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Research Article

IN VITRO STUDIES ON NEPHROPROTECTIVE EFFICACY OF *CYNODON DACTYLON* AND *GMELINA ASIATICA*

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ABSTRACT

Introduction: *Cynodon dactylon* and *Gmelina asiatica* plants are used since ages in ayurveda and siddha systems of medicine for treating various ailments. In the present paper, phytochemical analysis of these plant sources and their antioxidant and nephroprotective efficacy studies carried out through *in vitro,* method are presented and discussed. The phytochemical analysis of aqueous extracts of these plants revealed the presence of high concentration of secondary metabolites such as phenolic acids, flavonoids, and proanthocyanidin, which were determined using gallic acid and quercetin as standards.

Materials and Methods: Antioxidant activity of each extract was evaluated using *in vitro* assays such as (1,1-diphenyl picrylhyrazyl) radical scavenging assay, reducing power assay, nitric oxide scavenging activity, superoxide scavenging activity, nicotinamide adenine dinucleotide assay, lipid peroxidation assay and thiobarbituric assay. *In vitro* anti-inflammatory plant drugs were evaluated using *in vitro* methods like inhibition of protein denaturation, membrane stabilization assay and proteinase inhibitory assay. Acetyl salicylic acid and butyl hydroxyl toluene were used as standard for anti-inflammatory and anti-oxidant assays, respectively.

Results: Selected plants have shown potent protective activity against free radical which is evident through data obtained in various antioxidant assays. All the extracts tested revealed a protective effect on red blood cells against heat induced membrane damage and proteinase inhibition, which depicted its vital role in maintaining the integrity of the cell membrane. The promising results obtained through *in vitro* anti-oxidant and anti-inflammatory assay prompted us to evaluate nephroprotective potential of these plants using DNA fragmentation assay, epifluorescence assay and cytoprotective assay. Normal kidney cells (vero cells) were used for epiflourescence dual staining and DNA fragmentation assay using vitamin E as a positive control.

Conclusion: The data of the results obtained suggested that the selected plants can contribute in the development of human friendly antioxidant, anti-inflammatory, and nephroprotective agent. Attempts were also made to understand the propable mechanism of action through *in silico* approaches.

Keywords: Anti-inflammation, Antioxidant, *Cynodon dactylon,* DNA fragmentation, Ethnomedicine, *Gmelina asiatica*, *In vitro, In silico,* Nephroprotective.

INTRODUCTION

Plants as a source of medicine has been an ancient practice and is an important component of the health care system in India. The ethno botany and ubiquitous plants provide a rich resource for natural drug research and development. Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads, because of unmatched availability of chemical diversity [1]. According to World Health Organization, more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic, as well as infectious diseases [2]. Due to the development of adverse effects and microbial resistance to the available chemically synthesized drugs, recent medicinal researches are focusing toward ethnopharmacognosy. It was found that literally thousands of phytochemicals from plants are safe and are alternatives with less adverse effects [3]. In the present study, we evaluated the therapeutic potentials of selected medicinal plants like *Cynodon dactylon* (a hardy perennial grass) and *Gmelina asiatica* (a bush with yellow flowers), which are widely distributed in India, and various parts of these plants are bestowed with immense medicinal properties. Several biological activities have been already reported for *C. dactylon* such as [4], antiulcer [5], diuretic [6], antimicrobial [7], hepatoprotective [8], cardio protective [9] and immunomodulatory [7]. Leaves of *G. asiatica* are used in ayurvedic medicine [10] and also finds use in ethnomedicine [11]. The phytochemical analysis of aqueous extracts of these plants revealed the presence of many secondary metabolites like flavonoids, phenolic acids, and proanthocyanidins. Many beneficial biological activities such as, antimicrobial, antioxidant, anti-diarrheal, analgesic and wound healing activity have also been reported for this plant [12]. In the present study, focus is made toward understanding the mode of action for various therapeutic potential of these two plants employing *in vitro* and *in silico* approaches.

MATERIALS AND METHODS

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, thiobarbutric acid (TBA), potassium ferricyanide, ferricchloride, trichloroacetic acid (TCA), aluminum chloride, potassium persulfate, ammonium persulfate, ferrous sulfate, sodium salicylate, ammonium molybdate, sodium carbonate, aluminum chloride, sodium nitrate, sodium hydroxide, Folin-Ciocalteu's phenol reagent, ferrous chloride, sodium hydroxide, acetyl salicylic acid, bovine serum albumin, red blood cell (RBC), trypsin, acridine orange, ethidium bromide, gentamicin, vitamin E, phosphate buffer, nicotinamide adenine dinucleotide phosphate buffer.

Plant material

C. dactylon whole plant and *G. asiatica* leaves were collected from in and around SASTRA University, Thanjavur district. Identification and authentication was done by Dr. N Ravichandran, Botanist, CARISM;

SASTRA University, using herbarium samples deposited at Rapinat Herbarium St. Joseph College, Trichy.

Extraction

Each plant material was shade dried and coarsely powdered. Aqueous extract was made using 1 kg plant powder dissolved in 2500 mL of respective solvent and were extracted for 48 hr. After incubation, the extracted material was filtered, and the collected filtrate was distilled and concentrated using rota evaporator, followed by freeze drying. Appropriate amount of each extracted powder was then used for all biological assays.

Phytochemical screening

Preliminary phytochemical screening was carried out for the aqueous extract of *G. asiatica* (AEGA), and *C. dactylon* (AECD) extracts selected for the study to detect the presence of different primary and secondary metabolites present in them.

Determination of total phenolic content

The concentration of phenolic compounds in an aqueous extract was determined using the method of Singleton *et al.* [13]. Aqueous extract in various concentrations (10-1000 µg/mL) were used for the analysis. 50 µL of 10% Folin-Ciocalteu reagent and 400 µL of Na_2CO_3 (2% w/v) was added to 50 µL of each sample (three replicates) of plant extract. The resulting mixture was incubated at 5°C with continuous shaking for 15 minutes. The absorbance of each sample was measured at 765 nm using NanoDrop 2000 series spectrophotometer (Thermo Scientific, USA). The samples were prepared in triplicates for each analysis, and the mean value of the absorbance was obtained. The same procedure was repeated for the standard gallic acid. Based on the measured absorbance, the concentration of phenol was determined from the standard curve. Then, the phenolic content in different concentration was measured as gallic acid equivalent (GAE).

Estimation of total flavonoid content

The content of the flavonoid in the sample extract was measured using aluminum chloride colorimetric method [14]. About 200 µL of sample was mixed with 600 µL of methanol, 40 µL of 10% aluminum chloride, 40 µL of 1 M potassium acetate and 1.12 mL of distilled water and incubated at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 420 nm with UV NanoDrop 2000 series spectrophotometer (Thermo Scientific, USA). The content was determined from extrapolation of the standard curve which was made by preparing quercetin solution (0-0.8 mg/mL) in distilled water. The concentration of flavonoid was expressed as quercetin equivalent (QE) from the standard curve in terms of mg/mL.

Determination of proanthocyanidins

The content of the proanthocyanidin was determined based on the procedure Sun *et al.* [15]. The mixture of 600 µL of vanillin-methanol (4% v/v), 300 µl of hydrochloric acid was added to 100 µL of aqueous extract and vortexed. The resulting mixture was allowed to stand for 15 minutes at room temperature, followed by reading at the 500 nm absorbance using UV NanoDrop 2000 series spectrophotometer (Thermo Scientific, USA). Total proanthocyanidin content was expressed as GAE (mg/mL) from the standard curve.

Biological assays

Anti-inflammatory activity

Inhibition of protein denaturation assay

Methods of Mizushima and Kobayashi (1968) and Sakat *et al.* (2010) [16] were followed. The reaction mixture was consisting of 100 µL of test extract at different concentrations of (50, 100, 250, 500 µg/mL) and 500 µL of 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted by adding a small amount of 1 N HCl. The samples were incubated at 37°C for 20 minutes and then heated at 57°C for 20 minutes. After cooling the sample, the turbidity was measured using a NanoDrop 2000 series spectrophotometer (Thermo Scientific, USA) at 660 nm. The experiment was performed in triplicates. The percentage inhibition of the protein denaturation was calculated as follows:

$$\% \text{ inhibition} = ([\text{Abs control} - \text{Abs sample}]/\text{Abs control}) \times 100 \quad (1)$$

Membrane stabilization assay

Preparation of RBC suspension

Fresh whole human blood (10 mL) was collected and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 minutes and were washed three times with an equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline (Sadique *et al.*, 1989; Saket *et al.*, 2010) [16].

Heat induced hemolysis

Samples of the extract with different concentrations along with 1 mL of 10% RBC suspension, saline was taken as blank, while aspirin was taken as a positive control. All the centrifuge tubes containing reaction mixture were incubated at 56°C for 30 minutes in a regulated water bath. On termination of incubation, the tubes were cooled under running tape water. The reaction mixture was centrifuged at 2500 rpm for 5 minutes, and the supernatants were absorbed at 560 nm. The experiment was performed in triplicates for all the test samples. The percent inhibition of hemolysis by the extract was calculated as follows:

$$\% \text{ Inhibition of hemolysis} = 1 - (OD_2 - OD_1/OD_3 - OD_1) \times 100$$

Where OD_1=Absorbance of test sample unheated.
OD_2=Absorbance of test sample heated.
OD_3=Absorbance of the control sample heated.

Protease inhibitory assay

The assay was performed according to the modified method of Oyedepo *et al.* (1995) and Sakat *et al.* (2010) [16]. One mL aqueous sample extract of different concentration was added to the reaction mixture containing 0.06 mg trypsin, 1 mL of 20 mM Tris HCl buffer (pH 7.4). The above mixture was incubated at 37°C for 5 minutes and then 1 mL of 0.8% (w/v) casein was added. The mixture was inhibited for an additional 20 minutes, 2 mL of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged, and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicates. The percentage of inhibition of proteinase inhibitory activity was calculated using the following equation:

$$\% \text{ inhibition} = ([\text{Abs control} - \text{Abs sample})/\text{Abs control}) \times 100$$

Anti-oxidant capacity

Determination of DPPH radical scavenging activity

DPPH radical scavenging activity has been largely used as quick and reliable parameter to assess the *in vitro* general antioxidant activity of plant extracts. The ability of the extract to scavenge DPPH radicals were determined by the method of Gyamfi *et al.*, 2002 [17]. Different concentrations of extract like 1000, 500, 250, 12.5, and 62.5 µg/mL were used for studies. DPPH stock solution: 1.3 mg of DPPH was dissolved in 1mL of methanol. Two hundred µL of DPPH stock was added to different concentrations of extract after 20 minutes of incubation at 37°C in dark. Optical density of sample/control was measured at 517 nm using a NanoDrop 2000 series spectrophotometer (Thermo Scientific, USA). Percentage of inhibition was determined using the equation mentioned above.

Determination of reducing power

The reducing power of the extract was evaluated according to the method of Oyaizu 1986 [18]. Two hundred µL of sample dissolved in

distilled water was added to the mixture containing 500 µL of 0.2 M phosphate buffer (pH 6.6) and 500 µL of $K_3Fe(CN)_6$ (1% w/v). The above mixture was incubated at 50°C for 20 minutes, followed by the addition of 2.5 mL of TCA (10% w/v). The mixture was centrifuged at 3000 rpm for 10 minutes to collect the upper layer of the solution (500 µL), mixed with distilled water (500 µL) and 100 µL of $FeCl_3$ (0.1%, w/v). The absorbance was then measured at 700 nm against blank sample.

Scavenging activity of superoxide anion

Measurement of superoxide radical scavenging activity of sample extracts was done using the standard method (Nishikimi *et al.* 1972) [14]. The sample extract was added to the reaction mixture containing 200 µL of potassium ferricyanide in phosphate buffer (0.1 M, pH 7.4) and 200 µL of NADH in 166 µM phosphate buffer. Incubated at 25°C for 5 minutes and the absorbance were read at 560 nm against butylated hydroxytoluene as control. Percentage inhibition was calculated.

Hydrogen peroxide scavenging activity

Scavenging of hydrogen peroxide was measured by the method of Ruche *et al.* 1989 [14]. All the solutions were prepared freshly. Aqueous plant extract was prepared in different concentrations (10, 20, 50, 100, 200, 500, and 1000 µg/mL) and mixed with 150 µL of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4). The reaction mixture was incubated for 10 minutes. The absorbance of the solution was measured at 230 nm using NanoDrop 2000 series spectrophotometer (Thermo Scientific, USA) against a blank solution containing the plant extract without H_2O_2.

Lipid peroxidation inhibition assay

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissue. As freshly collected RBCs serve as a good source of lipid membrane to study lipid peroxidation, it was used and was resuspended to make 1% solution in phosphate buffer (37°C, pH 7.2). Selected concentrations (10-1000 µg/mL) of sample extract, each in triplicate, were mixed with 20 µL of 1.875 mM/L $FeSO_4$ and 20 µL of ascorbate (15.4 µg/mL). The reaction mixtures were incubated at 37°C for 1 hr, after which 200 µL of 0.1 mol/L ethylenediaminetetraacetic acid was added to bind the Fe^{2+}, thus preventing the reaction. TBA reagent (1 mL) was added, and the mixture was heated in boiling water bath for 15 minutes to form a stable, chromogenic reagent, N-methyl-2-phenylindole with malondialdehyde (MDA) [16]. One molecule of MDA reacts with two molecules of reagent to yield a stable chromophore. After that, the reaction mixture was centrifuged for 15 minutes at 3700 rpm and the absorbance was read out at 517 nm using NanoDrop 2000 series spectrophotometer (Thermo Scientific, USA). The values of IC_{50} were calculated using GraphPad Prism.

TBA method

The method of Ottolenghi modified by Kikuzaki and Nakatani [19] was used for the determination of free radicals present in the aqueous leaf extract of the selected plants. Four hundred µL of 20% TCA and 400 µL of 0.6% TBA were added to 200 µL of sample. The above mixture was placed in water bath for 10 minutes. After cooling was centrifuged at 3000 rpm for 20 minutes. The absorbance activity of the supernatant was measured at 552 nm and recorded after it has reached its maximum.

Nitric oxide (NO) scavenging activity

The NO radical scavenging activity of AECD and AEGA was determined using the method of Garratt *et al.* 1989 [20]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate NO which interacts with oxygen to produce nitrite ions determined by the use of Griess reagents. Four hundred µL of 10 mM sodium nitroprusside was added to 100 µL of phosphate buffer saline (pH 7.4) to which 100 µL of plant extract in various concentrations like 1000, 500, 200, 100, 50 and 10 µg/mL were added. Incubated the above mixture at 25°C for 150 minutes. 0.5 mL of the above mixture was added to the Griess reagent ([1.0 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid kept at room temperature for 5 minutes then to it added 1 mL of naphthylethylenediamine dichloride 0.1% w/v)]). The mixture was incubated at room temperature for 30 minutes. Then the absorbance was read at 570 nm.

***In vitro* nephroprotective activity**

Epiflourescence staining

Dual-staining procedure was used with epifluorescence microscopy which allows the detection of live cells and dead cells. In this method, normal kidney cells (vero cells) were suspended in 10 mL of phosphate buffered saline (PBS), and 200 µL of the suspension was incubated with gentamicin followed by the addition of 50 µL of the selected plant extracts in the concentration of 500 mg/mL. Another 200 µL of the suspension was incubated with gentamicin followed by the addition of vitamin E, which is used as a positive control. To the above suspensions 50 µL of ethidium bromide and 50 µL of acridine orange were added and was incubated for 1 hr. After the incubation the cells were viewed under epiflourescence microscope, in which the live cells emit green color and dead cells emit red color.

Cytoprotective assay

Cytoprotective assay is the assay employed to asses cell viability using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which colorimetrically measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The toxicity to the cells was caused by incubation of cells with gentamicin. The cells were then solubilized with an organic solvent (e.g. isopropanol), and the released, solubilized formazan reagent is measured spectrophotometrically [23]. Plate vero cells of 0.1×10^6 in 100 µL PBS were placed in 96 wells (flat bottom). The cells were then incubated with 50 µL of MTT solution in the concentration of 1 mg/mL for 48 hr at 37°C. 150 µL of dimethyl sulfoxide (DMSO) was added to the formazon product produced by the viable cells. The absorbance at 630 nm was measured using microplate spectrophotometer (Bio-Tek Instruments, USA).

Docking studies

Ligands data and preparation

To understand the mechanism behind. The *in vitro* nephroprotective screening docking analysis against nephrotoxic proteins were carried out using the molecules identified from *G. asiatica* using liquid chromatography mass spectrometry/mass spectrometry analysis. The three markers compounds of *G. asiatica* were used in our molecular docking studies. These inhibitors were employed in docking studies and for subsequent docking simulations.

Proteins data and preparation

The three-dimensional structure of the proteins Bcl2 and Fas was retrieved from the protein data bank (PDB) with PDB code: 4MAN and PDB code: 1WCH respectively, was selected for the docking simulations to tests the hypothesis that *G. asiatica* may alter the expression of Bcl-2 and Fas protein and prevent nephritis [26] and was prepared to use the graphical interface Maestro [24]. All water molecules were removed; the hydrogen atoms were added to the proteins. Preparation and refinement were done running ProteinPrep job on the structure in a standard procedure. Minimizations were performed until the average root mean square deviation of non-hydrogen atoms reached 0.3 Å [24].

Docking simulations

Molecules identified in the plant extract under study were docked into the binding site of the receptor PDB ID: 4MAN and PDB code: 1WCH using glide (Grid-Based Ligand Docking with Energetics) software from Schrodinger [25]. Grids were prepared for the proteins using receptor grid generation. The glide algorithm is based on a systematic search of

positions, orientations, and conformations of the ligand in the receptor binding site using funnel type approach. The search begins with a rough positioning and scoring phase that significantly limits the search space and reduces the number of poses to be selected for minimization on the pre-computed optimized potential for liquid simulations-2001 van der Waals and electrostatic grids for the protein. The 5-10 lowest-energy poses obtained from this stage are subjected to Monte Carlo simulations and the minimized poses accepted are then rescored using the GlideScore function, which is a more sophisticated version of ChemScore. This force field includes additional terms accounting for solvation and repulsive interactions. In order to provide a better correlation between good poses and good scores, glide extra-precision. Mode was subsequently used on the conformations selected from glide standard precision mode. Considering the clear dependence of the docking accuracy of ligands on the protein structure, docking simulations were carried out.

Statistical analysis

All assays were carried out in triplicates and results are expressed as mean±standard deviation. The IC_{50} values were calculated using Graphpad prism software.

RESULT AND DISCUSSION

The AEGA and AECD were prepared to examine the total phenolic content, flavonoid and proanthocyanidin concentration. *In vitro* anti-inflammatory activity, antioxidant activity and the nephroprotective activity were carried out and reported. The yield of the AEGA and AECD were 13.2% and 11.9%, respectively.

Phytochemical screening

The preliminary phytochemical investigation showed the presence of various phytochemical constituents such as phenolic acids, flavonoids, and proanthocyanidins. The result is expressed as the number of GAEs per gram of the plant extract for phenol and proanthocyanidins. Flavonoids were expressed as the number of QEs per gram of the plant extract, as quercetin is a flavonoid widely distributed in nature. The highest concentration of phenolic compounds such as flavonoids and proanthocyanidins were found in the AEGA when compared to AECD (Table 1). Flavonoids and proanthocyanidins are phenolic compounds and plant phenolics are the major group of compounds that act as primary antioxidants or free radical scavengers (Polterait, 1997). Phenolic antioxidants are generally believed to form phenoxyl radical upon donating a hydrogen atom that could quench active free radicals, which were also reported to have multiple biological effect.

Anti-inflammatory activity

Inhibition of albumin denaturation

Denaturation of proteins involves the disruption and possible destruction of both the secondary and tertiary structures of protein. Most of the biological proteins lose their biological functions when denatured. As protein denaturation is implicated in inflammation and as part of the investigation on the mechanism of the anti-inflammation activity, ability of plant extract to inhibit thermally induced protein denaturation was studied. The plant extracts were effective in inhibiting the heat-induced protein denaturation (Fig. 1). The IC_{50} value for AECD was 243.3 µg/mL (Fig. 1a') and for AEGA the IC_{50} value was 241.5 µg/mL (Fig. 1a). The effect of the extract was compared with aspirin, the standard drug used.

Table 1: Total phenols, total flavonoids, proanthocyanidin content of aqueous extracts of *G. asiatica* and *C. dactylon*

Plants	Total phenol (mg/GAE/g)[a]	Total flavonoids (µg QE/g)[b]	Proanthocyandins
G. asiatica	8.34	22	12.5
C. dactylon:	7.1	20.8	9.5

Values are expressed as mean (n=3). [a]GAE: Gallic acid equivalent, [b]QE: Quercetin equivalent, *G. asiatica: Gmelina asiatica, C. dactylon: Cynodon dactylon*

Membrane stabilizing activity

The human RBC membrane stabilization has been used as a method to study the *in vitro* anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane, and its stabilization implies that the extract may well stabilize lysosomal membranes. Membrane stabilization property of the test drug inhibits lysis and subsequent release of the cytoplasmic contents which in turn limits the tissue damage and exacerbation of the inflammatory response. Since the RBC membrane is similar to that of lysosomal membrane, inhibition of RBC hemolysis will provide good insight into the inflammatory process. Therefore, membrane stabilizing effect of AECD and AEGA was evaluated in human erythrocytes using heat-induced hemolysis. A dose-related inhibition of hemolysis was observed in the two extracts as shown in Fig. 1. The IC_{50} value of, AECD was 276.3 µg/mL (Fig. 1c'), whereas, AEGA showed IC_{50} of 270.3 µg/mL inhibition of RBC hemolysis (Fig. 1c). This might have contributed to the anti-inflammatory activity of the extracts.

Proteinase inhibitory activity

Proteinases are the key component of the inflammatory response as they are discharged from activated leukocytes and mast cells or generated through the coagulation cascade. Neutrophils are known to be a rich source of proteinase, which carries in their lysosomal granules many serine proteinases. The significant level of protection to such proteinase induced inflammation was provided by proteinase inhibitor. A significant antiproteinase activity was observed in two extracts at different concentrations (Fig. 1). The IC_{50} value of AECD was 125.7 µg/mL (Fig. 1b') and for the AEGA the IC_{50} was found to be 73.73 µg/mL (Fig. 1b). The results obtained from our studies on AEGA and AECD have revealed the potential anti-inflammatory activity of the test drug.

Antioxidant activity

DPPH scavenging assay

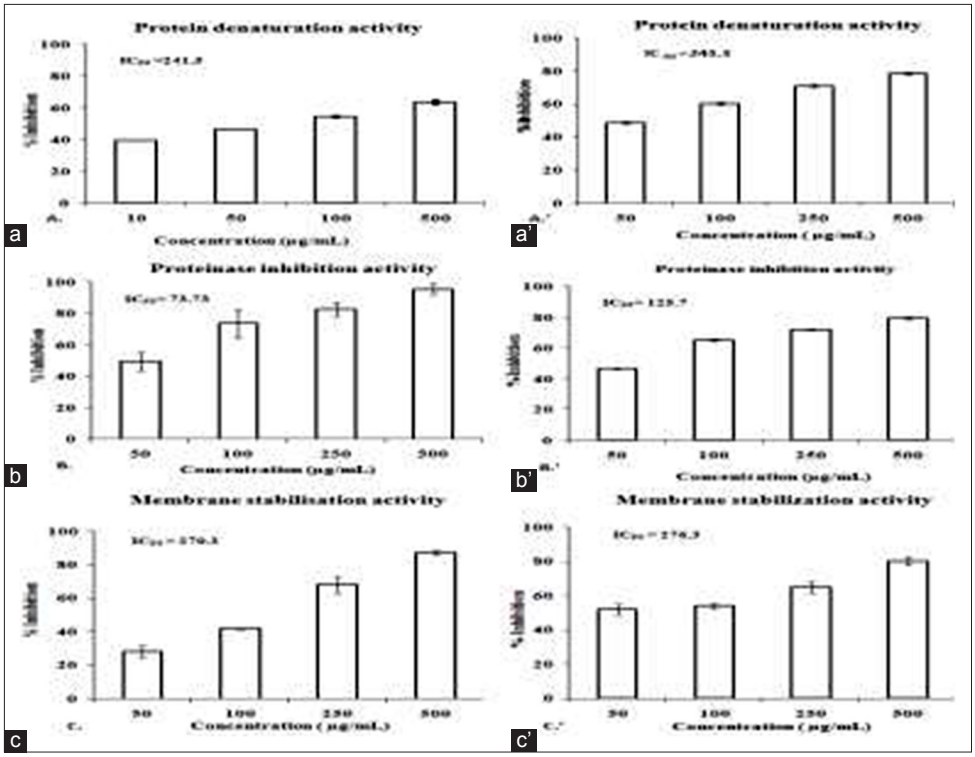
DPPH is a well-known radical and a trap ("scavenger") for other radicals. Therefore, rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. In its radical form, DPPH shows an absorbance at 517 nm and has a deep violet color in solution because of a strong absorption band centered at about 520 nm. However upon reduction with an antioxidant (Kumarasamy *et al.*, 2007) it becomes colorless or pale yellow when neutralized, which can be quantitatively measured from the changes in absorbance (Subhan *et al.*, 2008). Fig. 2 shows free radical scavenging activity of AEGA and AECD at different concentration. The IC_{50} of 206 µg/mL was found in *G. asiatica* (Fig. 2b), and IC_{50} value of 336.1 µg/mL was found in *C. dactylon* (Fig. 2a). These results indicated that AEGA and AECD exhibited the ability to quench the DPPH radical, which indicated that extract has good antioxidant potential with free radical scavenging activity.

Reducing power assay

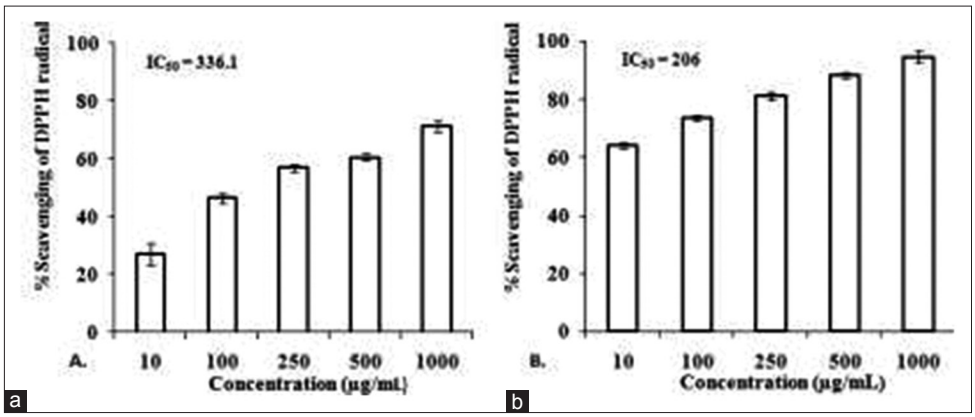
The antioxidant activity of phenolic compounds present in plants are mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). The reducing properties are generally associated with the presence of reductones (Duh *et al.*, 1999), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). The potential of the plant extracts to reduce Fe^{3+} to Fe^{2+} by electron transfer is an indication of their antioxidant ability. The ferric reducing activity of the AEGA and AECD is presented in Fig. 3. The observed change of yellow color of test solution to various shades of blue and green, depending on the concentration of the extract, is an indication of its antioxidant activity. The IC_{50} value of 48.4 µg/mL was observed in *G. asiatica* (Fig. 3b), which is significantly higher than the *C. dactylon* with an IC_{50} value of 51.3 µg/mL (Fig. 3a).

Super oxide radical scavenging activity

Superoxide is the major form of reactive oxygen species derived out of the electron transport chain process associated with the inner



AQ9 **Fig. 1: The anti-inflammatory activity of *Gmelina asiatica* (a-c) and *Cynodon dactylon* (a'-c'). (1a, and a') The percentage inhibition of protein denaturation by aqueous extract of *G. asiatica* (AEGA) and aqueous extract of *C. dactylon* (AECD) with an IC_{50} value of 241.5 µg/mL and 243.3 µg/mL respectively, (1b and 1b') the proteinase inhibition activity of AEGA and AECD with an IC_{50} value of 73.73 µg/mL and 125.7 µg/mL, (1c and c') the membrane stabilization activity of AEGA and AECD with an IC_{50} value of 270.3 µg/mL and 276.3 µg/mL**

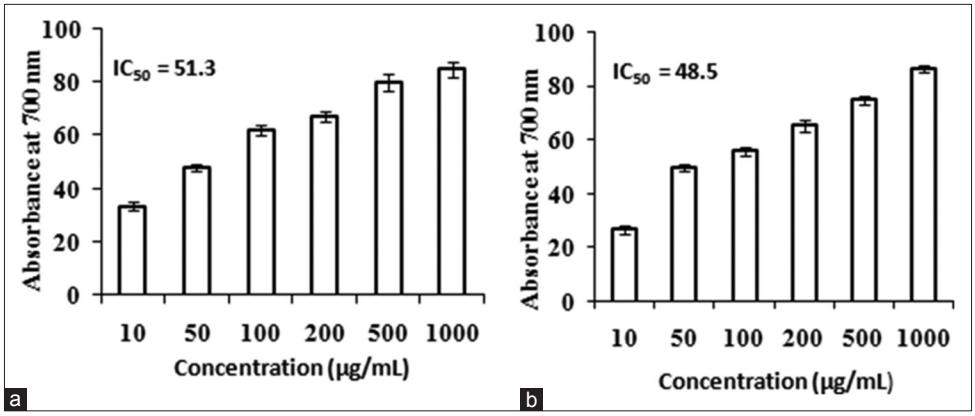


AQ9 **Fig. 2: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of aqueous extract of *Cynodon dactylon* (a) and *Gmelina asiatica* (b). The DPPH radical scavenging activity was found to be in a dose-dependent manner upon treatment with both the extracts. (a) The DPPH radical scavenging activity of aqueous extract of *C. dactylon* with an IC_{50} value of 336.1 µg/mL, (b) the DPPH radical scavenging activity of aqueous extract of *G. asiatica* with an IC_{50} value of 206 µg/mL**

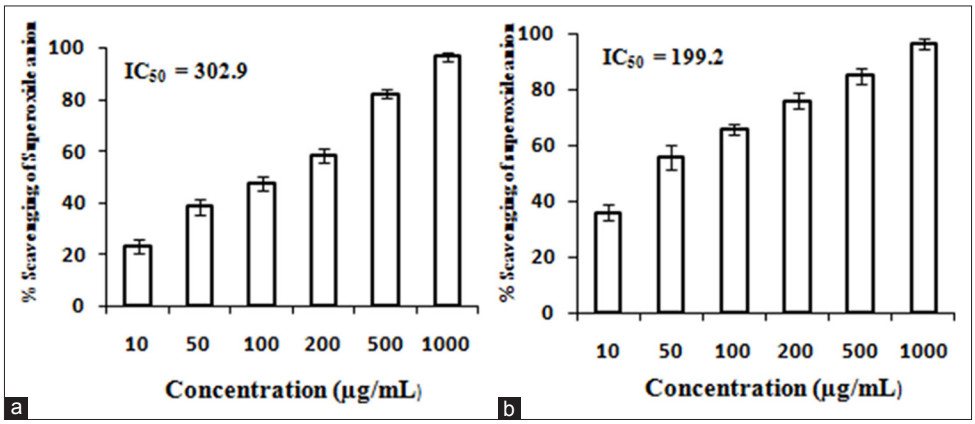
mitochondrial membrane. Superoxide form is the indication of the disturbance in the free flow of e^- across various e^- carriers, associated with the electron transport chain. Formed superoxides, being highly reactive, reacts with another superoxide, resulting in the formation of peroxides. There was a decrease of absorbance at 560 nm with the plant extracts, which indicates the consumption of superoxide anion in the reaction mixture thus suggesting a promising scavenging activity. The scavenging activity of this radical by the treatment of AEGA and AECD is presented in Fig 4. The maximum scavenging activity of superoxide radical with an IC_{50} value of 199.2 µg/mL was found in *G. asiatica* (Fig. 4b) whose scavenging activity is consequently higher than *C. dactylon* which showed a scavenging activity with an IC_{50} value of 302.9 µg/mL (Fig. 4a).

Hydrogen peroxide scavenging activity

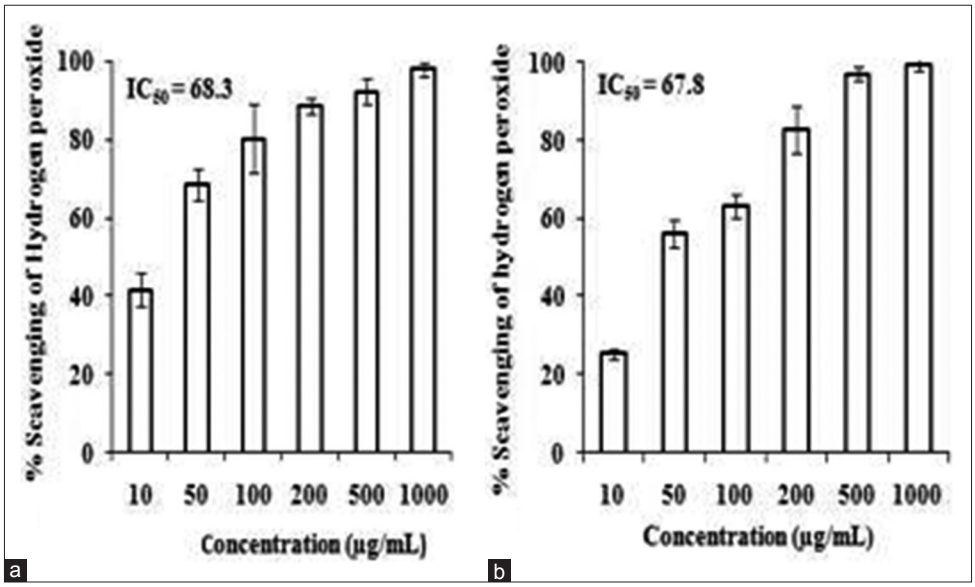
Hydrogen peroxide is a neutral molecule formed during the dismutation of superoxide by superoxide dismutase which freely diffuses out of the lipid bilayer, thereby targeting other biomolecules, namely proteins, lipids, etc. which comes in contact. However, it may be toxic if converted to hydroxyl radical in the cell. Scavenging of hydrogen peroxide by the plant extracts may be attributed to their phenolics, which donate electron to hydrogen peroxide, thus reducing it to water. The plant extracts were capable of scavenging hydrogen peroxide in a dose dependent manner which is presented in Fig. 5. The scavenging activity of both the plants was evaluated, and it was observed that in the selected plants significantly higher scavenging activity exists in the extract of *C. dactylon* at different concentration with an IC_{50} value of



AQ9 **Fig. 3:** Reducing power activity of aqueous extract of *Cynodon dactylon* (AECD) and Gmelina asiatica (AEGA). The reducing power activity was found to be in a dose dependent manner upon treatment with both the extracts. (a) The reducing power activity of AECD with an IC_{50} value of 51.3 µg/mL, (b) the scavenging activity of AEGA with an IC_{50} value of 48.5 µg/mL



AQ9 **Fig. 4:** Superoxide anion scavenging activity of the aqueous extract of *Cynodon dactylon* (AECD) and *Gmelina asiatica* (AEGA). The superoxide anion scavenging activity was found to be in a dose dependent manner upon treatment with both the extracts. (a) The superoxide anion peroxide scavenging activity of AECD with an IC_{50} value of 302.9 µg/mL, (b) the scavenging activity of AEGA with an IC_{50} value of 199.2 µg/mL



AQ9 **Fig. 5:** Hydrogen peroxide scavenging of aqueous extract of *Cynodon dactylon* (AECD) and *Gmelina asiatica* (AEGA). The hydrogen peroxide scavenging activity was found to be in a dose dependent manner upon treatment with both the extracts. (a) The hydrogen peroxide scavenging activity of AECD with an IC_{50} value of 68.3 µg/ML, (b) the scavenging activity of AEGA with an IC_{50} value of 67.8 µg/mL

68.3 µg/mL (Fig. 5a) and the IC_{50} value of *G. asiatica* was 67.8 µg/mL (Fig. 5b).

Lipid peroxidation assay

Lipid peroxides are the products of a chemical damage caused by oxygen free radicals to the polyunsaturated fatty acids of cell membranes. The extract showed inhibition of the peroxidation effect in all concentration on addition of Fe^{2+} to RBC cells which causes an increased lipid peroxidation. The anti-lipid peroxidation activity of AEGA and AECD is presented in Fig. 6. The highest anti-lipid peroxidation activity with an IC_{50} value of 135.5 µg/mL was found in the AEGA (Fig. 6b) which was significantly more higher than anti-lipid activity of AECD whose IC_{50} value was 165.7 µg/mL (Fig. 6a).

TBA method

The mechanism of damage occurring on peroxidation of membrane phospholipid is evident in the quantification of tissue MDA by TBA test. The thiobarbituric acid reactive substance formed during the TBA test leads to the estimation of the extent of lipid peroxidation. The *in vitro* free radical scavenging properties of fatty acids was observed in the AEGA and AECD using lipid peroxidation estimated by TBA method, which is presented in Fig. 7. The IC_{50} value of *G. asiatica* was 13.29 µg/mL (Fig. 7b), and the IC_{50} of the AECD was 13.66 µg/mL (Fig. 7a).

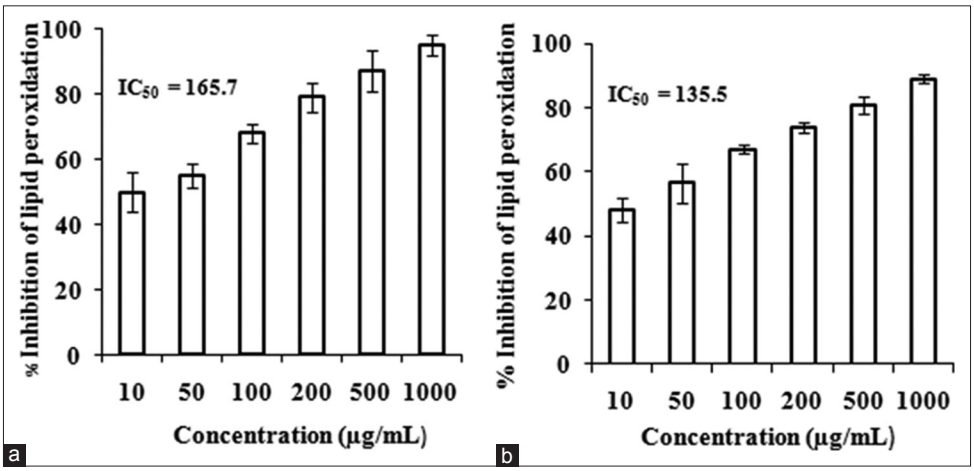
NO scavenging assay

In biological systems, NO is generated by the catalytic action of nitric oxide synthase (NOS) on L-arginine; in inflammatory cells, nitric oxide is produced by the inducible isoform of NOS. In living biological systems, a high level of NO and its oxidized derivatives such as peroxynitrite are known to cause toxic. The NO scavenging capacity of some antioxidants present in the AEGA and AECD was evaluated and is presented in the Fig. 8. The maximum NO scavenging capacity was found in *G. asiatica* with an IC_{50} value of 14.97 µg/mL (Fig. 8b) which was significantly more than *C. dactylon* whose IC_{50} value was 60.3 µg/mL (Fig. 8a).

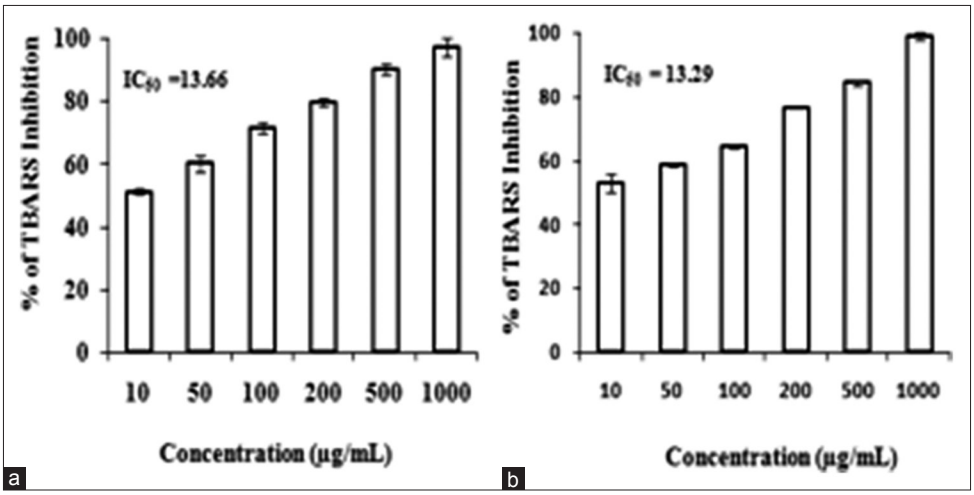
***In vitro* nephroprotective activity**

Epifluorescence staining

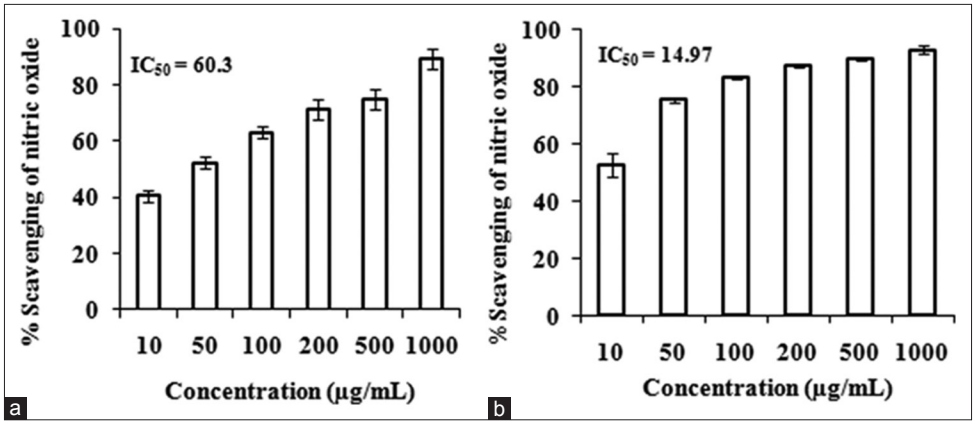
The cytoprotective effects of the AEGA and AECD against gentamicin induced toxic cells was examined under fluorescent microscope using epifluoresence staining and the data obtained are presented in the Fig. 9. The morphological changes observed in cells subjected to gentamicin are due to the chromatin condensation, cell shrinkage and other alterations, which are the characteristics of fragmentation in cells. Vitamin E is used as a positive control, showed green colored nuclei indicating the protective nature (Fig. 9e). Similarly, treatment with AEGA and AECD resulted in bright green color, with intact



AQ9 **Fig. 6: Inhibition of lipid peroxidation by the aqueous extract of *Cynodon dactylon* (AECD) and *Gmelina asiatica* (AEGA). The percentage of lipid peroxidation was found to be dose dependent manner in both the extracts. (a) The percentage inhibition of lipid peroxidation by AECD with an IC_{50} value of 165.7 µg/mL, (b) percentage inhibition of lipid peroxidation by AEGA with an IC_{50} value of 135.5 µg/mL**



AQ9 **Fig. 7: Thiobarbituric acid reactive substance (TBARS) inhibition activity by the aqueous extract of *Cynodon dactylon* (AECD) and *Gmelina asiatica* (AEGA). The percentage of TBARS inhibition was found to be increasing with increasing concentration in both the extracts. (a) The percentage of TBARS inhibition by AECD with an IC_{50} value of 13.66 µg/mL, (b) the percentage of TBARS inhibition by AEGA with an IC_{50} value of 13.29 µg/mL**



AQ9 **Fig. 8: Nitric oxide scavenging activity of aqueous extract of *Cynodon dactylon* (AECD) and *Gmelina asiatica* (AEGA). The scavenging activity was found to be in a dose dependent manner upon treatment with both the extracts. (a) The scavenging activity of AECD with an IC_{50} value of 60.3 µg/mL, (b) the scavenging activity of AEGA with an IC_{50} value of 14.97 µg/mL**

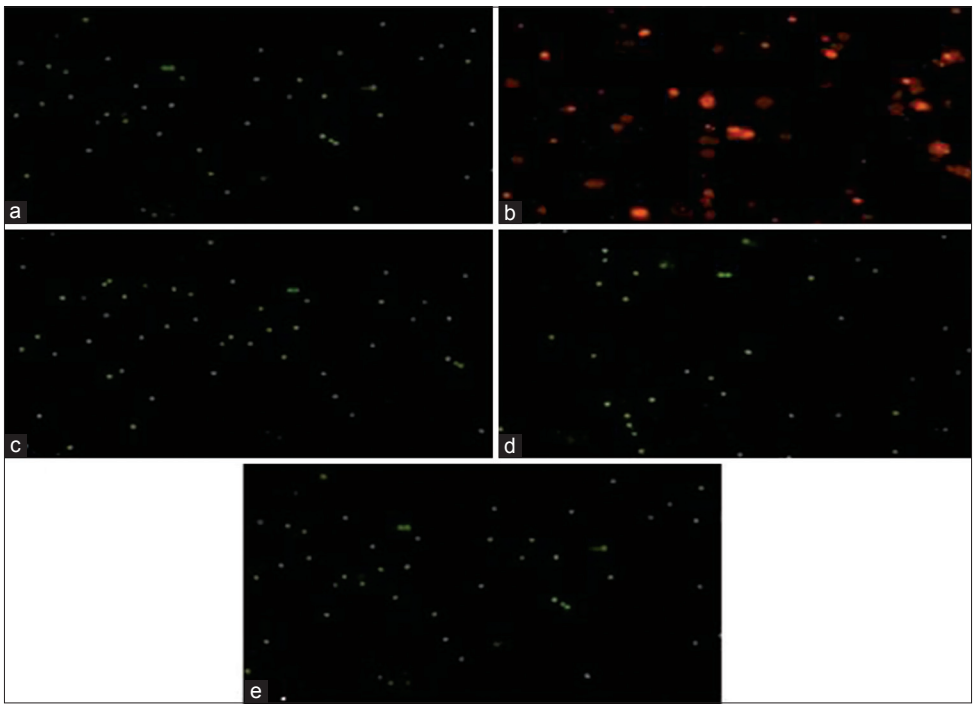


Fig. 9: Epiflourescent staining of the aqueous extract of *Gmelina asiatica* (AEGA) and *Cynodon dactylon* (AECD). (a) Vero cells emitting green fluorescence after treating it with acridine orange and ethidium bromide indicating that they are alive, (b) gentamicin induced vero cells emitting red fluorescence on treatment with acridine orange and ethidium bromide indicating that they are dead, (c) live gentamicin induced toxic vero cells after the treatment of cells with AEGA, (d) live gentamicin induced toxic vero cells after the treatment of cells with AECD, (e) live gentamicin induced toxic vero cells after the treatment of cells with vitamin E which is used as a positive control

chromatin (Fig. 9c and 9d). The data of the results using the staining assay employing fluorescent microscopy revealed the presence of green colored cells thus, confirm the cytoprotective nature of the plant extracts.

Cytoprotective assay

The viability of cells caused by the plant extracts against toxicity induced cell was assessed using MTT assay. This viability test is based on the ability of the mitochondrial succinate-tetrazolium reductase system to convert yellow tetrazolium salt MTT to purple formazon dye. This test helps to assess the presence of denotes the survival cells after toxic exposure. Here the toxicity was induced to cells by gentamicin. The percentage of survivability induced by the plant extracts is presented in Fig. 10. The formation of the formazon product through the reduction of MTT by the AECD (Fig. 10a) and AEGA (Fig. 10b) was observed to be in dose dependent manner which significantly denotes metabolically active cells.

Docking analysis

The docking results for all the natural inhibitory compounds under study are reported in (Table 2) and their structures are presented in Fig. 11. All compounds with higher GlideScore clearly showed the ability of the ligand selected for their efficacy binding. The scoring function takes a pose as input and returns a number indicating the likelihood that the pose represents a favorable binding interaction. Most scoring functions are due to physics-based molecular mechanic force fields that estimate the energy of the pose; a low (negative) energy indicates a stable system and thus a likely binding interaction. Among the natural

inhibitory compounds, dihydrogmelinol poses the highest negative energy indicating the probable binding interaction thus indicating the inhibition of the protein as shown in Fig. 12.

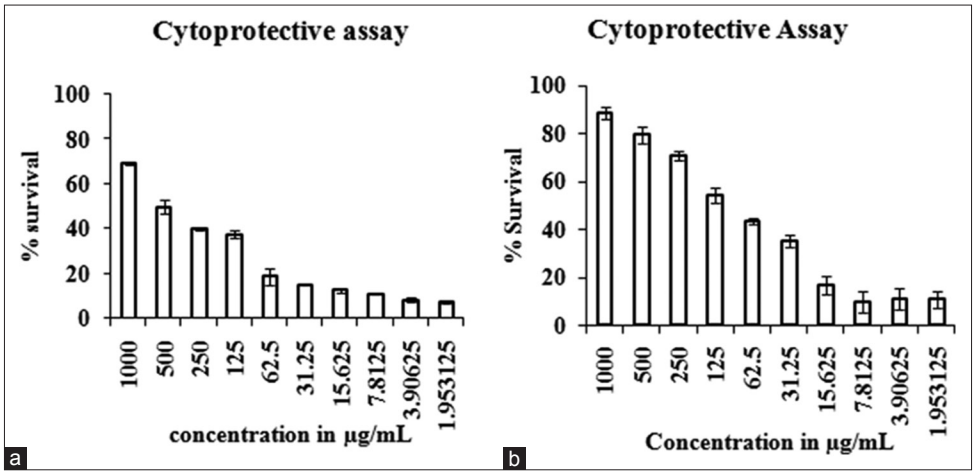
CONCLUSION

The present study, anti-oxidant, anti-inflammatory and nephroprotectiv activity of the AECD and AEGA were investigated. This study depicts the *in vitro* antioxidant, anti-inflammation

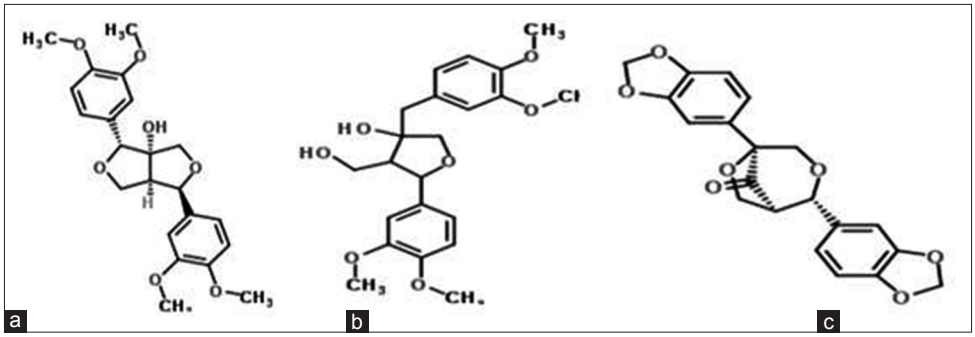
Table 2: Ranking of the poses of natural inhibitory molecules in the target Bcl2 and Fas protein

Nature inhibitory molecule	G-score (Bcl2)	G-score (Fas)
Dihydrogmelinol	−6.242	−7.965
Gmelanone	0.066	−6.104
Gmelinol	−3.771	−5.956

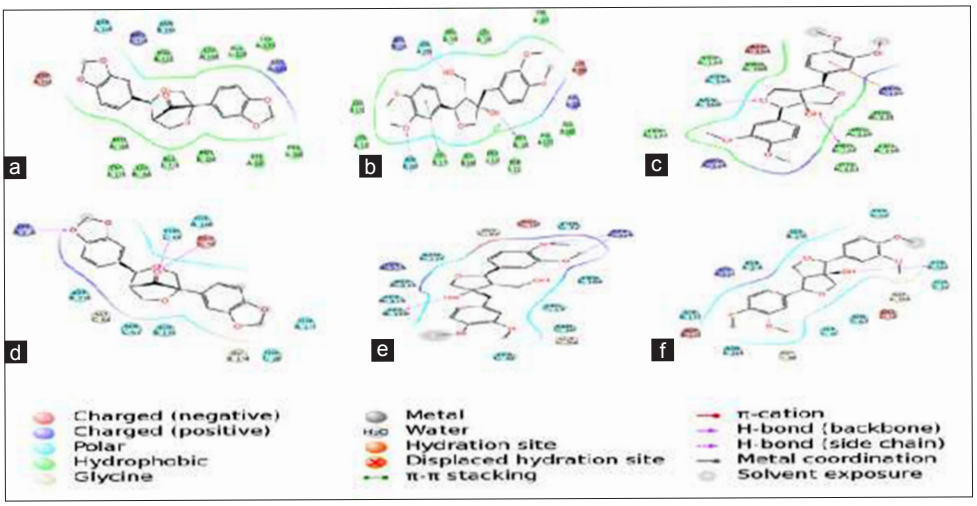
The G-score denotes the glide score of the docked structure



AQ9 **Fig. 10: Survival rates of the gentamicin treated cells after the incubation with aqueous extract of *Cynodon dactylon* (AECD) and *Gmelina asiatica* (AEGA). The survival rates were found to be in a dose-dependent manner upon treatment with both the extracts. (a) The protective activity of AECD with an IC_{50} value of 408.5 µg/mL, (b) The protective activity of AEGA with an IC_{50} value of 335.7 µg/mL**



AQ9 **Fig. 11: Structures of molecules found in the *Gmelina asiatica* extract and used for docking simulations. (a) Gmelinol, (b) dihydrogmelinol, (c) gmelanone**



AQ9 **Fig. 12: The docked structure of natural inhibitory compounds into the protein Bcl2 and Fas**

and nephroprotective potential of aqueous extracts of the leaf of *G. asiatica* and arial parts of *C. dactylon.* These therapeutic effects may be due to the presence of phenolic compounds such as flavonoids and proanthocyanidins. In order to validate, the possible mechanism of action the identified molecules from the plant extract were subjected to docking studies and their binding affinity with the targets further confirmed the nephroprotective potential of the selected plant drugs.

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