

Differential Free Radical Scavenging Activity and Radioprotection of *Caesalpinia Digyna* Extracts and its Active Constituent

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Caesalpinia digyna extracts/Free radical reactions/In vitro radioprotection/Polyphenolic content/Bergenin.

Two extracts E1 and E2 were prepared from the dried root of the plant *Caesalpinia digyna* by extracting with solvents of different polarity. The extracts were standardized with respect to a polyphenol, bergenin, by LC-MS analysis and they were subjected to free radical scavenging activity and *in vitro* radioprotection studies. Free radical reactions were carried out with superoxide, hydroxyl, and peroxy radicals and DPPH. In vitro radioprotecting activity was studied by following their effect on γ -radiation induced lipid peroxidation, protein carbonylation and DNA damage. The results indicated that E1 with higher free radical scavenging ability is also a more potent inhibitor of radiation induced damage to proteins, DNA and liposomes than E2. Comparing the results with those for bergenin indicated that bergenin alone is not responsible for the free radical scavenging ability and *in vitro* radioprotection. The studies also confirmed that the extracts enriched with bergenin are more effective than the isolated polyphenol, bergenin.

INTRODUCTION

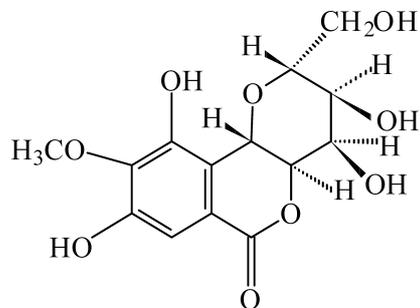
Currently, there is a growing interest in the development of natural products and plant extracts as antioxidants and anti-cancer agents.^{6,24} It has been postulated and verified by several researchers that such natural formulations are less toxic and have minimum side effects. It is therefore necessary to identify important medicinal plants and plant products and evaluate them by modern scientific tools so that such plant products can be explored as leads for the development of novel therapeutic agents. In the last one decade, we have been evaluating a number of plant extracts and in many studies, it has been found that the natural formulations are more active than the pure constituents.¹⁵

Members of the species of genus *Caesalpinia* like *Caesalpinia sappan*, *Caesalpinia bonducella*, *Caesalpinia pulcherrima* etc are known since ancient times for their medicinal properties and they are commonly used in Indian

folk medicine.¹¹ *Caesalpinia digyna* belonging to the same family is a large, scandent, prickly shrub growing wild in the shrub forests of the eastern Himalayas, Nilgiris, Ceylon, Malaya islands etc.²⁸ The plant is one of the constituents of Ayurvedic preparation known as *Geriforte* used for curing senile purities, antifatigue effect etc.¹⁷ Its root is known to possess anti-tubercular, astringent, and antidiabetic properties.^{2,22} The extract of the root also exhibited antioxidant, anti-inflammatory, neuroprotective & hepatoprotective activity.² This plant has many phytochemicals such as caesalpinine A, cellalocinine, ellagic acid, gallic acid, bergenin, bonducellin etc.⁸ In some of our earlier reports, bergenin has been isolated from the root extract of *Caesalpinia digyna*, purified and spectroscopically characterized.^{14,28} Bergenin is a hydrolysable tannin derivative, which exhibits antihepatotoxic, antiulcerogenic, anti-HIV, anti-arrhythmic, anti-malarial, anti-inflammatory, neuroprotective and immunomodulatory effects.^{9,16,21,23,27,29} It is an isocoumarin derivative with five hydroxyl groups and the chemical structure of bergenin is given in scheme-1.⁵ Although both the root and the active phenolic constituent are known to exhibit promising antioxidant and medicinal properties,²⁸ they have not been evaluated for their radioprotection capabilities. Therefore an attempt has been made to compare the radioprotection and free radical scavenging activities of the root extract with that of its major active constituent. For this, the root was extracted with methanol and acetone and two

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**Bergenin**

Scheme – 1

extracts E1 and E2 respectively have been prepared. They have been standardized with respect to bergenin content. The two extracts along with bergenin have been subjected for free radical scavenging activity and *in vitro* radioprotecting activity.

MATERIALS AND METHODS

Chemicals & general instruments

Thiobarbituric acid (TBA), butylated hydroxy toluene (BHT), 2, 2'-diphenyl picrylhydrazyl hydrate radical (DPPH), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS²⁻), phosphatidyl choline, cholesterol, bovine serum albumin (BSA), xanthine, ethylene diamine tetraacetic acid (EDTA), cytochrome-C and tris base were obtained from Sigma-Aldrich, USA. Xanthine oxidase was obtained from Calbiochem, U.S.A. The plasmid DNA (pBR322) was obtained from Bangalore Genei, India. IOLAR grade purity nitrous oxide (N₂O) gas was obtained from Indian oxygen Ltd, Mumbai. All the other chemicals were with the highest purity available and purchased locally. Solutions were made in fresh nanopure water. Absorption spectra were recorded on Hitachi U-2001 absorption spectrophotometer and ⁶⁰Co γ -source (dose rate 40 Gy/min) was used for irradiation. The absorbed dose was determined by standard Fricke dosimeter.^{10,26} LC-MS analysis was performed on HPLC Varian Prostar 140 coupled with MS Varian, IT 500 using methanol as eluant on a C18 column with mass detector.

Preparation and standardization of the extracts

The dried root of *Caesalpinia digyna* was chopped into small pieces and further dried in shade. The root was powdered and passed through sieves (mesh size 20) and 100 g of the powder was defatted with 600 ml of petroleum ether (60–80°C) and then extracted with 600 ml of methanol in a soxhlet extractor for 18–20 h. The extract was concentrated to dryness under reduced pressure and controlled temperature (40°–50°C). This is termed as extract E1. This extract

was again fractionated with acetone. This acetone soluble fraction is termed as extract E2. Acetone being less polar than methanol, E2 and E1 differ in the content of polar and non-polar constituents and E2 would contain less polar compounds than E1. Complete characterisation of the two extracts has not been attempted. Bergenin, the active constituent of the root was isolated in pure form from methanolic extract of the root and characterised by IR, NMR and mass spectrometry according to the procedure given elsewhere.^{28,30} Its purity was compared with Sigma-Aldrich sample (Catalogue No B6776).

Estimation of total phenolic content

The total phenolic content present in the extracts was measured using a modified colorimetric Folin-Ciocalteu method.³¹ Briefly, 0.5 ml of water and 0.125 ml of the aqueous solutions of the extracts (concentration range 5–22 μ g/ml) were added to a test tube. Folin-Ciocalteu reagent (0.125 ml) was added to the solution and allowed to react for 6 min. To this, 1.25 ml of sodium carbonate (7%) solution and 3 ml of water was added and the absorbance of the solution was measured at 760 nm after 90 minutes. The measurements were compared with a standard calibration curve plotted for gallic acid solution (1–10 μ g/ml) and the total phenolic content was expressed as percent (%) gallic acid equivalents.

Radical scavenging assays

The superoxide radical scavenging ability of the extracts and bergenin was studied spectrophotometrically using xanthine/xanthine oxidase method.⁴ Briefly, 1 ml total solution contained 38 mM tris- HCl buffer, 16 μ M xanthine, 10 μ M cytochrome C (Fe²⁺) and about 0.02 units/ml of xanthine oxidase enzyme. Under these conditions, the superoxide radicals generated during the reaction between xanthine and xanthine oxidase reduce cytochrome C (Fe³⁺) to produce cytochrome C (Fe²⁺) absorbing at 550 nm. When the extract or bergenin is added, and if these compounds react with superoxide radicals, the absorbance at 550 nm decreases. From the rate of reduction in absorbance at 550 nm (ΔA) the scavenging/inhibiting ability of superoxide radicals by the extracts/bergenin has been estimated according to equation 1.

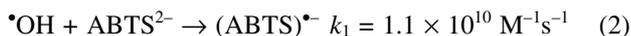
$$\% \text{inhibition} = \frac{\Delta A(\text{Uninhibited}) - \Delta A(\text{Inhibited})}{\Delta A(\text{Uninhibited}) - \Delta A(\text{Blank})} \times 100 \quad (1)$$

Here $\Delta A(\text{uninhibited})$, $\Delta A(\text{inhibited})$, and $\Delta A(\text{blank})$, represent absorbance at 550 nm in solutions containing (xanthine + xanthine-oxidase + cytochrome C), (xanthine + xanthine-oxidase + cytochrome C + extract/bergenin) and (xanthine + cytochrome C) respectively.

Relative rate constants for the scavenging of hydroxyl radical and peroxy radical by the extracts/bergenin were estimated by pulse radiolysis technique, utilizing 500 ns

pulses of 7 MeV electrons from a linear electron accelerator.¹²⁾ Aerated aqueous solution of 0.01 M KSCN was used for determining the dose per pulse.^{7,10)} which was kept at 13–15 Gy. Radiolysis of N₂O saturated water leads to the formation of hydroxyl radical predominantly.²⁶⁾ Rate constant for the reaction of hydroxyl radical with bergenin was determined by following the time dependent increase in absorbance due to the formation of transient phenoxyl radical of bergenin at 440 nm in presence of different concentration of bergenin. The observed pseudo first order rate constant was plotted against bergenin concentration and from the slope, the bimolecular rate constant was estimated.

Since plant extracts contain many active components whose concentrations are not known correctly, estimation of absolute rate constant is not possible. To overcome this problem, recently we had developed a method to estimate the comparative reactivity of the extracts based on competition kinetics¹⁵⁾ using a standard whose rate constant with hydroxyl radicals is known accurately. The reactivity of a compound towards a radical is directly related to the product of rate constant and the concentration of the compound. This reactivity parameter can be used to compare the relative ability of different extracts to react with a free radical, if the concentration of both the reactants is expressed in µg/ml. Here we employed ABTS²⁻ as the reference solute. In this method competition between ABTS²⁻ and the extracts towards hydroxyl radical was followed by monitoring the yield of ABTS^{•-} which absorbs at 645 nm.²⁵⁾ For this N₂O saturated solution of 15 µg/ml ABTS²⁻ at pH 7 (10 mM phosphate buffer) was pulse radiolysed, both in the absence and presence of varying concentration of either E1 or E2. Hydroxyl radical reacted quantitatively with ABTS²⁻ to produce ABTS^{•-}, but in the presence of either E1 or E2, a decrease in the absorbance of ABTS^{•-} was observed. From the extent of decrease, rate of scavenging of hydroxyl radical by the extracts (E1 or E2) was calculated with respect to ABTS²⁻. The reactions involved in this competition kinetic method are:



The absorbance due to ABTS^{•-} at 645 nm in the absence (A₀) and in the presence (A) of varying concentrations of extracts is related to the rate constants by the following equation 4:

$$\frac{A_0}{A} - 1 = \frac{k_2}{k_1} \frac{[\text{E1}] \text{ or } [\text{E2}]}{[\text{ABTS}^{2-}]} \quad (4)$$

Slope of the linear plot for [(A₀/A)–1] vs. [E1]/[ABTS²⁻] or [E2]/[ABTS²⁻], in which both the concentrations are expressed in µg/ml, is used to estimate the comparative ability of the extracts to react with the hydroxyl radical with respect to ABTS²⁻.

The kinetics of peroxy radical scavenging capacity of the extracts was also carried out by the competition kinetics method using pulse radiolysis technique. In this method trichloromethyl peroxy radicals (CCl₃O₂[•]) were generated by radiolysis of aerated aqueous solutions containing 48% of 2-propanol and 4% of CCl₄¹⁾ and the competition between ABTS²⁻ and the extracts towards peroxy radical was followed by monitoring the yield of ABTS^{•-} as explained above.

The reaction of DPPH with the extracts and bergenin was examined by steady state and kinetic measurements. For steady state experiments, 1 ml of 100 µM DPPH in methanol was mixed with equal volume of extract/bergenin solution prepared in methanol, and kept in dark for 20 minutes. The absorbance at 517 nm was monitored in presence and absence of different concentrations of extracts/bergenin. Kinetics of DPPH radical reactions were studied by monitoring the time dependent decay of DPPH absorbance at 517 nm using SX-18, MV stopped-flow spectrometer from Applied Photo Physics, UK.

In vitro radioprotection studies

Protein oxidation was carried out in BSA and the formation of carbonyl groups due to radiolysis in the presence and absence of different concentrations of extracts and bergenin was assayed by a standard DNPH-coupled spectrophotometric method.¹⁹⁾ One mg protein was dissolved in 1 ml of 10 mM phosphate buffer to which test compound (extracts or bergenin) was added. Control sample was prepared by substituting extracts/bergenin with buffer. Samples were saturated with N₂O and exposed to γ-radiation to an absorbed dose of 50 Gy. Following irradiation, the protein was precipitated with ice chilled 10% TCA. The pellet was suspended in 0.2% 2,2'-dinitrophenyl hydrazine (DNPH) in 2 N HCl and incubated at room temperature for 2 hr. Proteins were reprecipitated with TCA, excess DNPH was removed with several washes of 50% ethyl acetate in ethanol, the protein pellet was dissolved in 6N guanidine hydrochloride and the absorbance was measured at 370 nm (ΔA). The results are expressed in terms of formation of carbonyls/mg of protein.

Agarose gel electrophoresis was employed to follow damage to pBR322 DNA. Agarose gel (1%) was prepared in 130 mM tris-borate/2.5 mM EDTA (TBE) buffer. Ethidium bromide was included in the gel preparation at a concentration of 0.5 µg/ml to enable the visualization of the DNA bands in a UV transilluminator. The gel was submerged in an electrophoresis tank filled with TBE buffer. About 200 ng of pBR322 DNA was suspended in 20 µl of 10 mM sodium phosphate buffer, pH 7.4 and exposed to an absorbed dose of 50 Gy both in the absence and presence of varying concentration (82–328 µg/ml) of the extracts/bergenin. Control and samples were mixed with loading dye (0.25% bromophenol blue and 30% glycerol) and loaded into the wells.

Electrophoresis was carried out at 60 V for one and half hours to separate the open circular (OC) and the super coiled (SC) form of DNA. The movement of the DNA bands was visualized on a UV transilluminator. The band intensity was quantified by gelquant software (version 2.7 DNR imaging systems Ltd. Israel).¹⁹⁾

Lipid peroxidation (LPO) studies were carried out in phosphatidyl choline liposomal models, according to the methods reported earlier.^{3,20)} N₂O/O₂-purged liposomal solution was exposed to γ -radiation (to an absorbed dose of 240 Gy) in the absence and presence of different concentrations of extracts E1, E2 and bergenin at physiological pH 7.4 buffer. The extent of lipid peroxidation was estimated in terms of thiobarbituric acid reactive substances (TBARS) using 15% w/v trichloroacetic acid (TCA), 0.375% w/v TBA, 0.25 N hydrochloric acid, 0.05% w/v BHT as TBA reagent measuring the absorbance at 532 nm ($\epsilon_{532} = 1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$).

Percentage protection for lipid peroxidation and protein carbonylation was calculated according to the equation (5).

$$\% \text{Protection} = 100 - \left\{ \frac{\Delta A(\text{TestCompound})}{\Delta A(\text{Blank})} \times 100 \right\} \quad (5)$$

Here ΔA (test compound) and ΔA (Blank) indicate the absorbance either at 370 or 532 nm in the presence of extract/bergenin and in its absence respectively.

For the estimation of IC₅₀ value i.e. the concentration (in $\mu\text{g/ml}$) required to inhibit protein carbonylation, DNA damage in terms of conversion of SC to OC form and lipid peroxidation, by 50%, the changes in carbonyl levels, OC/SC ratio and TBARS levels respectively was plotted as a function of the concentration of extracts or bergenin, and from the plot, the concentration required to reduce the activity by 50% was identified as the IC₅₀ value.

RESULTS

Polyphenolic content and standardization of the extracts

The total phenolic content in E1 and E2, estimated in terms of gallic acid by Folin-Ciocalteu method was found to be one μg of the extract as equivalent to 0.42 ± 0.04 and $0.34 \pm 0.03 \mu\text{g}$ of gallic acid respectively. This number indicates that the extracts are rich in phenols and to further standardize them, LC-MS analysis was carried out.

Since the *Caesalpinia digyna* plant extracts contain bergenin as one of the major constituents, the two extracts have been standardized with respect to the bergenin content by LC-MS analysis. Initially LC-MS analysis of bergenin in methanol was carried out and the peak at mass units 327.8 was identified (Fig. 1). A calibration plot was made for the peak area as a function of bergenin concentration in the range of 20 to 100 ppm (Inset of Fig. 1) and used to estimate

bergenin content in the extracts. Figure 1 shows the LC-MS analysis of the two extracts confirming the presence of bergenin by mass peak at mass units 327.8 and from the calibration plot, the bergenin content was estimated to be $39 \pm 2.2\%$ and $27 \pm 1.9\%$ respectively in E1 and E2. The extracts thus standardized with bergenin content were studied for free radical scavenging ability and in vitro antioxidant activity.

Free radical scavenging studies of the extracts and bergenin

For these studies superoxide radicals generated from xanthine/xanthine-oxidase¹³⁾ method, hydroxyl and peroxy radicals generated by water radiolysis²⁶⁾ and DPPH (2,2'-diphenyl-1-picrylhydrazide), a stable free radical³²⁾ were employed.

The IC₅₀ values for the scavenging of superoxide radicals by the extracts and bergenin were estimated and listed in Table 1. It can be seen that E1 is more effective than E2 in scavenging the superoxide radicals and its activity is even more than that of pure bergenin. Using nanosecond pulse radiolysis technique, the reactions of hydroxyl radicals with the extracts and bergenin were carried out in microsecond time scale and the transient spectra obtained in all these reactions are given in Fig. 2. The transient spectra obtained from the reaction of hydroxyl radical with the extracts at 10 μs after the pulse, showed broad spectrum from 300 to 700 nm wavelength regions. Under similar conditions, the transient produced by the reaction of hydroxyl radical with bergenin exhibits absorption spectrum in the same wavelength region, but the maximum appeared much sharper at $\sim 425 \text{ nm}$. By comparing the spectra it was noticed that the hydroxyl radical reaction with the extracts and bergenin produced similar transients, but the transient obtained by the reaction with extracts showed much broader spectra due to the presence of other species which are not identified. The rate constant for the reaction of hydroxyl radical with bergenin at pH 7 was independently determined by pulse radiolysis as $3.3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. The reactivity of the extracts with hydroxyl radicals was estimated by employing competition kinetics and inset of Fig. 2 shows linear plots for the variation of the absorbance due to ABTS⁻ as a function of the ratio of concentrations of E1 or E2 with ABTS²⁻ according to equation 4. From the slope of the linear plots the relative reactivity of extracts towards hydroxyl radical was calculated and the values are listed in Table 2. In a similar fashion, the reactivity parameters of the extracts and bergenin with, CCl₃O₂[•] radical have been estimated and the results listed in Table 2.

DPPH exhibits a strong absorption maximum at 517 nm, and is purple in color. Its color turns from purple to yellow, when the odd electron of DPPH radical becomes paired due to reduction by antioxidant (A-H).¹⁸⁾



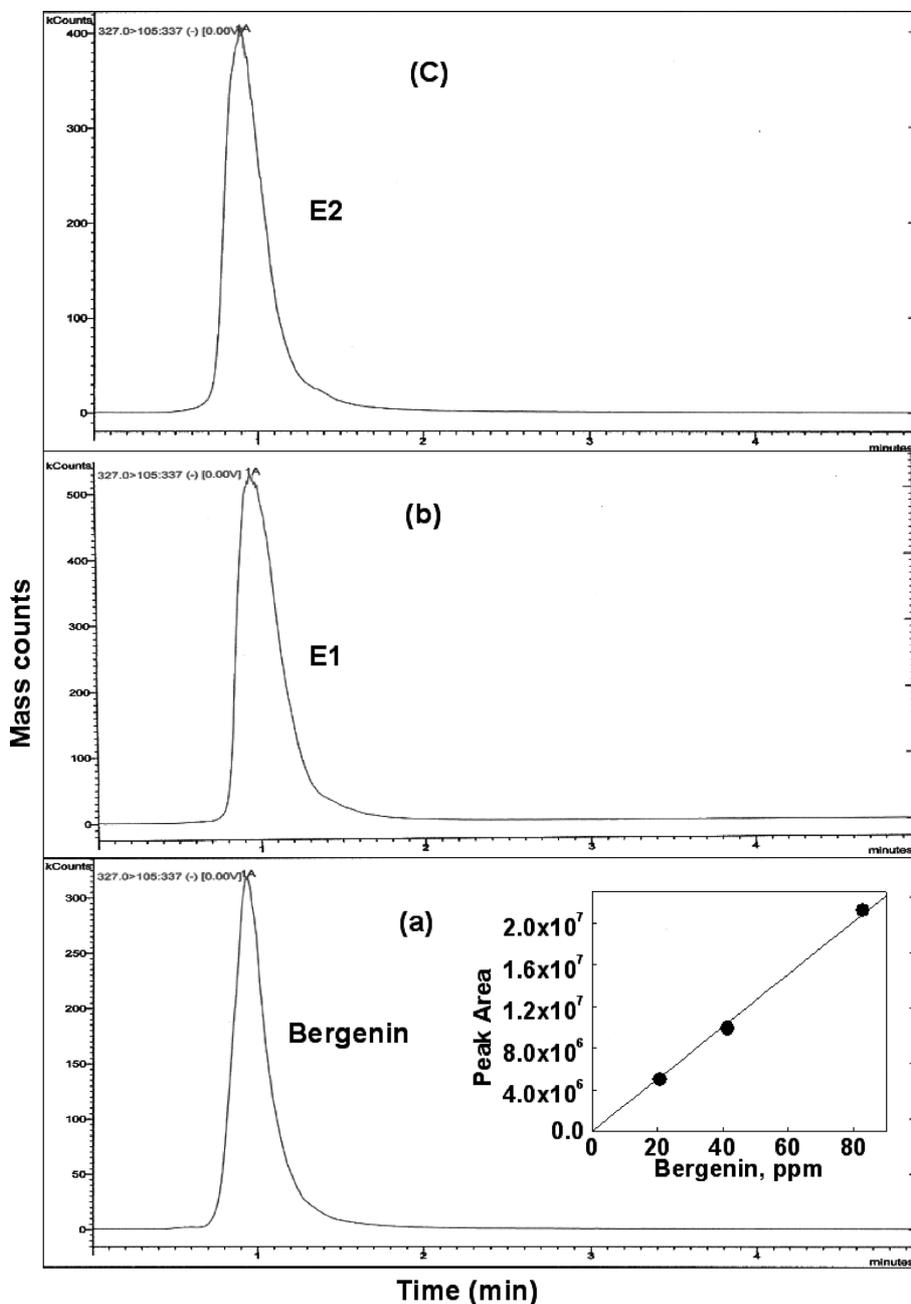


Fig. 1. LC-MS records of (a) bergenin (b) E1 (C) E2. The chromatograms were recorded by using analytical C18 column, methanol as eluant and mass detector set at mass unit 327.8. Inset shows calibration plot for estimation of bergenin.

Table 1. *In vitro* antioxidant activity of two different extracts of *Caesalpinia digyna* root and bergenin. E1- methanol extract, E2- acetone extract.

Fractions/ Compound	IC ₅₀ values (µg/ml) for scavenging/inhibition of free radicals and radiation induced damage to cellular organelles				
	DPPH	DNA Damage (50 Gy)	Inhibition of superoxide radicals	Protein carbonylation (50 Gy))	Lipid peroxidation (240 Gy))
E1	2.66 ± 0.13	69 ± 3.5	6.6 ± 0.3	3.4 ± 0.2	14.6 ± 0.7
E2	4.97 ± 0.24	124 ± 6	8.9 ± 0.4	3.7 ± 0.2	27.3 ± 1.4
Bergenin	377.5 ± 18.8	82 ± 4	23.2 ± 1.2	14.2 ± 0.7	34.0 ± 1.7

To study the reaction of DPPH radical with the extracts, both steady state and time resolved techniques were employed. Addition of E1, E2 or bergenin, changed the color of the DPPH solution. Following the absorbance changes at 517 nm due to DPPH radical in methanol in the absence and presence of different concentration of the extracts and bergenin, the IC_{50} value was estimated and listed in Table 1 for E1 and E2. These studies indicate that E1 is more reactive than either E2 or bergenin. The relatively large IC_{50} value for bergenin suggests that bergenin itself in pure form is not a good scavenger of free radicals but the extracts showed much better effect. The kinetics of DPPH radical reaction with bergenin and the extracts was also studied. DPPH being a stable radical showed no appreciable decrease in absorbance when monitored as a function of time. But in the presence of the extracts, the absorbance decreased. Inset (A) of Fig. 3 shows two representative absorption-decay traces at 517 nm in the absence and presence of the extract. By fitting this absorbance-decay profile to an exponential function, the observed decay rate constant

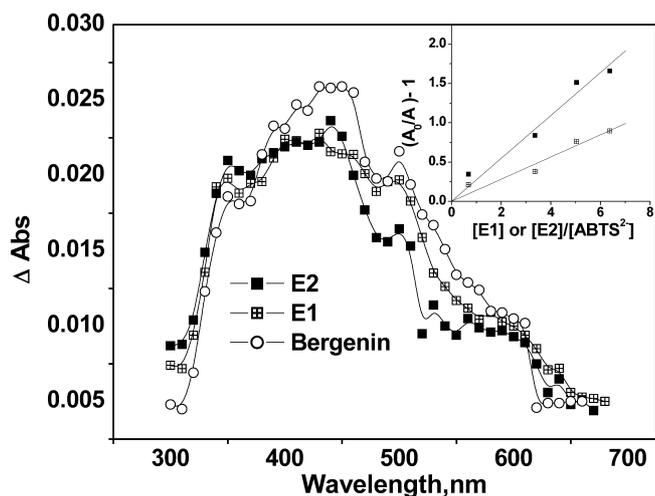


Fig. 2. Difference absorption spectrum of the transient obtained on pulse radiolysis of N_2O saturated aqueous solutions containing 100 $\mu\text{g/ml}$ of E1, E2 and bergenin at pH 7. Inset shows linear plot for competition kinetics between $ABTS^{2-}$ and E1 or E2 towards hydroxyl radicals. Lines show fitting according to equation (4).

(k_{obs}) is obtained. The k_{obs} values increased linearly with increasing extract concentration as given in Fig. 3. It can be seen from the figure that between the two, E1 showed higher reactivity than E2. Similar experiments have been carried out with bergenin (Inset B of Fig. 3), and the bimolecular rate constant for the reaction of bergenin with DPPH was found to be $88.1 \text{ M}^{-1}\text{s}^{-1}$. Comparing the reactivity of E1 and E2 and the above determined rate constant for bergenin, it was found that one microgram of E1 or E2 respectively is equivalent to 9 and 18.8 μg of bergenin in exhibiting reaction with DPPH radicals.

In vitro radioprotection studies

In vitro radioprotecting ability of the extracts was evaluated in terms of inhibition of γ -radiation induced protein carbonylation in BSA, DNA damage in plasmid pBR322 and lipid peroxidation in liposomes. BSA was subjected to irradiation in the absence and presence of different concentrations of the extracts and protein carbonylation was assessed. From these results, the IC_{50} values were estimated and the

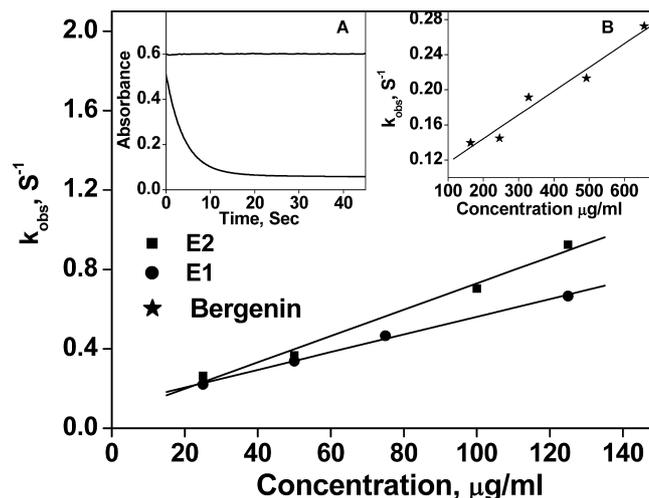


Fig. 3. Variation in observed rate constant for the decay of DPPH in presence of the extracts. Inset (A) shows representative decay trace of DPPH radical (125 μM) in absence and presence of E1 (100 $\mu\text{g/ml}$). Inset (B) shows linear plot for the variation of observed rate constant for the decay of DPPH radical on reaction with bergenin.

Table 2. Comparative rate constants for the reaction of different radicals with the extracts and bergenin. E1- methanol extract, E2- acetone extract.

Extracts/Compound	Hydroxyl radical ($\cdot\text{OH}$) (Times of $ABTS^{2-}$)	Peroxy radical ($\text{CCl}_3\text{O}_2^{\cdot}$) ^a (Times of $ABTS^{2-}$)	DPPH radical $k_{\text{obs}}(\text{s}^{-1})$ (at 125 $\mu\text{g/ml}$)
E1	0.14 ± 0.09	1.8 ± 0.1	0.92 ± 0.09
E2	0.26 ± 0.10	4.1 ± 0.2	0.66 ± 0.07
Bergenin	$3.3 \pm 0.2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$	$4.2 \pm 0.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$	0.14 ± 0.03^a

^a Calculated at 250 $\mu\text{g/ml}$.

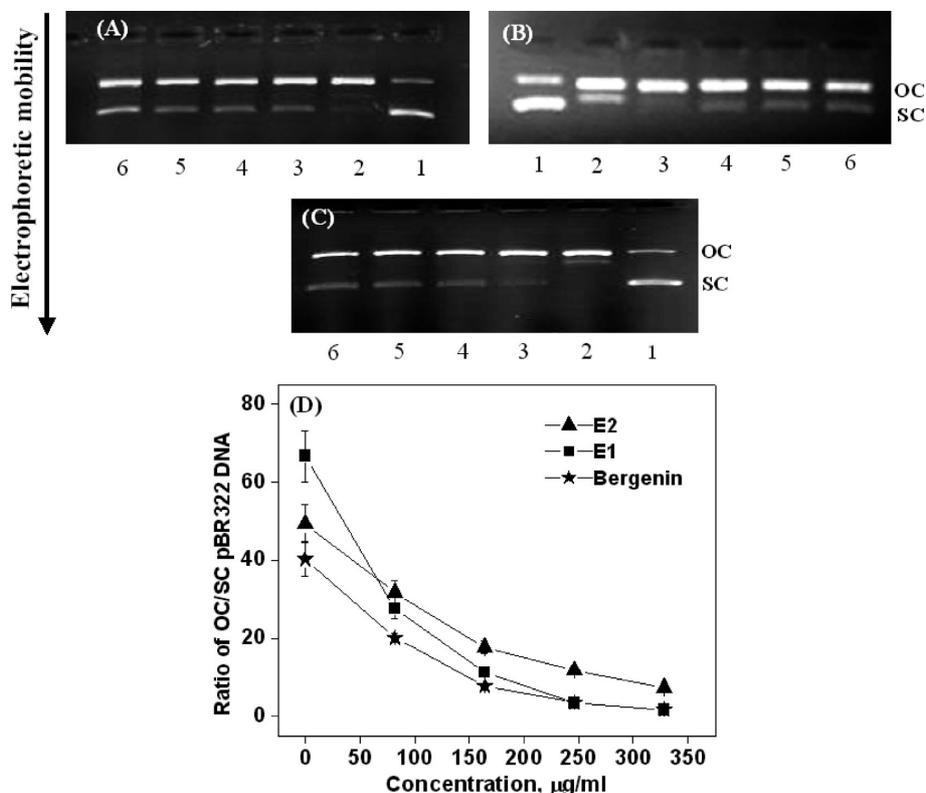


Fig. 4. Agarose gel electrophoretic pattern, depicting super coiled and nicked open circular forms of plasmid DNA protection by varying concentrations of E1 (A), E2 (B) and bergenin (C) against 50 Gy γ -radiation induced strand breaks. The arrow indicates the direction of electrophoretic mobility. Lane 1: pBR 322 control, Lane 2: pBR 322 exposed to γ -radiation of 50 Gy; lanes 3 to 6 exposed to radiation dose of 50 Gy in presence of the 82, 164, 246, 328 μ g/ml of extracts and bergenin. (D) Line graph showing ratio of OC/SC form as a function of increasing concentrations of E1, E2 and bergenin.

values are listed in Table 1 along with that for bergenin under similar conditions. The results indicated that both E1 and E2 exhibit almost similar protection towards BSA against radiation-induced damage. E1 is slightly more potent than E2. Bergenin however is not so effective as compared to either of the extracts tested.

Similarly, effect of the extracts or bergenin on γ -radiation induced damage in pBR322 DNA was studied. On exposure to γ -radiation, the SC form of DNA got converted to the OC form and in presence of extracts or bergenin, there is a decrease in the damage to the SC form. Figure 4A, 4B, and 4C show agarose gel electrophoresis pattern where, lane 1 is the unirradiated control, lane 2 is irradiation control and lanes 3, 4, 5, and 6 show the pattern for the irradiated plasmid DNA in the presence of varying concentrations of E1, E2, and bergenin. The intensities of OC and SC form of plasmid DNA were measured under different treatment condition and the ratio of OC/SC was plotted as a function of the concentration of extracts or bergenin (Fig. 4 D). From this the IC_{50} values were estimated and listed in Table 1. In these studies E1 was found to be superior to E2. However bergenin under these conditions showed similar protection

as E1.

Inhibition of lipid peroxidation by the extracts was monitored by TBARS formation in liposomal solution in the absence and presence of different concentrations of E1, E2 and bergenin after exposing to γ radiation. The IC_{50} values for the inhibition of TBARS are listed in Table 1. The results too indicated that among the three, E1 exhibits higher protection towards liposomes than either E2 or bergenin against radiation-induced damage.

DISCUSSION

Caesalpinia digyna, a herb used in Indian folk medicine, is known to possess antioxidant activity and the plant is rich in polyphenols like bergenin. Since herbal extracts are being considered as alternate therapeutic agents, the dried plant root is extracted with solvents of varying polarity and two active extracts have been prepared and they have been standardized with respect to bergenin. The bergenin content in these extracts was nearly 30%. These extracts were subjected to free radical reactions and in vitro radioprotection ability and the results were compared with those of bergenin.

The two extracts E1 and E2 and bergenin scavenged free radicals like hydroxyl radicals, superoxide radicals and DPPH to a different extent. Bergenin itself is a good scavenger of hydroxyl radicals but not so effective in scavenging other free radicals like superoxide radical, and DPPH. Reaction with hydroxyl radicals is non-specific, while reaction with the other radicals is more specific and considered to be important in evaluating the antioxidant activity of new compounds. From these studies, it can be considered that the extracts are more effective inhibitors of antioxidant-specific free radicals than bergenin. Similarly the *in vitro* radioprotecting ability of the extracts when compared, indicated that E1 exhibits higher radioprotection than the other extract. Bergenin in comparison is equally effective in inhibiting DNA damage but is less effective than the two extracts in inhibiting radiation induced damage to proteins and lipid peroxidation to liposomes. Both protein carbonylation and lipid peroxidation proceed through free radical mechanism, therefore it is not surprising that extracts that scavenge specific free radicals are more effective inhibitors of protein carbonylation and lipid peroxidation. Radiation induced DNA damage too can be mediated through free radicals. But bergenin showing good protection to DNA, and also being a good scavenger of hydroxyl radicals indicates that hydroxyl radical scavenging reaction is more important for the DNA protecting ability. Inhibition of radiation induced DNA damage by bergenin and bergenin rich extracts indicates that such extracts can be explored as potent radio protectors and therefore warrants future *in vivo* studies.

Our estimation of total polyphenolic content in the extracts indicate that they contain some polyphenolic substances other than bergenin, and these compounds may be responsible for the higher free radical scavenging power of the extract. Antioxidants derived from polyphenols on reaction with oxidizing free radicals generate the phenoxyl radicals. The reactivity of these phenoxyl radicals depends on the substitution and chemical structure and this determines whether free radical scavenging leads to antioxidant action or not. If the resultant antioxidant radicals are reactive they can even induce more damage to the biomolecules. The spectra of the transient obtained on reaction of hydroxyl radicals with the extracts and bergenin by the pulse radiolysis method indicate that other phenols present in the extract are also reactive towards the free radicals. In such cases, the reactions can lead to synergism and thereby increase the efficacy of the antioxidant mixture. Therefore an antioxidant rich formulation can be more potent than any one of the constituents.

In conclusion, all these results clearly confirm that the two extracts of *Caesalpinia digyna* containing bergenin, exhibit superior free radical scavenging ability and radioprotecting ability than their major polyphenolic constituent, bergenin. These results further support to the currently developing concepts that the plant extracts in natural formulations show

more potent biological activity than their active constituent.

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REFERENCES

1. Alfassi, Z. B., Mosseri, S. and Neta, P. (1987) Halogenated alkyl peroxy radicals as oxidants: Effects of solvent and of substituents on rates of electron transfer. *J. Phys. Chem.* **91**: 3383–3385.
2. Badami, S., Moorkoth, S., Rai, S. R. M. S., Kannan, E. and Bhojraj, S. (2003) Antioxidant activity of *Caesalpinia sappan* heartwood. *Biol. Pharmaceut. Bull.* **26**: 1534–1537.
3. Barik, A., Mishra, B., Shen, L., Mohan, H., Kadam, R. M., Dutta, S., Zhang, H.-Y. and Priyadarsini, K. I. (2005) Evaluation of a new copper (II)-curcumin complexes as superoxide dismutase mimic and its free radical reactions. *Free Radic. Biol. Med.* **39**: 811–822.
4. Barik, A., Mishra, B., Kunwar, A., Kadam, R. M., Shen, Li., Dutta, S., Padhye, S., Satpati, A. K., Zhang, H. Y. and Priyadarsini, K. I. (2007) Comparative study of copper II curcumin complexes as superoxide dismutase mimics and free radical scavenger. *Eur. J. Med. Chem.* **42**(4): 431–439.
5. Boonsri, S., Chantrapromma, S., Fun, H. K., Karalai, C., Kanjana-Opas, A. and Anjum, S. (2005) 2,3-Dihydro-7-hydroxy-3-[(4-methoxyphenyl)-methylene]-4H-1-benzopyran-4-one. *Acta Crystal. E* **61**: 3930–3932.
6. Bors, W., Heller, W., Michel, C. and Stettmaier, K. (1996) *Flavonoids and Polyphenols : Chemistry and Biology* In Handbook of Antioxidants. New York Ed Candies, E., Packer, L., Decker, p. 409.
7. Buxton, G. V. and Stuart, C. R. (1995) Re-evaluation of the Thiocyanate Dosimeter for Pulse Radiolysis. *J. Chem. Soc. Faraday Trans.* **91**: 279–281.
8. Chaudhry, G. R., Sharma, V. N. and Dhar, M. L. (1954) Chemical examination of the roots of *Caesalpinia digyna*. *J. Sci. Ind. Res. Part B* **13**: 147–148.
9. Da Silva, T. B. C., Alves, V. L., Mendonca, L. V. H., Conserva, L. M., Da Rocha, E. M. M., Andrade, E. H. A. and Lemos, R. P. L. (2004) Chemical Constituents and Preliminary Antimalarial Activity of *Humiria balsamifera*. *Pharmaceut. Biol.* **42**: 94–97.
10. Fielden, E. M. (1984) Chemical dosimetry of pulsed electron and X-ray sources in the 1-20 MeV range. In *The Study of Fast Processes and Transient Species by Electron Pulse Radiolysis* (pp 59). Boston: (Baxendale, J. H., Busi, F., Eds.; D. Reidel).
11. Gautam, R., Saklani, A. and Jachak, S. M. (2007) Indian medicinal plants as a source of antimycobacterial agents. *J. Ethnopharmacol.* **110**: 200–234.
12. Guha, S. N., Moorthy, P. N., Kishore, K., Naik, D. B. and Rao, K. N. (1987). One electron reduction of thionine studied

- by pulse radiolysis. Proc. Indian Acad. Sci. (Chem. Sci.) **99**: 261–271.
13. Halliwell, B. and Gutteridge, J. M. C. (1989) Free radic. Biol. Med. Oxford: Clarendon Press.
 14. Hay, J. E. and Haynes, L. J. (1958) Bergenin, a C-glycopyranosyl derivative of 4-O-methyl gallic acid. J. Chem. Soc. **453**: 2231–2238.
 15. Kaviarasan, S., Naik, G. H., Gangabhairathi, R., Anuradha, C. V. and Priyadarsini, K. I. (2007) In vitro studies on anti-radical and antioxidant activities of fenugreek (*Trigonella foenum graecum*) seeds. Food Chem. **103**: 31–37.
 16. Kim, H. S., Lim, H. K., Chung, M. W. and Kim, Y. C. (2000) Antihepatotoxic activity of Bergenin, the major constituent of *Mallotus japonicus*, on carbon tetrachloride-intoxicated hepatocytes. J. Ethnopharmacol. **69**: 79–83.
 17. Kulkarni, R. D. and Khandeparkar, U. K. (1981) Antifatigue effect of indigenous drug “Geriforte” in rats. Ind. Drugs **10**: 346–350.
 18. Kumar, S. S., Priyadarsini, K. I. and Sainis, K. B. (2002) Free radical scavenging activity of vanillin and o-vanillin using 1,1-diphenyl –2-picrylhydrazyl (DPPH) radical. Redox Rep. **7**: 35–40.
 19. Mishra, H. S., Khairnar, N. P., Barik, A., Priyadarsini, K. I., Mohan, H. and Apte, S. K. (2004) Pyrroloquinoline and Quinone a reactive oxygen species scavenger in bacteria. FEBS Lett. **58**: 26–30.
 20. Naik, G. H., Priyadarsini, K. I. and Mohan, H. (2006) Free radical scavenging reactions and phytochemical analysis of triphala an ayurvedic formulations. Curr. Sci. **90**: 1100–1104.
 21. Nazir, N., Koul, S., Qurishi, M. A., Taneja, S. C., Ahmad, S. F., Bani, S. and Qazi, G. N. (2007) Immunomodulatory effect of bergenin and norbergenin against adjuvant- induced arthritis – A flow cytometric study. J. Ethnopharmacol. **112**: 401–405.
 22. Patel, M. R., Bellare, R. A. and Delivala, C. V. (1966) Anti-tubercular action of *Caesalpinia digyna* Rottl. Roots. Indian J of Exp. Biol. **4**: 214–215.
 23. Piacente, S., Pizza, C. and Detommasi, N. (1996) Constituents of *Ardisia Japonica* and their invitro anti HIV activity. J. Nat. Prod. **59**: 565–569.
 24. Pokorny, J., Yanishlieva, N. and Gordon, M. (2001) *Antioxidants in Food*. CRC press, New York.
 25. Scott, S. L., Chem, W. J., Bakae, A. and Espenson, J. (1993) Spectroscopic parameters, electrode potentials, acid ionization constants and electron exchange rates of the 2,2'-azinobis(3-ethyl benzo thiazoline-6-sulfonate) radicals and ions. J. Phys. Chem. **97**: 6710–6714.
 26. Spinks, J. W. T. and Woods, R. J. (1991) *Introduction to radiation chemistry*. New York: Wiley, p. 243.
 27. Sridhar, C., Krishnaraju, A. V. and Subbaraju, G. V. (2006) Anti-inflammatory constituents of *termanius libialis*. Int. J. Pharma. Sci. **68**: 111–114.
 28. Srinivasan, R., Chandrasekar, M. J. N., Nanjan, M. J. and Suresh, B. (2007) Antioxidant activity of *Caesalpinia digyna* root. J. Ethnopharmacol. **113**: 284–291.
 29. Takahashi, H., Kosaka, M., Watanabe, Y., Nakade, K. and Fukuyama, Y. (2003) Synthesis and Neuroprotective activity of Bergenin and Norbergenin. Bioorg. Med. Chem. **11**: 1781–1788.
 30. Taneyama, M., Yoshida, S., Kobayashi, M. and Hasegawa, M. (1983) Isolation of norbergenin from *saxifraga stolonifera*. Phytochem. **22**: 1053–1054.
 31. Wolfe, K., Wu, X. and Liu, R. H. (2003) Antioxidant Activity of Apple Peels. J. Agric. Food Chem. **51**: 609–614.
 32. Yokozawa, T., Chen, C. P., Dong, E., Tanaka, T., Nonaka, G. I. and Nishioka, I. (1998) Study on the inhibitory effect of tannins and flavonoids against DPPH radical. Biochem. Pharmacol. **56**: 213–222.

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