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Original Article

ANTI-PROLIFERATIVE AND ANTIOXIDANT POTENCY OF LEAF METHANOL EXTRACT OF *CRYPTOSTEGIA GRANDIFLORA* R. BR

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ABSTRACT

Objective: The study aim to investigate the traditional medicinal importance of *Cryptostegia grandiflora* leaf methanol extract (CGLME) using anti-proliferative and antioxidant activity.

Methods: *In vitro* and *in vivo* antioxidant methods were followed for identification of antioxidant property, HPLC was done for detection of flavonoids, DNA binding and MTT assay was carried out to identify the potential antiproliferative property of flavonoids.

Results: CGLME antioxidant property could be ascribed to the presence of considerable quantity of multiple phenol molecules since they have shown increased level of hepatic antioxidant enzymes viz, Catalase (13.09±0.20), Peroxidase (17.17±0.07) and Superoxide dismutase (0.17±0.01) compare to CCl_4 control and protective against Fenton's reagent induced DNA molecule nicking (pUC19). Further, antioxidant property supported by Cyclic voltammetry with oxidation potential of CGLME scan rates from +50 to +300mV. CGLME has also shown anti-proliferative activity against colorectal adenocarcinoma (Caco-2) cell line at a concentration of CTC_{50} 750µg/mL (75.7%).

Conclusion: Significant amount of catecholamine like phenol molecules in CGLME may have contributed to antioxidant property. Further, research warrants isolation of active principles to understand responsible molecules for antioxidant and anti-proliferative property.

Keywords: HPLC analysis, Cyclic voltammeter, Antioxidant activity, DNA nicking assay, DNA binding assay, Anti-proliferative activity.

INTRODUCTION

Antioxidants are specific chemical compounds obtained from natural products particularly of plant origin which quenches free radicals and reduce the cell damage caused by them [1]. As antioxidants, the molecules can interfere with the oxidation process by reacting with free radical, chelating catalytic metals and also by acting as oxygen scavengers. Oxidation is an essential biological process for energy production in many living organisms. The potentially reactive derivatives of oxygen, attributed as reactive oxygen species (ROS) were detoxified by the antioxidants when they are present in the body. However, overproduction of ROS and/or an inadequate antioxidant defense can easily affect and persuade oxidative damage to various biomolecules including protein, lipids, lipoproteins and DNA [2]. However, excessive ROS, produced *in vivo* during some oxidative reactions are not only strongly associated with lipid peroxidation but also involved in the development of some diseases [3] such as atherosclerosis, cardiovascular, neurodegenerative and cancer like diseases [4,5,6]. Eradication of cancer cells in premature stage is an essential part of chemoprevention, measuring anti-proliferative dose against cancer cells provide useful insight on the chemoprotective potential of natural extracts. Due to this ability, presence of natural antioxidants in medicinal plant extracts may play an important role in prevention of normal cell from becoming a cancer cell.

The crude extract of CGLME contains catechol group substances, which are responsible for maintaining the integrity of native form of DNA [7]. The quantitative determination of catecholamine like substances in CGLME by cyclic voltammeter technique may also supports the antioxidant potentialities of the plant. The plant extract selection for anti-proliferative activity is based on basic research findings on ethnomedicinal knowledge (anti-viral, antitumor, anti-bacterial and antioxidant assay). However, CGLME has shown significant analgesic activity [8] and it has helped us to further explore its *in vitro* and *in vivo* antioxidant activity.

MATERIALS AND METHODS

Chemicals and reagents

Petroleum ether, Chloroform, Methanol (Merck), Ascorbic acid, Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Nitroblue tetrazolium (NBT), Folin–Ciocalteau Reagent (FCR), Potassium iodide, Butylated hydroxyl toluene (BHT), Malondi aldehyde (MDA), Potassium ferricyanide ($K_3Fe(CN)6$), Phospho molybdenum, Sodium phosphate, Sodium acetate, Phosphate Buffered Saline (PBS), Fetal Bovine serum (FBS), 3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetra zolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich (St. Louis, MO USA). EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E. Merck Ltd., Mumbai, India. Gallic acid, ascorbic acid, ferrous chloride, hemoglobin and potassium persulfate were procured from Himedia (India). pUC19 and agarose were procured from GeNeiTM (Bangalore, Karnataka, India). Water was purified on a Milli-Q system from Millipore (Millipore, Bedford, MA, USA). All the chemicals used were of analytical grade.

Determination of total phenolic content

Total phenol content was measured according to the method of Matthaus [9]. To a sample of CGLME (1 to 5µg), 1mL of FCR (ten folds diluted) was added and mixed thoroughly for 3 min. and 3mL of 2% Na_2CO_3 was added. This was incubated at room temperature for 2 hrs and then absorbance was measured at 740nm. Total phenolic content was calculated and expressed as equivalent to gallic acid.

High Performance of Liquid Chromatography (HPLC) analysis

Bio-active constituents in the CGLME were evaluated using HPLC with the flow rate of 1.5mL/min. and 30 min. run time. The C_{18} column with symmetric 5µm particle size was used to run sample along with standards delphinidin (D), malvidin (M) and anthocyanin (A).

Cyclic voltammeter study

Cyclic voltammeter was performed with Model CHI-660c electrochemical work station. The electrochemical cell contained carbon paste electrode (CPE) as a working electrode, platinum counter electrode and saturated calomel electrode as reference electrodes.

CPE was prepared by blending the fine particles of graphite powder with silicon oil as a binder by hand mixing in a mortar and pestle. The resulting fine mixture of carbon paste was inserted into hollow cavity of the Teflon tube which is connected externally with a copper wire. After filling the Teflon tube, the tip was smoothened with the butter paper. The ratio of the graphite powder and silicon oil was calibrated with the cyclic voltammograms of potassium ferrocyanide.

***In vitro* antioxidant activity**

DPPH radical scavenging activity

The free radical scavenging activity of CGLME was measured using DPPH according Blois method [10]. To different concentration of CGLME (20-100µg), 1mL of 0.1mM DPPH in methanol was added and kept in dark for 30 min. at room temperature. Absorbance was measured at 517nm along with parallel control and the inhibition concentration was calculated using the formula given below.

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

Where A_0 = absorbance of the control, A_1 = absorbance of the CGLME.

Determination of ferrous ion chelating ability

The Decker and Welch [11] method was used to investigate the ferrous ion chelating ability of CGLME. 1mL of CGLME at different concentration was mixed with 2mM $FeCl_2$ (0.05mL) and 5mM ferrozine (0.2mL). The mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the test solution along with parallel control was measured at 562nm. EDTA was used as standard for comparison. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated from the following formulae.

$$\% = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 = absorbance of the control, A_1 = absorbance of the extract.

Determination of reducing power capacity

The reducing power of CGLME was determined according to the method of Oyaizu [12]. A series of extract concentration (100-500µg/mL) was mixed with phosphate buffer (2.5 mL, 0.2M, pH 6.6) and potassium ferricyanide (2.5mL, 1%). The mixture was incubated at 50 °C for 20 min. TCA (2.5mL of 10%) was added to the mixture, which was then centrifuged at 3000rpm for 10 min. The upper layer of the solution (2.5mL) was mixed with distilled water (2.5mL) and $FeCl_3$ (0.5mL. 0.1%). The absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated increased reducing power and butylated hydroxytoluene was used as a standard.

Determination of total antioxidant assay

The total antioxidant activity of CGLME was determined by phosphomolybdenum method as described by Prieto et al [13]. 0.3mL of CGLME (50-250µg) was mixed with 3mL of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The reaction mixture was incubated at 95 °C for 90 min. and cooled to room temperature. Absorbance of the solution was measured at 695 nm. The total antioxidant capacity was expressed as the number of equivalents of ascorbic acid (AAE).

DNA nicking assay

The ability of CGLME to protect super coiled pUC19 DNA from harmful effects of hydroxyl radicals generated by Fenton's reagent was assessed by the DNA nicking assay as described by Lee et al [14] with slight modifications. The reaction mixture contained 20µg of plasmid DNA, 5µL Fenton's reagent (30 mM H_2O_2, 50mM ascorbic acid, and 80mM $FeCl_3$) followed by the addition of test material CGLME and resorcinol as a positive control. The final volume of the mixture was brought to 30µL using distilled water. The mixture was then incubated for 30 min. at 37 °C and the DNA sample was ran in 1% agarose gel electrophoreses in a horizontal slab gel apparatus for 1.5 hr. (40V/20 mA) using 1X TBE buffer. After electrophoresis, the gels were stained with 0.6lgm/mL of a solution of ethidium bromide for 30 min. followed by another 30 min. The gels were then photographed under gel document.

DNA binding studies

The concentration of Ct-DNA (in nucleotide phosphate, NP) was measured by using its known extinction coefficient at 260nm ($6600 M^{-1}Cm^{-1}$) as described by Reichmann et al [15]. Tris–HCl buffer (5mM Tris, pH 7.1, 50mM NaCl) was used for absorption titration experiments and viscosity measurements. Absorption titration experiments were carried out by varying the DNA concentration and maintaining the complex concentration constant. Absorbance values were recorded after each successive addition of DNA solution with equilibration (ca. 10 min.). The absorption data was analyzed for the valuation of intrinsic binding constant Kb using reported procedure [16].

***In vivo* antioxidant activity**

Experimental animals

Healthy Swiss albino mice weighing 20-22gm. were procured from Central Animal House, National College of Pharmacy, Shimoga, Karnataka, India and housed at 23±2 ºC, humidity 55–60%. These animals were fed with standard commercial pellet diet (Durga Feeds and Foods, Bangalore) and water *ad libitum*. All the animals were acclimatized for one week and all experiments were carried out according to the Institutional Animal Ethical Committee (IAEC) guidelines (Re: NCP/IAEC/CL/07/12/2010-11).

Preparation of liver homogenate

Liver homogenate (10%) was prepared with 0.15M KCl and centrifuged at 8000rpm for 10 min. The cell-free supernatant was used for following experiments. Total protein was estimated by Lowry et al [17] with bovine serum albumin as standard.

Carbon tetrachloride (CCl_4)-induced oxidative toxicity

The antioxidant property of the CGLME was further evaluated using *in vivo* studies. Mice were randomly divided into five groups, each containing four animals (n=4). Group I was given distilled water with 0.2% DMSO (1mL/kg b. w., p. o.) daily for 5 days and olive oil on 2nd and 3rd day. Group II (CCl_4 control) was given 0.2% DMSO (1mL/kg b. w., p. o.) solution for 5 days and a 1:1 mixture of CCl_4 and olive oil (2mL/kg b. w., s. c.) on 2nd and 3rd day. Groups III, IV and V (test group) were orally given 250, 500, and 750 mg/kg b. w., respectively for 5 days and a 1:1 mixture of CCl_4 and olive oil (2mL/kg b. w.,, s. c.) on 2nd and 3rd day. Group VI animals were orally administered with Vitamin E (100mg/kg b. w.). At the end of the experiment (on day 6), the animals were sacrificed by light ether anesthesia. Liver samples were immediately dissected; blood was cleaned off with ice-cold saline and stored in a refrigerator for further investigation.

Catalase activity (CAT)

CAT was estimated by the method of Claiborne [18]. The assay mixture consisted of 1.95mL of phosphate buffer (0.005M, pH 7.0), 1.0mL H_2O_2 (0.019M), 0.005mL of liver homogenate (10% w/v). Absorbance was recorded at 240nm. CAT activity was calculated in terms of nanomole consumed/min/mg protein.

Peroxidase activity

The peroxidase assay was carried out according to the method of Nicholos [19]. Briefly, to 0.5mL of liver homogenate 1mL of KI (10mM) and sodium acetate (40mM) solutions were added and absorbance was read at 353nm. And further 20mL of H_2O_2 (15mM) was added to initiate reaction; change in absorbance in 5 min. was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change the optical density by 1unit/min. The

specific activity was expressed in terms of units per milligram of protein.

Estimation of lipid peroxidation assay (LPO)

Lipid peroxides were estimated according to the method of Buege and Aust [20]. In brief, the lipid peroxidation was initiated by adding 100mL of 0.2mM ferric chloride to the mixture of 0.5mL liver homogenate and 0.15M KCl at 37 ◦C for 30 min. and stopped by adding 2mL of ice cold mixture of 0.25N HCl containing 15% TCA, 0.30% TBA and 0.05% BHT. The mixture was heated at 80 °C for 60 min. and the samples were cooled, centrifuged and absorbance of the supernatant was measured at 532nm. The results were expressed as MDA equivalents, which were calculated by using an extinction coefficient of 1.56. Lipid peroxidation was expressed as MDA equivalents in nanomoles per mg of protein.

Estimation of superoxide dismutase assay (SOD)

To determine the SOD activity, the Riboflavin-NBT assay of Lai et al (2008) was adapted [21] with slight modifications. The homogenate (0.1mL) was mixed with 0.1mL of 67mM phosphate buffer (pH 7.8) containing 0.01M EDTA and 0.1mL of 1.5mM NBT. After incubation at 37 °C for 5 min. 3mL of 1.2mM riboflavin was added. The reaction mixture was kept in foil-lined box and illuminated with a 25W light tube for 15 min. The inhibition of NBT reduction was determined by measuring the absorbance at 560nm.

The negative control (water instead of the sample) and parallel blank (addition of water instead of the riboflavin solution) were evaluated simultaneously. In addition, standards were also evaluated to determine the SOD activity. Unit of SOD activity was expressed as the amount of enzyme required to inhibit the reduction of NBT using the formula SOD acitivity = decrease in OD × 2/ S-B where decrease in absorbance = (S-B)-(T-C). Where, S-absorbance of standard, B-absorbance of blank, T-absorbance of test and C-absorbance of control.

Anti-proliferative activity

Preparation of test solutions

Each test material was weighed, mixed to obtain the desired concentration by dissolving in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1mg/mL concentration and sterilized by filtration. Serial two fold dilutions were prepared for carrying out cytotoxic studies.

Preparation of cell line and culture medium

Human adenocarcinoma-2 (Caco-2) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated FBS, penicillin (100IU/mL), streptomycin (100µg/mL) and amphotericin B (5µg/mL) in humidified atmosphere of 5% CO_2 at 37 °C until confluence. The confluent cells were dissociated with Trypsin Phosphate Versene Glucose (TPVG) solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25cm² culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Anti-proliferative activity by MTT assay

To determine the anti-proliferative activity, the rapid colorimetric assay was adopted as reported by Francis and Rita [22] with some modifications. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 X 10^5 cells/mL using DMEM, containing 10% FBS. To each well of the 96 well micro-titer plates, 0.1mL of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hr. when a partial monolayer was formed and the supernatant was flicked off, wash the monolayer once with medium and 100µl of different concentration of test samples were added on to the partial monolayer in micro titer plates. The plates were then incubated at 37 ºC for 3 days in 5% CO_2 atmosphere and microscopic examination was carried out and noted for every 24 hr. interval. After 72 hr., the drug solution in each well was discarded and 50µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3hr. at 37 ºC in 5% CO_2 atmosphere. The supernatant was removed and 100µl of propanol was added and the plates were gently shaken to solubilize the formazan. The absorbance was measured using a microplate reader at a wavelength of 540nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) was generated using dose-response curves for each cell line.

Statistical analysis

All experiments were performed in triplicate and an ANOVA (Ananlysis of Variance) test (using EZ ANOVA statistical software) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by T test ($P < 0.05$). The results represented as Mean ± SE.

RESULTS AND DISCUSSION

Total phenolic content

Preliminary phytochemical investigation of CGLME showed presence of several bioactive compounds like glycosides, alkaloids, steroids, tannins, saponins and high amount of phenolic compounds. The phenolic content present in CGLME was found to be 725.8µg GAE/mg of dry extract (Figure 1). Phenolics are very important plant secondary metabolites; chemically they are aromatic hydroxylated compounds having one or more aromatic rings with one or more hydroxyl group. There is a positive correlation between total phenolics and antioxidant property of many plants attributed to free radical scavenging activity [23] with their ability to donate electrons and hydrogen atoms, making them very good antioxidants [24].

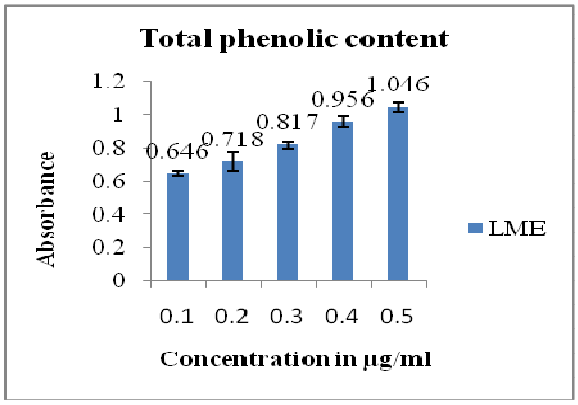


Fig. 1: Determination of total phenolic content.

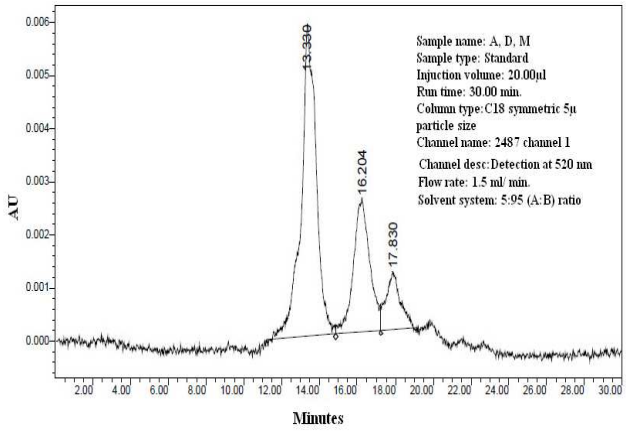
HPLC analysis

The HPLC of CGLME revealed the presence of potential antioxidant flavonoid molecules such as delphinidin, malvidin and anthocyanine. Delphinidin eluted at 13.25 min. retention time (RT) with an area of 14.3%, while malvidin and anthocyanine eluted at 16.2 and 17.8 min. RT with an area of 48.4 and 13.0% respectively. It indicates the presence of 75.8% of flavonoids in the CGLME (Figure 2), since it is well known that the reducing power of plant extracts depends on the concentration and variety of different flavonoid components as reported by many investigators on plant origin flavonoid components, which are responsible for antioxidant potential of variety of plant extracts [25].

Cyclic voltammeter analysis

Figure 2. depicts the cyclic voltammograms of CGLME containing the catechol, resorcinol and dopamine as neurotransmitter (Figure 3A). Cyclic voltammogramm of CGLME has revealed that it contains dopamine and catechol moiety resorcinol. The CPE was characterized by using cyclic voltammetric technique and is confirmed that no peak was found at the buffer solution containing 1mL of methanol in the absence of the extract of interest (Figure

3B). The CGLME was analyzed electrochemically using immobilization technique. The immobilized analyte was left for 2 min. to allow it to adsorb strongly. Later the electrode was immersed in the phosphate buffer solution with cycling potential from 0 to 0.4V versus saturated calomel electrode (SCE).



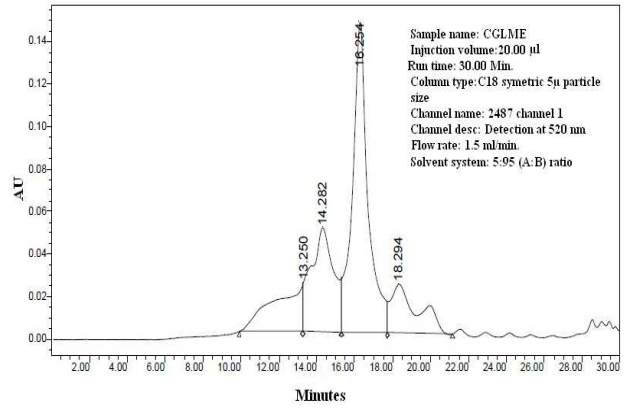


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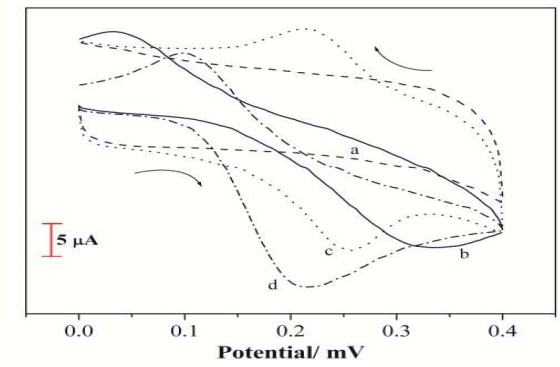
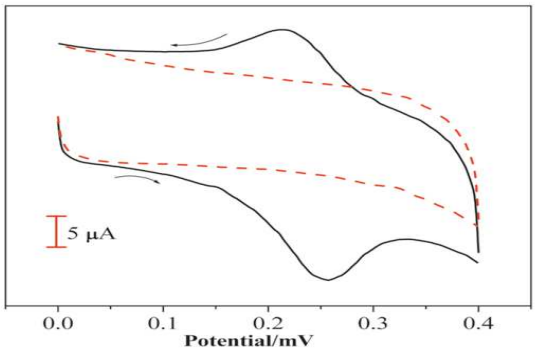
Fig. 2: A. HPLC chromatogram of standards Anthocyanin (A), Dulphindin (D) and Malvidin (M) on symmetric C18 column; Solvent system (A:B), Solvent A- Acetonitrile, Solvent B- 10% Acetic acid in water (5:95). B. HPLC chromatogram of CGLME on symmetric C18 column. Solvent system (A:B), Solvent A-Acetonitrile, Solvent B-10% Acetic acid in water (5:95).

The cyclic voltammograms of CGLME on CPE at different scan rate is shown in the Figure 3C. The observation was made to investigate the kinetics of the electrode reaction, wherein with the increased scan rate, the redox peak current also increased gradually indicating the direct electron transfer between CGLME and the CPE surface. The relationship between the scan rate and anodic currents was constructed as shown in Figure 3D. With the range from 50 to 300mV/s the redox peak current was proportional to the scan rate and the correlation coefficient was 0.9995, which indicates that the electron transfer reaction was adsorption controlled process [26]. The reproducibility of the proposed method for determining CGLME was tested in the phosphate buffer solution (pH 7.0) for ten repetitive times. It showed good reproducibility of modified electrode with a relative standard deviation of 2.4%. After each determination the modified electrode was washed with phosphate buffer solution and scanned using cyclic voltammeter in the blank phosphate buffer solution until the redox response wave of extract disappeared at 100mV/s in the potential range of 0 to 0.4V. After atmospheric exposure of modified electrode to one week, it was found that the electrochemical activity of the CPE remain almost same with the determination of CGLME, which indicated the good reproducibility and stability of the electrode.



A.



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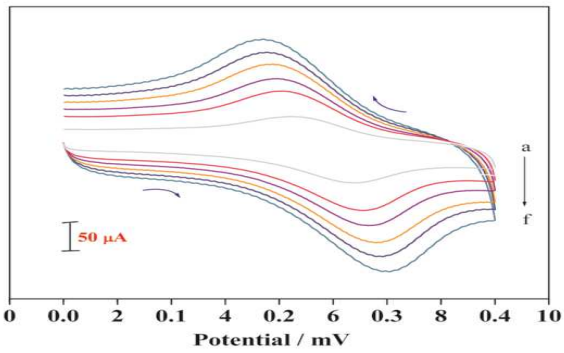

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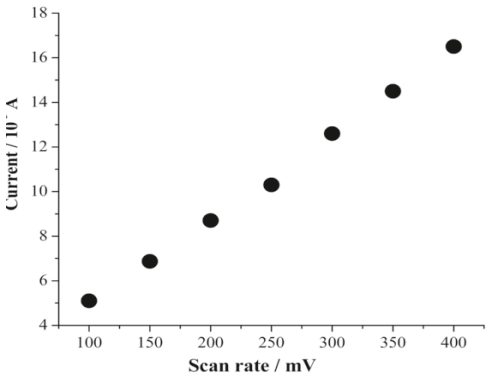
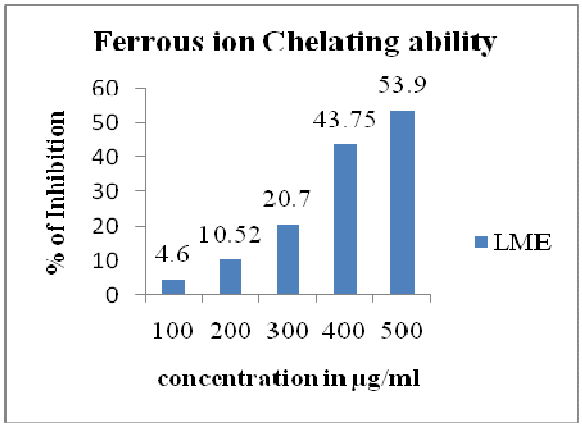


D.

Fig. 3: Behavior of cyclic voltammetry (CV) with CGLME, A. CV's of 1ml methanol alone, b.10µg CGLME, c. 1mM resorcinol and d. 1mM dopamine. B. CGLME (solid line) and methanol in (dotted line) at scan rate 50 mVs^{-1}. C. At different scan rates (a-f: 50-300mV/S) in 0.1PBS (pH7.0) at bare carbon paste electrode. D. Graph of scan rate versus current.

***In vitro* antioxidant assay**

In vitro antioxidant activities of *C. grandiflora* (20-100 µg) leaves extract of petroleum ether (CGLPE), chloroform (CGLCE) and methanol extracts (CGLME) were conducted, among them CGLME has shown significant activity. Based on antioxidant property, CGLME was selected for further *in vitro* and *in vivo* antioxidant assays to explore its reducing potential using different parameters since each *in vitro* antioxidant parameter will measure the different profile of CGLME at various concentration depending on the ability of collective multiple phytoconstituents action. The antioxidant potential of all the extracts were analyzed by DPPH radical scavenging assay and it was more in CGLME (82.20% at 100µg/mL) followed by CGLPE (45% at 100µg/mL) and CGLCE (28.80% at 100µg/mL). Many investigators [27] have demonstrated that the DPPH activity of plant extracts is ascribed to their hydrogen donating ability of different phytoconstituents. DPPH radical reacts with suitable reducing agent, wherein unstable nitrogen centered free radical accepts an electron or hydrogen atom to become a stable diamagnetic molecule. DPPH is a hydrophobic oxidizing free radical reduced by antioxidants. A freshly prepared DPPH solution exhibits a deep purple color and upon reacting with the antioxidants of CGLME the purple color disappears which can be measured by using spectrophotometer [28]. The reduction of DPPH radical in solution is based on the antioxidant potency of CGLME and was measured at 517nm [10]. DPPH free radical-scavenging ability of CGLME is shown in Fig. 4A (82.2% at100µg/mL) with linearly increased percentage inhibition. In metal chelating ability, the iron chelating (Figure 4B**)** measures the ability of antioxidant molecule to compete with ferrozine in chelating ferrous ions [29]. Ferrozine can quantitatively form complex with Fe^{2+}, the formation of the complex decreases as the CGLME competes with the ferrozine. Therefore, measurement of the rate of color reduction helps to estimate (53.9% at 500µg/mL) the chelating activity of the CGLME [30].



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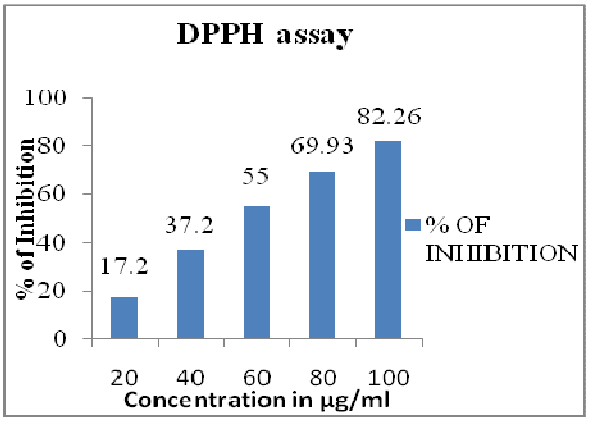

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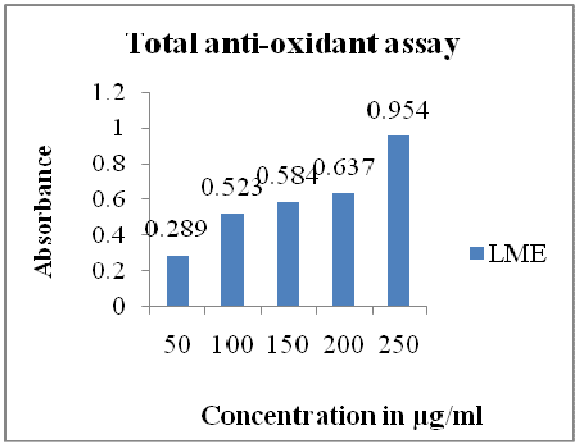

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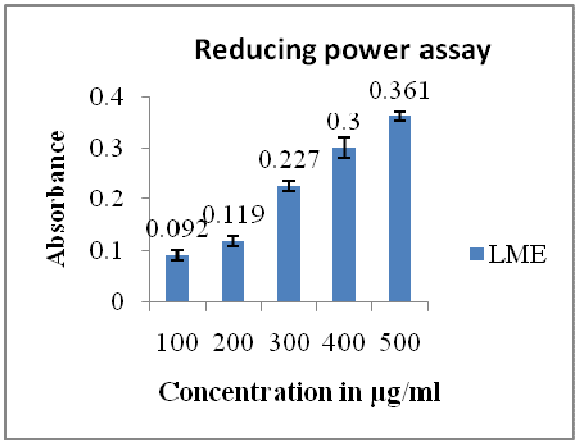

D

Fig. 4: Determination of *in vitro* antioxidant assays of CGLME, A. DPPH radical scavenging assay, B. Metal ion chelating ability, C. Reducing power of assay, D. Total antioxidant assay.

Some phenolic compounds exhibit antioxidant activity through the chelation of metal ions [31]. The chelating activity of CGLME was evaluated against Fe^{2+} to estimate the potential antioxidant activities where the Fe^{2+} can make a complex with ferrozine. The Fe^{2+} ions were captured by the CGLME, where the complex (red colored) formation is interrupted, as a result the red color of the complex is decreased. Thus, the chelating effect of the CGLME can be determined by measuring the rate of color reduction demonstrating the CGLME chelating ability.

The reducing property is generally associated with the presence of reductones [32]. The reductones antioxidant action is based on the breaking of free radical chain reaction by the donation of a hydrogen atom. They also react with certain precursors of peroxide thus preventing peroxide accumulation. Flavonoids play a key role since they can terminate radical chain reactions by converting free radicals to more stable products [33]. The reduction of Fe^{3+} / ferricyanide complex to the ferrous form occurs due to the presence of reductones in solution. The extent of Perls Prussian blue color formation indicates the reducing ability of CGLME measured at 700nm. The reduction power of the CGLME increases linearly as indicated with consistent increase in Prussian blue color formation **(**Figure 4C**)**. The reducing power of CGLME components may serve as a significant indicator of its potential antioxidant activity [34,35]. The CGLME has shown dose-dependent increase in reducing power as it measures the reductive ability of antioxidant molecules in the extract. Total antioxidant capacity of CGLME was found to be 0.954 absorbance at 250µg/mL as shown in Figure 4D. A measure of total antioxidant capacity helps to understand the redox-potential

associated with the reducing efficiency of molecules. According to Jayaprakasha et al [36] the antioxidant activity of any plant extract like CGLME depends on the presence of polyphenols which may act as reductones. In the present study CGLME (250µg/mL) has shown maximum absorbance at 740nm.

DNA nicking assay

DNA nicking assay was used for screening the efficiency of CGLME in scavenging the hydroxyl radicals (OH^-) produced by Fenton type of reaction, which interacts and breaks double stranded super coiled DNA into linear form at phosphodiester bond between adjacent nucleotides.

The effect of CGLME on induced oxidation to pUC19 plasmid DNA as shown in Figure 5. Nick was created by the interaction of free hydroxyl radicals generated from the Fenton reaction, thereby super coiled pUC19 plasmid DNA was broken into three forms such as supercoiled, open circular and linear form [37] as shown by the agarose gel electrophoresis (Figure 5). The 10µg/mL of CGLME showed to be good against creating the nick in DNA molecule eventually an increased native form of DNA was observed. Thus, the synergistic efficiency [38] of all different antioxidant molecules in CGLME could be involved in protecting the DNA molecule against hydroxyl radicals toxicity. Previous scientific study [14] has been demonstrated that redox-active metal ions may bind and form complexes with phenolic antioxidant molecules, thus prevents the reduction of redox-active metal ions with H_2O_2. The phenolic molecules may have significantly protected induced oxidation damage to pBR322 plasmid DNA by Fenton type of reaction through a mechanism of electron or hydrogen-atom transfer [39].

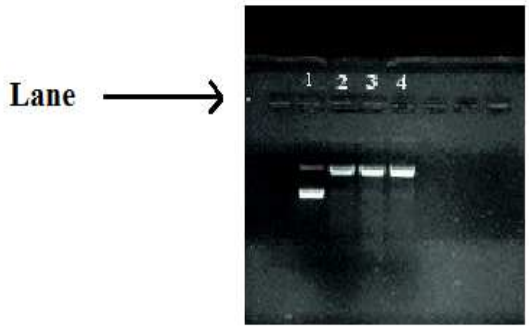

Fig. 5: The effect of CGLME on the integrity of pUC19 plasmid DNA in the presence of Fenton's reagents. Lane1: pUC19+FR; Lane2: pUC19+ H_2O; Lane: 3 pUC19+ FR+10µg; Lane 4: pUC19 + FR+ standard antioxidant compound (Resorcinol) Here, FR=Fenton's Reagent (H_2O_2+Fe_3++ Ascorbic acid).

DNA binding assay

Absorption titration experiments were carried out by varying DNA concentration (0 to 100µM) with steady amount of CGLME (0.5µM). Absorption spectra was recorded after successive addition of different concentration of DNA with parallel equilibration (≈10 min) in order to obtain the intrinsic binding constant,

Kb: [DNA] / (εa- εf) = [DNA] / (εb- εf) + 1/ Kb (εb - εf)

Where εa, εf and εb are the apparent, free and bound CGLME extinction coefficients respectively. A plot of [DNA]/ (εa- εf) versus [DNA] gave a slope of 1/ (εb-εf) and an intercept on y axis equal to 1/Kb (εb -εf), where Kb is the ratio of the slope to the intercept y [40]. To further illustrate the DNA binding strength of a plant extract, the intrinsic binding constant Kb was determined for CGLME which was found to be $3.18 \times 10^4 M^{-1}$. The binding constant of this CGLME was lower in comparison to typical classical intercalators (Ethidium-DNA, $1.4 \times 10^6 M^{-1}$) [41].

The DNA binding property of CGLME was often characterized by absorption spectra by following changes in the absorbance and shift in the wavelength. The interaction of CGLME with Ct-DNA was monitored by the red shift (the bathochromic effect) in UV–visible spectra as shown in Figure 6. The absorption maximum wavelength was shifted from 268 to 284nm in the spectra of Ct-DNA/ CGLME.

Proportionate increase in Ct-DNA led to strong hypochromism of absorption intensities in the CGLME studied. The hypochromicity, characteristic intercalation [20] has been usually credited to the interaction between the electronic states of molecules present in the plant extract and those of DNA bases [42]. The percentage hypochromicity of CGLME was determined using the following equation $(\varepsilon_f - \varepsilon_b)/\varepsilon_f \times 100$, where ε_f is the extinction coefficient of the free extract and ε_b is the extinction coefficient of the bound extract. The percentage hypochromism of CGLME was found to be 18.6. The half-reciprocal plot for binding of CGLME with Ct-DNA was as presented in Figure 6.

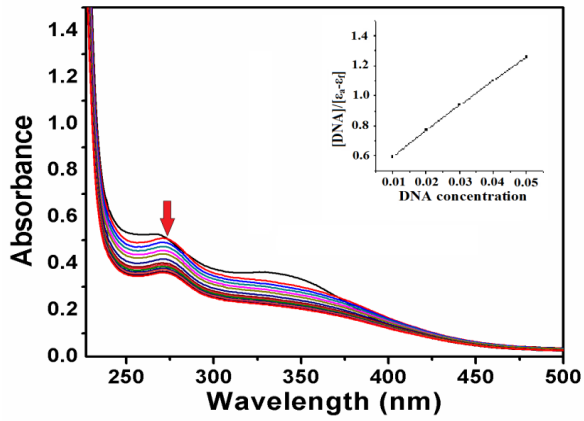

Fig. 6: Absorption spectra of CGLME in Tris-HCl buffer upon addition of ct- DNA. [Complex] = 0.5 µM, [DNA] = 0.1 µM. Arrow shows the absorbance changing upon increase of DNA concentration. The inner plot of [DNA]/εa-εf vs [DNA] for the titration of Ct-DNA with CGLME.

***In vivo* antioxidant assay**

Free radicals being produced in normal or pathological condition may contain one or more unpaired electrons. ROS are various forms of activated oxygen species, either in the form of superoxide (O_2^-), non-free radical species (H_2O_2), hydroxyl radical (OH^+) or singlet oxygen (O_2) [43] is a product of normal/pathological disturbance in a metabolism and attacks biological molecules like lipids, proteins, enzymes, DNA and RNA leading to cell or tissue injury [44].

The results of *in vivo* antioxidant enzyme assay of CGLME were as shown in Table 1. Mice was treated by intra peritoneal injection of Carbon tetrachloride (CCl_4), as a result the raising the serum levels of liver non-specific enzymes such as ALT, AST, ALP and LDH indicating severe hepatic cell necrosis [45]. Liver is not only a target organ of CCl_4 intoxication but it also affects other several organs like kidneys, heart, lungs, brain and blood [46]. Enormous generation of free radical metabolites such as CCl_3 and CCl_3COO which incite interaction with lipid membrane causing the damage to cellular macro molecules leading to elevation of lipid peroxidation in liver [47].

Generally, the major effect of CCl_4 free radical on liver antioxidant enzymes (catalase, peroxidase and superoxide dismutase) has been shown to be reduced due to inactivation during the catalytic cycle [48]. However, treatment with any antioxidant molecule could apparently reduce the lipid peroxidation with concomitant decrease in enzyme inactivation to restore enzyme activity [49]. The activities of catalase, peroxidase and superoxide dismutase in the liver were significantly lower in mice treated with CCl_4 when compared to

normal control group. The diminished level of catalase, peroxidase and superoxide dismutase in CCl_4 alone intoxicated group have been significantly restored by CGLME (P<0.001). The results indicate the significant increase in the levels of antioxidant and prophylactic enzymes in *in vivo* (mice) at 500mg/kg b. w.

SOD has been reported as one of the most effective enzyme in intracellular antioxidant defense system [50]. It plays an important role in the conversion of superoxide anion into hydrogen peroxide and molecular oxygen, thus diminishing the toxic effect and protecting the cells from peroxidative process in liver [51]. The decrease in percentage inhibition of superoxide anion by SOD may be due to hepatocellular damage by CCl_4. However, an increase in the percentage inhibition of superoxide after administration of CGLME explicitly implies an efficient protective mechanism of this plant. This may be attributed to the presence of secondary metabolites such as polyphenols, flavonoids etc., that are well known potent free radical scavengers.

Table 1: *In Vivo* antioxidant activity of CGLME

Groups	Treatment	Catalase activity µ mol/mL	Lipid peroxidation mol/min/mg	Total peroxidase mol/min/mg	Superoxide dismutase u/mg/protein
I	Normal control	10.46±0.61	9.57±0.07	4.62±0.16	0.15±0.01
II	CCl_4/Control 2mL/kg b.w.	7.41±0.30	19.24±0.40	3.3±0.07	0.13±0.00
II	CGLME 250mg/kg b.w.	12.18±0.20	16.54±0.09**	4.14±0.01*	0.14±0.01
IV	CGLME 500mg/kg b.w.	13.09±0.20**	17.17±0.07**	4.45±0.14	0.17±0.01*
V	CGLME 750mg/kg b.w.	16.09±0.20**	13.17±0.10**	5.19±0.04*	0.16±0.01
VI	Vitamin E 100mg/kg b.w.	11.96±0.18	15.12±0.03	4.32±0.24	0.15±0.02

Effect of CGLME on *In vivo* antioxidant activity, each value are in Mean ± S.E (n = 4 mice). Where, *p<0.05 and **p<0.01, the oral administration of CGLME to Group III= 250 mg/kg b. w.; Group IV = 500 mg/kg b. w.; Group V = 750 mg/kg b. w. and Group VI= Vitamin E 100 mg/kg b. w. Results are expressed as percentage inhibition of the control.

Table 2: Anti-proliferative activity of CGLME

S. No.	Plant extract	CGLME in (µg/mL)	% inhibition	CTC50 (µg/mL)
1.	CGLME	1000	75.7±0.301	750.00±0.00
		500	23.1±0.505	
		250	18.9±0.700	
		125	17.3±0.307	
		62.5	13.9±0.502	

Values were expressed as mean ± SD (n=3).

Anti-proliferative activity

The anti-proliferative study was performed for CGLME against Caco-2 cell line at different concentrations to determine the CTC_{50} of (sample concentration tolerated by 50% of the cell cultures) CGLME. Results are tabulated in Table 2.

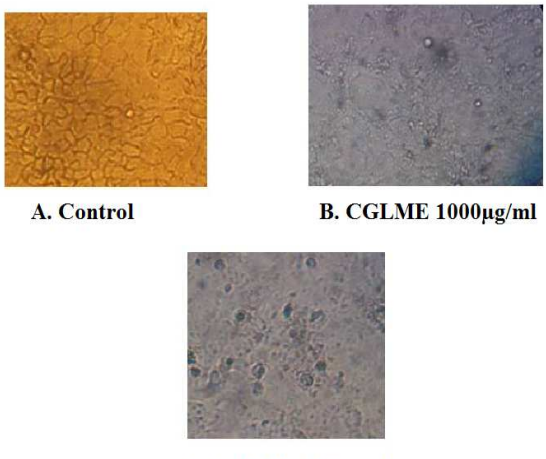

Fig. 7: Absorption spectra of CGLME in Tris-HCl buffer upon addition of Ct-DNA. [Complex]= 0.5µM, [DNA] = 0.1µM.

Arrow shows the absorbance changing upon increase of DNA concentration. The inner plot of [DNA]/εa-εf vs [DNA] for the titration of Ct-DNA with CGLME.

The percentage growth inhibition has been increased from 13.9 to 75.7 for 62.5 to 1000µg/mL CGLME and moderate activity was observed at 1000µg/mL against Caco-2 cancer cell line (Figure 6) with CTC50 value of 750.0µg/mL. Previous studies reported the anti-proliferative activity of alcoholic extract of *C. grandiflora* against KB cell line [52] and methanol extract showed partial antiviral activity at 182µg/mL [53]. Generally, anti-proliferative activity of plant extracts could be due to synergistic effect of secondary metabolites like alkaloids and terpenoids [54]. The traces of these molecules in CGLME may have been responsible for anti-proliferative activity against Caco-2 cell line.

CONCLUSION

The present investigation gathered experimental evidence that CGLME has shown significant *in vitro*, *in vivo* antioxidant property in various models and anti-proliferative activity due to catecholamines and allied molecules present in CGLME. Further research warrants isolation of active molecules and molecular mechanism on relative to antioxidant and anti-proliferative property.

REFERENCES

1. Tuba AK, Gulcin I. Antioxidant and radical scavenging properties of curcumin. Chemico-Biol. Inter 2008; 174: 27-37.
2. Koneru A, Sathyanarayana S, Mukkanti K, Khan KA. *In vitro* antioxidant activity of Itrifal Kishneezi: A unani formulation. American Journal of Drug Discovery Development 2011; 1: 121–128.
3. Moskovitz J, Yim MB, Choke PB. Free radicals and disease. Archives of Biochemistry and Biophysics 2002; 397: 354–359.
4. Kaliora AC, Dedoussis GV, Schmidt H. Dietary antioxidants in preventing atherogenesis. Atherosclerosis 2006; 187(1): 1–17.
5. Sun Y. Free radicals, antioxidant enzymes and carcinogenesis. Free Radic Biol Med 1990; 8(6): 583-599.

6. Stadtman ER. Protein oxidation and aging. Science 1992; 257(5074): 1220-1224.
7. Chaudhuri SK, Huang L, Fullas F, Brown DM, Wani MC, Wall ME. Isolation and structure identification of an active DNA strand-scission agent, (+)-3, 4-dihydroxy-8, 9-methylenedioxy-pterocarpan. J. Nat. Prod 1995; 58(12): 1966-1969.
8. Santhosh KH, Manjunatha H, Krishna V, Pradeepa K, Rajesh Gupta KP. Analgesic activity of *Cryptostegia grandiflora* (Roxb.) R.Br. leaves methanol extract using mice. Asian Pacific Journal of Tropical Disease 2012; S494-S498.
9. Matthaus B. Antioxidant activity of extracts obtained from residues of different oilseeds. J Agric Food Chem 2002; 50: 3444–3452.
10. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958; 181: 1199-1200.
11. Decker EA, Welch B. Role of ferritin as a lipid oxidation catalyst in muscle food. J. Agr. Food Chem 1990; 38: 674-677.
12. Oyaizu M. Studies on products of browning reaction Anti oxidative activities of products of browning reaction prepared from glucosamine. Jpn. J. Nutr 1986; 44: 307-315.
13. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a Phosphomolybdenum Complex: Specific application to the determination of vitamin E. Analytical Biochemistry 1999; 269: 337-341.
14. Lee JC, Kim HR, Kim J, Jang YS. Antioxidant property of an ethanol extract of the stem of Opuntia ficus-indica var. Saboten. J. Agric. Food Chem 2002; 50(22): 6490-6496.
15. Reichmann ME, Rice SA, Thomas CA, Doty P. A further examination of the molecular weight and size of desoxypentose nucleic acid. J. Am. Chem. Soc 1954; 76: 3047-3053.
16. Wolfe A, George HSJ, Meehan T. Polycyclic Aromatic Hydrocarbons Physically Intercalate into Duplex Regions of Denatured DNA. Biochemistry 1987; 26(20): 6392-6396.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin–phenol reagent. J. Biol. Chem 1951; 193: 265-275.
18. Claiborne A. Catalase activity. Handbook of Methods for Oxygen Radical Research, CRC Press Inc. USA 1985; 283–284.
19. Nicholos MA. A spectrophotometric assay for iodide oxidation by thyroid. Analytical Biochemistry 1962; 4(4): 341–345.
20. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978b; 52: 302-10.
21. Lai LS, Chang PC, Chang CT, Isolation and characterization of superoxide dismutase from wheat seedlings. J. Agric. Food. Chem 2008; 56(17): 8121–8129.
22. Francis D, Rita L. Rapid colorometric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of Immunological Methods 1986; 89: 271-277.
23. Vinson JA, Yong H, Xuchui S, Zubik L. Phenol antioxidant quantity and quality in foods: vegetables. J. Agric. Food Chem 1998; 46: 3630-3634.
24. Gow Chin Y, Pin Der D, Cherng Liang T. Relationship between antioxidant activity and maturity of peanut hulls. J. Agric. Food Chem 1993; 41(1): 67-70.
25. Krzysztof Pawlak, Wieslawa Bylka, Beata Jazurek, Irena Matlawska, Maria, Sikorska, Henryk Manikowski, Grazyna Bialek-Bylka. Antioxidant activity of flavonoids of different polarity, assayed by modified ABTS cation radical decolorization and EPR technique. Acta Biologica Cracoviensia Series Botanica 2010; 52(1): 97–104.
26. Chandrashekar BN, Kumara Swamy BE, Pandurangachar M, Sathisha TV, Sherigara BS. Colloids and Surfaces B. Biointerfaces 2011; 88: 413–418.
27. Kazuko S, Kuniko F, Keiko Y, Takashi N. Antioxidative properties of xanthan on autooxidation of soybean oil in cyclodextrin. J. Agric. Food Chem 1992; 40(6): 945-948.
28. Surendra KRS, Naveen Goyal. *In Vitro* Antioxidant Activity of root extracts of *Heliotropium eichwaldi* Stued. Research Journal of Pharmaceutical Biological and Chemical Science. 2012; 3(1): 444.
29. Elmastas M, Gulcin I. Radical scavenging activity and antioxidant capacity of bay leaf extract. J. Iran. Chem Soc 2006; 3: 258-266.
30. Chua MT, Tung YT, Chang ST. Antioxidant activities of ethanolic extracts from the twigs of Cinnamomum osmophloeum. Bioresource Technology 2008; 99: 1918–1925.
31. Zhao H, Fan W, Dong J, Lu J, Chen J, Shan L, Linc Y, Kong W. Evaluation of antioxidant activities and total phenolic contents of typical malting barley varieties. Food Chem 2008; 107(1): 296-304.
32. Pin-Der Duh. Antioxidant activity of budrock (Arctium lappa Linn): It's scavenging effect on free radical and active oxygen. J. Am. Oil Chem. Soc 1998; 75: 455-461.
33. Ming-Zhu Jiang, Hui, Yan, Yan Wen, Xiang-Mei Li. *In vitro* and *in vivo* studies of antioxidant activities of flavonoids from Adiantum capillus-veneris L. African Journal of Pharmacy and Pharmacology 2011; 5(18): 2079-2085.
34. Gulcin I, Buyukokuroglu ME, Oktay, Kufrevioglu OI. Antioxidant and analgesic activities of turpentine of Pinus nigra Arn subsp. pallsiana (Lamb.) Holmboe. J. Ethnopharmacology 2003; 86: 51-58.
35. Shimon Meir, Joseph Kanner, Bezalel Akiri, Sonia Philosoph-Hadas. Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. J. Agric. Food Chem 1995; 43: 1813-1819.
36. Jayaprakasha GK, Selvi T, Sakariah KK. Antibacterial and antioxidant activities of grape (Vitis vinifera) seed extracts. Food Res Int 2003; 36(2): 117-122.
37. Thampi BS, Manoj G, Leelamma S, Menon VP. Dietary fibre and lipidperoxidation: effects of dietary fibre on levels of lipids and lipid peroxides in high fat diet. Ind. J. Exp. Biol 1991; 29(6): 563-567.
38. Zhou J, Li P, Cheng N, Gao H, Wang B, Wei Y, Cao W. Protective effects of buckwheat honey on DNA damage induced by hydroxyl radicals. Food and Chemical Toxicology 2012. 50: 2766–2773.
39. Singh BN, Singh BR, Singh RL, Prakash D, Dhakarey R, Upadhyay G, Singh HB. Oxidative DNA damage protective activity, antioxidant and anti-quorum sensing potentials of *Moringa oleifera*. Food and Chemical Toxicology 2009; 47: 1109–1116.
40. Prakash D, Singh BN, Upadhyay G. Antioxidant and free radical scavenging activities of phenols from onion (*Allium cepa*). Food Chem 2007; 102: 1389–1393.
41. Bhojya Naik HS, Prabhakara MC. Mixed ligand Ni (II) complexes: DNA binding, oxidative and photo-cleavage studies. Main Group Chemistry 2008; 7: 97-107.
42. Lepecq JB, Paoletti C. A fluorescent complex between ethidium bromide and nucleic acids: Physical-Chemical characterization. J. Mol. Biol. 1967; 27: 87-106.
43. Subhadradevi V, Asokkumar K, Umamaheswari M, Sivashanmugam A, Sankaranand R. *In vitro* antioxidant activity of *Vetiveria zizanioides* root extract. Tanzania Journal of Health Research. 2010; 12: 276.
44. Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chemistry. 2004; 84: 551–562.
45. Asha VV. Preliminary studies on the hepatoprotective activity of Manorial *Subangulata and Naragamia alata*. Indian J. Pharmacol 2001; 33: 276-279.
46. Ahmad FF, Cowan DL, Sun AY. Detection of free radical formation in various tissues after acute carbon tetrachloride administration in gerbil. Life Sci 1987; 41(22): 2469-2475.
47. Gulçin I, Kufrevioglu OI, Oktayb M, Buyukokuroglu ME. Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.). J. Ethnopharmacology 2004; 90*:* 205–215.
48. Srinivasan R, Chandrasekar MJ, Nanjan MJ, Suresh B. Antioxidant activity of *Caesalipinia digyna* root. J. Ethnopharmacology 2007; 113: 284-291.
49. Dietrich M, Block G, Hudes M, Morrow JD, Norkus EP, Traber MG, Cross CE, Packer L. Antioxidant supplementation decreases lipid peroxidation biomarker F (2)-isoprostanes in plasma of smokers. Cancer Epidemiol Biomarkers Prev 2002; 11(1): 7-13.
50. Curtis SJ, Moritz M, Snodgrass PJ. Serum enzymes derived from liver cell fractions. I. The response to carbon tetrachloride intoxication in rats. Gastroenterology 1972; 62: 84-92.

51. Aravind T, Bhojya Naik HS, Prakash Naik HR, 1, 2, 3-Triazole Fused Quinoline-Peptidomimetics: Studies on Synthesis, DNA Binding and Photonuclease Activity. Int. J. Pept Res Ther 2009; 15: 273–279.
52. Raymond WD, Younas Malik M, Charles DH, Shah NM, John ET, Wolfgang K. Antitumor agents V: Cytotoxic cardenolides from *Cryptostegia grandiflora* (Roxb.) R. Br. Journal of Pharmaceutical Sciences 1972; 61(4): 570–573.
53. Vijayan P, Raghu C, Ashok G, Dhanaraj SA, Suresh B. "Antiviral activity of medicinal plants of Nilgiris. Indian J Med Res 2004; 120(1): 24-29.
54. Sini KR, Haribabu Y, Sajith MS, Surya Sreekumar K, *In-vitro* cytotoxic activity of *Orthosiphon thymiflorus* Roth.) Sleensen leaf extract against Dalton Lymphoma Ascites cell line. Journal of Chemical and Pharmaceutical Research 4(1): 917-921.