it caused damage to plasmid DNA resulting in the formation of open circular and linear forms (Fig. 3 &4).

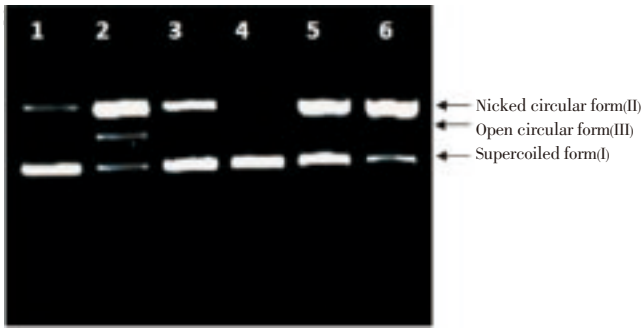

Figure 4. Effect of purpurin on the protection of supercoiled pBR322 DNA against hydroxyl radical generated by the fenton's reaction.
Lane 1: pBR322 DNA
Lane 2: pBR322 DNA + Fenton's reagent (DNA damage control),
Lane 3: pBR322DNA + Fenton's reagent + Purpurin (50 μg/mL),
Lane 4: pBR322 DNA + Fenton's reagent + Purpurin (100 μg/mL),
Lane 5: pBR322 DNA + Fenton's reagent + Purpurin (150 μg/mL),
Lane 6: pBR322 DNA + Fenton's reagent + Purpurin (200 μg/mL)

4. Discussion

4.1. Antioxidant activity

Bioactive compounds are well known for the prevention/ treatment of various types of human ailments viz. cardiovascular diseases, cancer etc. In last few decades, evaluation of antioxidant action of various natural products has received much attention. Antioxidants are beneficial in retarding oxidative degradation of food stuffs having high lipid content. Number of reactive oxygen species (ROS) are formed in human body such as superoxide anion, hydroxyl radical and hydrogen peroxide etc. Oxidative damage caused by ROS may result in DNA lesions, loss of functions of enzymes, increased cell permeability, disturbed cell signaling and necrotic cell death or apoptosis [26-29]. Purpurin occurs as a glycoside in the madder root (*Rubia*) together with alizarin [30]. Both purpurin and alizarin are dye molecules and possess antimutagenic potential [31-32]. The free radical scavenging activities of both compounds was checked using DPPH assay. The feature of an antioxidant molecule is its potential to trap free radicals. The antioxidant donates protons to DPPH radical resulting in change in color from purple to yellow. The decrease in absorption is taken as the measure of the extent of radical scavenging. In the present study, both molecules showed good radical scavenging potential. Alizarin scavenged DPPH radicals with IC_{50} of 20.55 μg/mL while Purpurin showed IC_{50} of 72.96 μg/mL (Table 4). Jin et al. [33] reported these two hydroxyanthraquinones (alizarin and prupurin) along with pseudopurpurin may acts as antioxidant theoretically based on functional density theory. Shikonin and its derivatives have also been reported to possess radical scavenging activity [34]. In ABTS assay, alizarin and purpurin showed IC_{50} values of 4.91 and 4.60 μg/mL respectively (Table 4). In Ferric ion reduction potential assay, alizarin and purpurin showed IC_{50} values of 62.17 and 71.27 μg/mL respectively in comparison to ascorbic acid (Table 4). Anthraquinones, juglanthraquinone A and juglanthraquinone B and 9,10-dihydro-4,8-dihydroxy-9,10-dioxoanthracene-2-carboxylic acid isolated from the stem bark of *Juglans mandshurica* showed good radical scavenging potential in DPPH and ABTS assay [35]. Anthraquinones, morindone isolated from the cell suspension culture of *Morinda elliptica* found to be active radical scavenger [36]. In TAC assay, alizarin and purpurin showed 8.28 and 10.12% antioxidant activity in comparison to ascorbic acid. Anthraquinones isolated from *Reynoutria sachalinensis* showed very high antioxidant potential [37]. Svetlana et al. [38] studied density functional theory (DFT) calculations to estimate the antioxidant activity of purpurin a naturally occurring anthraquinone pigment and antioxidant activity of purpurin was elucidated by its bond dissociation enthalpy and ionization potential. Our study provides practical proof to these density functional theory based study. Anthraquinones can acts as very good electron and hydrogen donors. Further, radical structures of these are also stable due to resonance delocalization. There are three main mechanism by which anthraquinones act as antioxidant viz. a) hydrogen atom transfer (HAT) mechanism which is based on direct free radical scavenging by transfer of the hydrogen atom to a radical; b) The chain breaking mechanism that leads to indirect hydrogen abstraction i.e named electron transfer-proton transfer (ET-PT) mechanism; c) a sequential proton loss-electron transfer (SPLET) mechanism [38]. Antioxidant activity of these compounds is difficult to understand not only because of different methods used to determine their antioxidant power but also due to different perspectives on the molecular mechanism of radical scavenging and the structural dependence of the antioxidant action [39]. Further, it is also well known that the behavior of hydroxyl moieties in compounds mainly depends on the electronic effects of the adjacent groups and the overall geometry of compounds. Both these molecules are dyes and possess good radical scavenging activity; they can be used as food additives and colorant in sweets, various food delicacies etc. Further, these phytoconstituents are of natural origin and are safer than synthetic food additives. These phytochemicals can also protect food from oxidative deterioration.

4.1. Genoprotective activity

Hydroxyl radicals are generated under aerobic conditions by Fenton's reagent resulting in oxidative damage viz. single and double strand breaks in DNA, base modification and cross linking of DNA with proteins. So, tissue exposed to hydroxyl radicals may lead to cancer, cardiovascular diseases, cataract and neurological disorders [40]. Hydroxyl radicals can react with the ribose moiety of nucleic acids by abstracting a hydrogen atom [41]. Many resultant radical intermediates can cause cleavage of the phosphodiester linkage at the 3′-position. Sometimes, hydroxyl radicals can also form adducts with the DNA bases, resulting in oxidation and ring opening [42,43]. Alizarin was found to be very good scavenger of hydroxyl radical in turn protecting plasmid DNA from damage. It protected pBR322 plasmid DNA even at low concentration of 50 μg/mL. Numerous reports are

available which showed that medicinal plants were effective in providing protection against hydrogen peroxide induced DNA damage[25,43,44]. On the other side, purpurin showed protection to pBR322 DNA only at low concentration of 50 and 100 μg/mL. Above these concentrations it itself caused damage to DNA. At these concentrations, purpurin did not cause any damage to DNA. This damaging effect is enhanced, when fenton´s reagent is also added to reaction mixture. This may because of that the purpurin may acts as prooxidant at higher concentration. It may acts as DNA protective at lower concentrations and DNA damaging at higher concentrations. It may be proposed that at higher concentrations, purpurin may bind to DNA and iron ions forming complex. It may lead to the reaction which results in the production of reactive oxygen species which may acts as DNA cleaving agent There are several reports which showed that many phytochemicals which are capable of generating ROS in presence of transition metals such as iron and copper. Presence of iron in living system may result in prooxidant action of phytoconstituents and consequently their cytotoxic effect [45,46].

The present study provides a better understanding of the antioxidant properties of the both anthraquinone molecules and also suggests the use of these anthraquinones in foods and as nutraceuticals.

Conflict of interest statement

We declare that we have no conflict of interest.

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