Project Report

In vitro study of the antioxidant, iron chelating and DNA protective properties of medicinal plants, their formulations and usefulness to combat cancer

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1. **Project title:** *In vitro* study of the antioxidant, iron chelating and DNA protective properties of medicinal plants, their formulations and usefulness to combat cancer.

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4. **Other Scientific Staff engaged in the study:** None

5. **Non-Scientific Staff engaged in the study:** None

6. **Implementing Institution and other collaborating Institutions:** Bose Institute

7. **Date of commencement:** 16/4/2008 (16th April 2008).

8. **Duration:** 2 years

9. **Date of completion:** 15/4/2010 (15th April 2010)

10. **Objectives as approved:**
    (i) Searching for the antioxidant, iron chelator and DNA protective activities from crude extracts and purified fractions of five medicinal plant, i.e. *Terminalia chebula, Terminalia belerica, Emblica officinalis, Caesalpinia crista, Cajanus cajan*, Amrit Bhallataka, Guduchi (*Tinospora cordifolia*) and their formulations by using biochemical approaches *in vitro* system.
    (ii) Study of the anticancer activity of the crude extracts and purified fractions from the same plants and their formulations by selective killing of Ehrlich’s Ascites Carcinoma (EAC) cell and various types of human cancer cell lines *in vitro* systems.
    (iii) Study of the authenticity of the herbal formulations possessing antioxidant, iron chelating, DNA protective and anticancer properties based on chemical fingerprinting by HPTLC/ HPLC.

11. **Deviation made from original objectives if any, while implementing the project and reasons thereof:** Not Applicable
12. Progress report during April 2008-April 2010 (Experimental work giving full details of experimental set up, methods adopted, data collected supported by necessary tables, charts, diagrams and photographs):

**Introduction:**

Potentially harmful reactive oxygen species (ROS), which include reactive molecules such as superoxide radical (O$_{2}^{-}$), hydroxyl radical (OH$^{-}$), hydrogen peroxide (H$_{2}$O$_{2}$) and nitric oxide (NO$^{+}$), evolved as a consequence of normal aerobic metabolism (Aruoma et al. 1989; Halliwell 1991). ROS directly bring about damage of cellular macromolecules, especially DNA, which undergo strand breakage, change and release of bases as well as modification of sugar moieties (Henle and Linn 1997; Marnett 2000), thus promoting a series of pathological events, viz. cancer and ageing (Retsky et al. 1993). On the other hand ROS along with pro-oxidants give rise to emphysema, cirrhosis, arteriosclerosis, inflammation, genotoxicity and other diseased conditions (Braaca et al. 2002). Various food supplements containing antioxidants act through different mechanisms - such as chelating metals that catalyze the formation of free radicals and also by scavenging the same, thus becoming vital for human body due to their ability to combat oxidative damage. Use of synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) have been restricted due to their possible carcinogenic properties (Branen 1975). Thus, major attention is being given to isolation of natural antioxidants. Presence of natural antioxidants from different kinds of plant materials have been reported (Ashokkumar et al. 2008; Owolabi et al. 2008) and it has been found that these phytocompounds are of great interest (Bongiorno et al. 2008; Naik and Panda 2008). Many indigenous herbal plants have been used as medicines in India (Packer and Ong 1998; Jovanovic and Simic 2000).

In case of cancer treatment, chemotherapy is supposed to be the only remedy. Chemotherapeutic drugs are chemically designed to target cells that are dividing and growing rapidly. Once they reach the cancer cells, they act to retard their growth, eventually resulting in their destruction. Since chemotherapy also affects normal actively dividing cells, most patients experience some degree of side effects. Recent development of herbal medicines for treatment of cancer has aroused keen interest in research. Several Indian medicinal plants have shown anticancer activity (Saha et al. 2006; Saleem et. al. 2002). So, it will be interesting to study the antioxidant, iron chelating, DNA protective and finally anticancer activites of the proposed medicinal plants in this project proposal.
**Methodology:**

**Collection & Authenticity of medicinal plants:**

The plant materials were collected from Bankura district of West Bengal, India and the genus and species of the medicinal plants are authenticated through the Central Research Institute (Ayurveda), Kolkata, India, where specimens for each plant were deposited. A list of the specimen numbers for the plants is provided below:

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Name of the plant</th>
<th>CRI (Ayurveda) specimen No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Terminalia chebula</em></td>
<td>CRHS 113/08</td>
</tr>
<tr>
<td>2</td>
<td><em>Terminalia belerica</em></td>
<td>CRHS 114/08</td>
</tr>
<tr>
<td>3</td>
<td><em>Emblica officinalis</em></td>
<td>CRHS 115/08</td>
</tr>
<tr>
<td>4</td>
<td><em>Caesalpinia crista</em></td>
<td>CRHS 121/08</td>
</tr>
<tr>
<td>5</td>
<td><em>Cajanus cajan</em></td>
<td>CRHS 119/08</td>
</tr>
</tbody>
</table>

**Preparation of crude extract:**

The powder (100 g) of the dried fruits, leaves or stem of the proposed plants was stirred using a magnetic stirrer with 500 ml mixture of ethanol:water (6:4) (hydro-alcoholic extract) for 15 hours; then the mixture was centrifuged at 2850 x g and the supernatant decanted. The process was repeated again with the precipitated pellet. The supernatants were collected, concentrated in a rotary evaporator at room temperature and freeze dried in a lyophilizer. The dried extract was stored at -20°C until use.

**Antioxidant and Phytochemical studies:**

**Antioxidant activity:**

Antioxidant capacity was measured based on the scavenging of ABTS$^+$ radical cation by the sample in comparison to trolox standard (Hazra et al. 2008). ABTS solution was mixed with potassium persulfate to generate ABTS$^+$ radical cation. Then 10 μl sample solution was mixed with 1 ml ABTS$^+$ solution and the absorbance was measured at 734 nm. All experiments were repeated six times. The percentage inhibition of absorbance was calculated and plotted as a function of concentration of standard and sample to determine the trolox equivalent antioxidant concentration (TEAC).

**Reducing power:**

The Fe$^{3+}$-reducing power of the extract was determined by a standard method (Hazra et al. 2008). In a phosphate buffer solution (0.2 M, pH 6.6), different concentrations (0.0-0.4 mg/ml) of the extract were mixed with potassium hexacyanoferrate (0.1%), followed by incubation. After incubation, the upper portion of the solution was diluted, and FeCl$_3$ solution (0.01%) was
added. The reaction mixture was left at room temperature for colour development and the absorbance was measured at 700 nm. All tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a positive control.

**Determination of total phenolic content:**
The amount of total phenols present in the plant extracts, as given in Table 1, was determined using Folin-Ciocalteu (FC) reagent by a formerly described method (Hazra et al. 2008). The phenolic content was evaluated from gallic acid standard curve.

**Determination of total flavonoid content:**
The amount of total flavonoids was determined with aluminium chloride (AlCl₃) according to an earlier method (Hazra et al. 2008). The flavonoid content was calculated from quercetin standard curve and the values for each plant material have been given in Table 1.

**Reactive Oxygen Species (ROS) scavenging capacities:**

**Hydroxyl radical:**
The scavenging assay for hydroxyl radical was performed by a standard method (Hazra et al. 2008). Hydroxyl radical was generated by the Fenton reaction using a Fe³⁺-ascorbate-EDTA-H₂O₂ system. The assay quantifies the 2-deoxyribose degradation product, by its condensation with TBA. All tests were carried out six times. Mannitol, a classical .OH scavenger, was used as a positive control. Percent inhibition was evaluated by comparing the results of the test and blank solution.

**Superoxide radical:**
Measurements of superoxide anion scavenging activities of the sample and standard quercetin were done based on the reduction of NBT according to a previously described method (Hazra et al. 2008). Superoxide radical is generated by a non-enzymatic system of phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH). These radicals reduce nitro blue tetrazolium (NBT) into a purple colored formazan which was measured spectrophotometrically at 562 nm. All tests were performed six times.

**Nitric oxide radical:**
Sodium nitroprusside (SNP) gives rise to nitric oxide that under interaction with oxygen produce nitrite ions measured by Griess Illosvoy reaction (Hazra et al. 2008). The chromophore generated was spectrophotometrically measured at 540 nm against blank sample. All tests were performed six times. Curcumin was used as a standard.
Hydrogen peroxide scavenging:
FOX-reagent method was used to determine this activity of the sample and the reference compound sodium pyruvate, as previously described (Hazra et al. 2008). The absorbance of the ferric-xylenol orange complex was measured at 560 nm. All tests were carried out six times.

Peroxynitrite scavenging:
Peroxynitrite (ONOO\(^-\)) synthesis was done 12 hrs before the assay, according to Beckman et al. (1994). Acidic solution (0.6 M HCl) of 5 ml H\(_2\)O\(_2\) (0.7 M) was mixed with 5 ml of 0.6 M KNO\(_2\) on an ice bath for 1 s and 5 ml of ice-cold 1.2 M NaOH was added to the reaction mixture. Excess H\(_2\)O\(_2\) was adsorbed by granular MnO\(_2\) and the reaction mixture was left at -20°C. The concentration of the peroxynitrite solution was measured spectrophotometrically at 302 nm (ε = 1670 M\(^{-1}\) cm\(^{-1}\)).

Evans blue bleaching assay was used to measure the peroxynitrite scavenging activity (Hazra et al. 2008). The percentage of scavenging of ONOO\(^-\) was calculated by comparing the results of the test and blank sample. All tests were performed six times. Gallic acid was used as reference compound.

Singlet oxygen scavenging:
Singlet oxygen (1\(^O_2\)) production, and at the same time, its scavenging by the sample and the reference compound lipoic acid can be monitored by \(N,N\)-dimethyl-4-nitrosoaniline (RNO) bleaching, using a earlier reported method (Hazra et al. 2008). Singlet oxygen was generated by a reaction between NaOCl and H\(_2\)O\(_2\) and the bleaching of RNO was read at 440 nm. All tests were performed six times.

Hypochlorous acid scavenging:
According to a previously described method (Hazra et al. 2008), hypochlorous acid (HOCl) was prepared just before the experiment by adjusting the pH of a 10% (v/v) solution of NaOCl to pH 6.2 with 0.6 M H\(_2\)SO\(_4\) and the concentration of HOCl was determined by taking the absorbance at 235 nm using the molar extinction coefficient of 100 M\(^{-1}\) cm\(^{-1}\). The scavenging activities of the plant extract and the standard, ascorbic acid, a potent HOCl scavenger was evaluated by measuring the decrease in the absorbance of catalase at 404 nm. All tests were performed six times.

Iron chelation and DNA protective activites:

Fe\(^{2+}\) chelating:
The ability of chelating of ferrous ion by the sample in comparison to the standard EDTA was evaluated according to Hazra et al. (2008). In a Heps buffer (20 mM, pH 7.2) medium, the plant extract was added to ferrous sulfate solution (12.5 μM) and the reaction was started by the
addition of ferrozine (75 μM). The mixture was shaken vigorously and left standing for 20 min at room temperature. The absorbance was read at 562 nm. All tests were performed six times.

**Lipid peroxidation inhibition:**

The antioxidant and iron chelating capacity of the plant extracts were alternatively measured by lipid peroxidation inhibition, following a method (Kizil et al. 2008), with slight modification. Brain homogenate was prepared by centrifuging Swiss Albino mice brain (20 ± 2 gm) with 50 mM phosphate buffer (pH 7.4) and 120 mM KCl, at 3000 rpm for 10 min. A 100 μl aliquot of the supernatant homogenate was mixed with plant extract of various concentrations (2.5-25 μg/ml), followed by addition of 0.1 mM FeSO₄ and 0.1 mM ascorbic acid, each of 100 μl and incubated for 1 hr at 37 °C. 500 μl 28% TCA was used to stop the reaction and then 380 μl 2% TBA was added with heating at 95 °C for 30 min, to generate the colour. Then, the samples were cooled on ice, centrifuged at 8000 rpm for 2 min and the absorbance of the supernatant was taken at 532 nm. All tests were repeated 6 times. Trolox was used as the standard.

**DNA protection:**

The protection of the pUC-18 plasmid DNA damaged by Fenton reaction generated OH radicals was studied by quantifying the decrease of supercoiled DNA after oxidative attack, following an earlier reported method (Hermes-Lima et al. 1998), with minor modifications. In Hepes buffer (pH 7.2), FeSO₄ solution, plant extracts of varying concentrations, DNA (10 μg/ml final concentration) and water were added to make an initial reaction mixture. Finally, H₂O₂ solution was added to start the reaction. After 10 min, the reaction was stopped by adding Desferal as stopping reagent followed by loading buffer. 25 μl of each reaction mixture was loaded in 1% agarose gel. After migration, the gel was stained with ethidium bromide and visualized in a UV transilluminator. The DNA bands were quantified through densitometry and the following formulae were used to calculate the percentage of protection.

\[
\% \text{ SC} = \left[ \frac{1.4 \times \text{ SC}}{(\text{OC} + (1.4 \times \text{ SC}))} \right] \times 100
\]

where, SC = supercoiled; OC = open circular; 1.4 = correction factor

\[
\% \text{ protection} = 100 \times \left[ \frac{(\text{control SC} - \text{chelator SC})}{(\text{control SC} - \text{no chelator SC})} - 1 \right]
\]

**Cytotoxicity and Anticancer activities:**

**Animals:**

The Animal Ethical Clearance Committee of the Institute (Registration number: 95/1999/CPCSEA) approved the use of adult male Swiss Albino mice, weighing 20-25 gm for experimentation. The animals were obtained from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. They were housed in standard environmental conditions of temperature
(25 ± 2°C), humidity (60 ± 5%) and under light and dark cycles of 12-h. The mice were fed standard laboratory diet and were given sterilized water ad libitum.

Isolation of Cancer cells (EAC):
The transplantable cancer cell namely Ehrlich’s Ascites Carcinoma (EAC) cells were used in the present study. The EAC cells were maintained in vivo in Swiss albino mice, by interperitoneal (ip) transplantation of 2 x 10⁶ cells /mouse after every 10 days. EAC cells 9 days old were used for the experiment. These tumors in the ascites form are fast growing and kill the host animals within the period of three-four weeks approximately after tumor transplantation with 2 x 10⁶ cells. The tumor uptake by the host was manifested by a very high initial growth rate followed by exponential growth up to a period of 10th day post transplantation (log phase) followed by gradual decline in this growth rate with progressive accumulation of ascites fluid (lag phase). By the 21st day post transplantation, the tumor volume reaches its maximum.

The mice were killed by cervical dislocation (acceptable method of euthanasia), dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000 rpm for 5 min. It was then resuspended in excess RPMI-1640 medium (with 10% HI FBS) and taken in sterile Petri dishes and incubated in a CO₂ incubator (37°C; 5% CO₂) for 1 h. The cells of macrophage lineage adhered to the bottom of the Petri dishes. The non-adherent population was aspirated out gently and washed repeatedly with PBS. The cells were centrifuged at 3000 rpm for 5 min, resuspended in medium and counted in a haemocytometer.

Isolation of Splenocyte:
Spleens from normal healthy mice were dissected after sacrifice of the mice and washed twice with sterilized PBS. The spleen was then taken in a Petri dish containing RPMI-1640 medium (with 10% HI FBS) and incubated in a CO₂ incubator (37°C; 5% CO₂) for 1 h. The non-adherent splenocyte population was aspirated out gently, centrifuged at 3000 rpm for 5 min, resuspended in medium and counted in a haemocytometer.

Cytotoxicity and Cell Viability:
For the estimation of the cytotoxic activities of the plant extracts, a colorimetric assay was employed for quantification of cell proliferation, cell viability, and cytotoxicity using a kit (Cell Proliferation Reagent WST-1) from Roche Diagnostics GmbH. The cytotoxicity of the various concentrations of 60% ethanolic crude of the different plants were evaluated in both EAC and Splenocyte cells, where separate experiments were set up for each cell line. Briefly, 1 x 10⁴ viable cells from each cell line were seeded into a 96-well flat bottomed plate with requisite amount of RPMI-1640 medium. Thereafter, the cells were treated with various concentrations of
each plant extract (in triplicates) and were left undisturbed and allowed to grow for 16 h. Then, 10 μl/well Cell Proliferation Reagent WST-1 was added and again incubated for one and a half hour. It was shaken thoroughly for 1 min on a shaker. The absorbance of the samples was measured against a background control as blank using a microplate (ELISA) reader at 450 nm.

Anticancer activity based on quantification of apoptotic cell by flow cytometry:
For the determination of cell cycle phase distribution, around 1.5-2 × 10⁶ cells were cultured in several 24-well plates containing RPMI-1640 medium (with 10% HI FBS) and treated with various concentrations of each plant extract (in triplicates) and were left undisturbed and allowed to grow for 16 h in a CO₂ incubator (37°C; 5% CO₂). Post treatment, the cells were centrifuged to discard any medium, fixed with chilled methanol and diluted with sterilized PBS. After dilution, Triton X-100 and RNAse A were added to permeate the cell wall and digest the RNA during incubation for 3 hrs at 37°C. Nuclear DNA of EAC cells was labeled with ethidium bromide and phase distribution was determined on FACS, fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using CellQuest software (Becton Dickinson). A total of 10,000 events were acquired and analysis of flow cytometric data was performed using ModFit software. A histogram of DNA content (x-axis, red fluorescence) versus counts (y-axis) has been displayed.

Statistical analysis:
All data are reported as the mean ± SD of six measurements, except in DNA protection where three measurements are done. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). The IC₅₀ values were calculated using the formula Y = 100*A1/(X + A1) where A1 = IC₅₀, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The IC₅₀ values were compared by paired t-Test. The results with a value of p < 0.05 were considered significant.

Results:
Antioxidant and Phytochemical studies:
Antioxidant activity:
The reaction between ABTS and potassium persulfate results in the production of a blue colored chromophore, ABTS⁺. After addition of the plant extracts this pre-formed radical cation was converted to ABTS in a dose dependant manner. The results (Figure 1) are compared with trolox and the TEAC value demonstrates the extract as a potent antioxidant. The respective TEAC values of the plants are provided in Table 1.
Figure 1: Antioxidant capacities of (a) Terminalia chebula (b) Terminalia belerica (c) Emblica officinalis (d) Caesalpinia crista (e) Cajanus cajan (f) Tinospora cordifolia

Reducing power:
Most of the plants showed reducing power, which alternatively proved their antioxidant property.
Determination of total phenolic and flavonoid content:

It is also found that the plant extracts showed significant amount of flavonoid and phenolic contents. Flavonoids show their antioxidative action through scavenging or chelating process. Phenolic content is also very important plant constituent because of their scavenging ability due to their hydroxyl groups. Both of these compounds have good antioxidant potential and their effects on human nutrition and health are significant. The amount of total phenolics and flavonoids present in the plant extracts are given in Table 1.
Table 1: TEAC values, Phenolic and Flavonoid contents of plant extracts:

<table>
<thead>
<tr>
<th>Antioxidant values</th>
<th>60% ethanolic crudes of</th>
<th>Terminalia chebula</th>
<th>Terminalia belerica</th>
<th>Emblica officinalis</th>
<th>Caesalpinia crista</th>
<th>Cajanus cajan</th>
<th>Tinospora cordifolia</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEAC Values</td>
<td>1.499 ± 0.005</td>
<td>1.49 ± 0.004</td>
<td>4.6 ± 0.05</td>
<td>0.152 ± 0.002</td>
<td>0.611 ± 0.003</td>
<td>0.359 ± 0.0035</td>
<td></td>
</tr>
<tr>
<td>† Phenolic content</td>
<td>0.202 ± 0.008</td>
<td>0.18 ± 0.002</td>
<td>0.193 ± 0.004</td>
<td>0.0801 ± 0.001</td>
<td>0.083 ± 0.001</td>
<td>0.0822 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>‡ Flavonoid content</td>
<td>0.108 ± 0.006</td>
<td>0.087 ± 0.005</td>
<td>0.168 ± 0.003</td>
<td>0.0473 ± 0.001</td>
<td>0.081 ± 0.001</td>
<td>0.0841 ± 0.008</td>
<td></td>
</tr>
</tbody>
</table>

† Phenolic content (mg/ml Gallic acid equivalent per 100 mg plant extract)
‡ Flavonoid content (mg/ml Quercetin equivalent per 100 mg plant extract)

Reactive Oxygen Species (ROS) scavenging capacities:

*Hydroxyl radical:*

Hydroxyl radical is one of the reactive free radicals formed in biological systems, causing DNA strand breakage, which brings about carcinogenesis, mutagenesis and cytotoxicity. Ferric-EDTA premixture is incubated with ascorbic acid and H₂O₂ at pH 7.4. Thus, hydroxyl radicals are formed, which cause 2-deoxy-2-ribose damage and generate malondialdehyde (MDA) like product. This compound forms a pink chromogen upon heating with TBA at low pH. Addition of the extracts to the reaction mixture removes hydroxyl radicals and prevents further damage (Figure 3). The activities of the extracts of *Terminalia chebula* and *Emblica officinalis* were so less that the figures were not provided. The fact is also supported by their IC₅₀ values (Table 2).

Figure 3: Fenton inhibition activities of (a) *Terminalia belerica* (b) *Caesalpinia crista* (c) *Cajanus cajan* (d) *Tinospora cordifolia*
Superoxide radical:
Superoxide anion is also implicated as harmful reactive oxygen species (ROS). It has detrimental effect on the cellular components in a biological system. It indirectly initiates lipid oxidation by generating singlet oxygen. The 60% ethanolic extract of nearly all the plants (except *Tinospora cordifolia*) scavenged superoxide radical, one of the most detrimental ROS, quite successfully (Figure 4), as is also corroborated in their IC$_{50}$ values (Table 2).

Figure 4: Superoxide scavenging capacities of (a) *Terminalia chebula* (b) *Terminalia belerica* (c) *Emblica officinalis* (d) *Caesalpinia crista* (e) *Cajanus cajan* (f) *Tinospora cordifolia*
Nitric oxide radical:
The production of nitric oxide radical at a sustained level results in direct tissue toxicity and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis. The reaction of NO with superoxide radical generates highly reactive peroxynitrite anion (ONOO-) which is highly toxic for living cell. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extracts directly compete with oxygen to react with nitric oxide, thus inhibiting nitrite formation. Nearly all the plants showed (Figure 5) excellent nitric oxide scavenging results; some of them well above than the standard in activity. The IC$_{50}$ values of the respective plants also reflect the fact (Table 2).

Figure 5: Nitric oxide scavenging capacities of (a) *Terminalia chebula* (b) *Terminalia belerica* (c) *Emblica officinalis* (d) *Caesalpinia crista* (e) *Cajanus cajan* (f) *Tinospora cordifolia*
Hydrogen peroxide scavenging:

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membrane rapidly, once inside the cell, \( \text{H}_2\text{O}_2 \) can probably react with \( \text{Fe}^{2+} \) and possibly \( \text{Cu}^{2+} \) ions to form hydroxyl radical and this may be the origin of many of its toxic effects. Neither of the plants showed any reasonable activity in scavenging hydrogen peroxide against the standard. The figures of the plants other than Terminalia belerica and Caesalpinia crista (Figure 6) are not at all representable, as can also be found in their IC\(_{50}\) values (Table 2).

Figure 6: Hydrogen peroxide scavenging capacities of (a) Terminalia belerica (b) Caesalpinia crista
Peroxynitrite scavenging:

Peroxynitrite (ONOO\(^-\)) is relatively stable amongst all free radicals but once protonated forms highly reactive peroxynitrurous acid (ONOOH). The generation of excess ONOO\(^-\) leads to oxidative damage and tissue injury. Peroxynitrite bleaches Evans blue by oxidizing it. According to the present results, the extracts inhibit Evans blue bleaching through peroxynitrite scavenging and it is observed that the figures (Figure 7) and the \(\text{IC}_{50}\) values (Table 2) of the extracts of the plants showed that some of the plants have considerable scavenging ability of peroxynitrite anion, while others have minor.

Figure 7: Peroxynitrite anion scavenging capacities of (a) Terminalia chebula (b) Terminalia belerica (c) Emblica officinalis (d) Caesalpinia crista (e) Cajanus cajan (f) Tinospora cordifolia
Singlet oxygen scavenging:

Another ROS, singlet oxygen which is a high energy form of oxygen, is generated in the skin upon UV-irradiation. Singlet oxygen induces hyperoxidation, oxygen cytotoxicity and decreases the antioxidative activity. The plant extracts showed considerable activity in scavenging singlet oxygen species, as is evident from the figures (Figure 8) and from the IC$_{50}$ values of the plants (Table 2).

Figure 8: Singlet oxygen scavenging capacities of (a) *Terminalia chebula* (b) *Terminalia belerica* (c) *Emblica officinalis* (d) *Caesalpinia crista* (e) *Cajanus cajan* (f) *Tinospora cordifolia*
Hypochlorous acid scavenging:
At the sites of inflammation, the oxidation of Cl⁻ ions by the neutrophil enzyme myeloperoxidase results in the production of another harmful ROS, hypochlorous acid. HOCl has the ability to inactivate the antioxidant enzyme, catalase through break down of heme-prosthetic group. The inhibition of catalase inactivation in the presence of the extracts signifies its HOCl scavenging activity and from the results obtained, it is anticipated that a reasonable dose-dependent efficiency in scavenging the hypochlorous acid is observed for all the plant extracts, as is corroborated in the figures (Figure 9) and from the IC₅₀ values (Table 2).

Figure 9: Hypochlorous acid scavenging capacities of (a) Terminalia chebula (b) Terminalia belerica (c) Emblica officinalis (d) Caesalpinia crista (e) Cajanus cajan (f) Tinospora cordifolia
Iron chelation and DNA protective activities:

Fe$^{2+}$ chelating:
The two oxidation states of iron, Fe (II) and Fe (III) donate or accept electrons through redox reactions that are significant for biological reactions, but they also may be harmful to cells. In excess, iron helps superoxide anion (O$^{2-}$) and hydrogen peroxide to convert into the extremely reactive hydroxyl radical (OH) (Haber-Weiss reaction) that cause severe injury to membranes,
proteins and DNA. It decomposes lipid hydro-peroxides into peroxyl and alkoxyl radicals responsible for the chain reaction of lipid peroxidation. Nearly all the plants showed (Figure 10) reasonable Fe$^{2+}$-chelation activities, with some having quite superior efficacies, as is reflected in their IC$_{50}$ values (Table 2).

Figure 10: Fe$^{2+}$ chelating activities of (a) Terminalia chebula (b) Terminalia belerica (c) Emblica officinalis (d) Caesalpina crista (e) Cajanus cajan (f) Tinospora cordifolia and (g) EDTA
Lipid peroxidation inhibition:

Lipid peroxidation is initiated through iron catalysed generation of ferryl-perferryl complex or hydroxyl radicals that accelerates peroxidation by decomposing lipid hydro-peroxides into peroxy and alkoxyl radicals. The hydroxyl radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids that eventually yield carbonyl products like malondialdehyde (MDA), which generate a pink chromogen with TBA. The 60% ethanolic crude extracts of the plant materials showed good activities in inhibiting lipid peroxidation (Figure 11). The trend of IC_{50} values (Table 2) of the plant extracts also support this result.

Figure 11: Lipid peroxidation inhibition capacities of (a) *Terminalia chebula* (b) *Terminalia belerica* (c) *Embilica officinalis* (d) *Caesalpinia crista* (e) *Cajanus cajan* (f) *Tinospora cordifolia*
DNA protection:
At the cellular level, subjecting cells to oxidative stress can result in severe metabolic dysfunction, including DNA damage with a characteristic pattern of modification of all bases, production of base-free sites, deletions, strand breaks, DNA-protein cross-links, and chromosomal rearrangement. An important reaction involved in DNA damage involves generation of hydroxyl radical through Fenton chemistry. Hydroxyl radical is known to react with all components of the DNA molecule: the purine and primidine bases as well as the deoxyribose backbone. When DNA was exposed to Fenton reaction, \( \text{H}_2\text{O}_2 \) will be generated to hydroxyl radicals, and then the supercoiled (SC) form of DNA would cleave to give rise open-circular (OC) form. The ability of the plant extracts to protect the DNA supercoil can be expressed by the concentration of sample required for 50% protection, designated as the \([P]_{50}\) value. It is evident from the figures (Figure 12) and the respective \([P]_{50}\) values (Table 2) that the extracts have considerable efficacy in protecting DNA samples.

Figure 12: DNA protection capacities of (a) *Terminalia chebula* (b) *Terminalia belerica* (c) *Emblica officinalis* (d) *Caesalpinia crista* (e) *Cajanus cajan* (f) *Tinospora cordifolia*. 

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(c) ![Plant extract vs Trolox (standard)](image)

(d) ![Plant extract vs Trolox (standard)](image)

(e) ![Plant extract vs Trolox (standard)](image)

(f) ![Plant extract vs Trolox (standard)](image)
Subdivisions in each figure indicate (1) the agarose gel picture and (2) the % of protection graph against sample in μg.

(a)-1

(a)-2

(b)-1

(b)-2

(c)-1

(c)-2
Table 2: Comparison of the antioxidant capacities, free radical scavenging and other activities of 60% ethanolic crudes of *Terminalia chebula*, *Terminalia belerica*, *Emblica officinalis*, *Caesalpinia crista*, *Cajanus cajan* and *Tinospora cordifolia*:

<table>
<thead>
<tr>
<th>Name of Assay</th>
<th>60% ethanolic crudes of</th>
<th>Standard</th>
<th>Values of Standard compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Terminalia chebula</em></td>
<td><em>Terminalia belerica</em></td>
<td><em>Emblica officinalis</em></td>
</tr>
<tr>
<td>TEAC Values (Antioxidant activity)</td>
<td>1.499 ± 0.005</td>
<td>1.49 ± 0.004</td>
<td>4.6 ± 0.05</td>
</tr>
<tr>
<td>† Phenolic content</td>
<td>0.202 ± 0.008</td>
<td>0.18 ± 0.002</td>
<td>0.193 ± 0.004</td>
</tr>
<tr>
<td>‡ Flavonoid content</td>
<td>0.108 ± 0.006</td>
<td>0.087 ± 0.005</td>
<td>0.168 ± 0.003</td>
</tr>
<tr>
<td>§ [P]50 value for DNA protection</td>
<td>1.153 ± 0.178</td>
<td>1.593 ± 0.159</td>
<td>1.446 ± 0.07</td>
</tr>
</tbody>
</table>

* Hydroxyl radical (OH·)
  175480.9 ± 5888.2***
  4872.88 ± 1719.04**
  311139.8 ± 14430***
  700.25 ± 52.23**
  665.22 ± 43.66*
  1152.69 ± 116.56***
  Mannitol 571.45 ± 20.12

* Superoxide (O₂⁻)
  14.03 ± 0.261***
  20.23 ± 0.31***
  27.03 ± 4.02***
  139.4 ± 17.23***
  49.99 ± 4.02**
  293.21 ± 23.09***
  Quercetin 42.06 ± 1.35

* Nitric oxide (NO)
  38.72 ± 10.37***
  47.38 ± 6.62***
  66.55 ± 20.67*
  52.21 ± 7.28***
  57.89 ± 15.67*
  199.33 ± 27.47***
  Curcumin 90.82 ± 4.75

* Hydrogen peroxide (H₂O₂)
  20277.29 ± 3213.42***
  50.70 ± 3.55***
  19459.22 ± 3642.93***
  80.72 ± 8.58***
  20590.66 ± 3347.7***
  7336.45 ± 7372.5 NS
  Sodium pyruvate 3.24 ± 0.30

* Peroxynitrite (ONOO⁻)
  1056.60 ± 84.49***
  1108.81 ± 26.38***
  817.96 ± 15.82 NS
  4229.17 ± 523.79***
  3145.22 ± 203.89***
  5590.89 ± 580.20***
  Gallic acid 876.25±56.96

* Singlet oxygen (¹O₂)
  95.0 ± 10.38***
  139.43 ± 6.69***
  45.91 ± 9.06 NS
  425.24 ± 48.27***
  841.86 ± 636.98*
  132.7 ± 14.08***
  Lipoic acid 46.16±1.16

* Hypochlorous acid (HOCl)
  295.03 ± 28.79**
  235.5 ± 24.54***
  415.62 ± 26.11***
  410.48 ± 81.22*
  187.42 ± 19.15***
  241.69 ± 14.64***
  Ascorbic acid 235.96±5.75

* Iron chelation
  19.12 ± 1.24***
  40.57 ± 0.53***
  108.02 ± 0.83***
  634.6 ± 18.57***
  273.17 ± 6.38***
  414.66 ± 3.84***
  EDTA 1.27 ± 0.05

* Lipid peroxidation
  3.97 ± 0.079***
  3.04 ± 0.21***
  3.24 ± 0.068***
  217.63 ± 39.78***
  38.28 ± 1.34***
  43.9 ± 1.88***
  Trolox 6.76 ± 0.17

† Phenolic content (mg/ml Gallic acid equivalent per 100 mg plant extract)
‡ Flavonoid content (mg/ml Quercetin equivalent per 100 mg plant extract)
§ [P]50 signifies Concentration of sample in μg required for 50% protection of DNA supercoil
◆ IC₅₀ Values are in μg/ml, except in H₂O₂ scavenging where the unit is in mg/ml.
  * p< 0.05; ** p< 0.01; *** p< 0.001; NS = Non significant
Cytotoxicity and Anticancer activities:

Cytotoxicity and Cell Viability:

Biological activity of any plant is most certainly depends on its cytotoxic property. For that purpose, the cytotoxicity effect of the plant extracts were studied on both normal cells (splenocyte) and cancer cell (EAC). Results (Figure 13 and Table 3) showed that all the plants have no cytotoxic effect on normal cell. However, most of the plants showed significant cytotoxic activity on EAC cells in varying fashion with superior results for *Emblica officinalis*, *Caesalpinia crista*, *Cajanus cajan* and *Tinospora cordifolia*.

Figure 13: Cell Viability and cytotoxic capacities of (a) *Terminalia chebula* (b) *Terminalia belerica* (c) *Emblica officinalis* (d) *Caesalpinia crista* (e) *Cajanus cajan* (f) *Tinospora cordifolia*. Subdivisions in each figure indicate activities for (1) Splenocyte and (2) EAC cells. All the data of the tests for splenocyte was found to be statistically non-significant (paired t-test). All the data were taken with concentration of 0, 10, 30, 50, 80, 100, 150 and 200 µg/ml except for *Terminalia chebula* and *Terminalia belerica*, for their cytotoxicities in EAC cell, where the concentrations are 0, 10, 30, 50 and 80 µg/ml.

*Terminalia chebula*:

*Terminalia belerica*:

*Emblica officinalis*:
Caesalpinia crista:

Cajanus cajan:

Tinospora cordifilia:
Table 3: IC<sub>50</sub> values (in μg/ml) for the cytotoxic effect on EAC cell:

<table>
<thead>
<tr>
<th>Name of the Assay</th>
<th>60% ethanolic crudes of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Terminalia chebula</td>
</tr>
<tr>
<td>Cytotoxicity on EAC cell</td>
<td>344.06 ± 22.09</td>
</tr>
</tbody>
</table>

Anticancer activity based on quantification of apoptotic cell by flow cytometry:

Since anticancer activity of any compound should induce apoptosis of cancer cell, our \textit{in vitro} studies have shown that the plant extracts were effective in imparts growth inhibition, cell cycle deregulation and apoptosis in EAC cells. Results in Figure 14 indicate that the extracts of three plants, viz., \textit{Terminalia belerica}, \textit{Cajanus cajan} and \textit{Tinospora cordifolia} selectively induced apoptosis of cancerous EAC cell in a dose dependent manner.

Figure 14: Anticancer activity studied through Cell Cycle Analysis of the plant extracts of (a) \textit{Terminalia chebula} (b) \textit{Terminalia belerica} (c) \textit{Emblica officinalis} (d) \textit{Caesalpinia crista} (e) \textit{Cajanus cajan} (f) \textit{Tinospora cordifolia}. Subdivisions in each figure indicate the activities of the plants in (1) The Cell Cycle Analysis for each studied dose (no. on top of each figure represents the ascending order of doses) and (2) Dose dependent curve for percentage of analysis. All the data of the tests for splenocyte was found to be statistically non-significant (paired \textit{t}-test). All the data were taken with concentration of 0, 10, 30, 50, 80, 100, 150 and 200 μg/ml except for \textit{Terminalia chebula} and \textit{Terminalia belerica}, where the concentrations are 0, 10, 30, 50 and 80 μg/ml.
Terminalia chebula:

(a)-1

(a)-2

% of Apoptosis

Concentration of sample (µg/ml)
Terminalia belerica:

(b)-1

(b)-2

% of Apoptosis

Concentration of sample (µg/ml)
**Emblica officinalis:**

(c)-1

(c)-2

% of Apoptosis

Concentration of sample (µg/ml)
Caesalpinia crista:

(d)-1

(d)-2

% of Apoptosis

Concentration of sample (µg/ml)
Tinospora cordifolia:

(f)-1

(f)-2

Percentage of Apoptosis vs. Concentration of Sample (μg/ml)
References:


13. Detailed analysis of results indicating contributions made towards increasing the state of knowledge in the subject:

Plant materials were collected from Bankura district of West Bengal and authenticated from CRI, Kolkata. The fruits of Terminalia chebula, Terminalia bellerica and Emblica officinalis, leaves of Caesalpinia crista and Cajanus cajan and the stem of Tinospora cordifolia were finely powdered and the hydro-alcoholic crude extract (ethanol:water::6:4) of these plant materials were prepared. The hydro-alcoholic crude extract of each plants were tested for total antioxidant activity, reducing power, different free radical scavenging activity, iron chelating property, lipid peroxidation and DNA protection studies and determination of phenolic and flavonoid content. The plant materials’ IC\text{50} values of each activity have been summarized in the Table 2.

The TEAC values of the plants followed the order Emblica officinalis > Terminalia chebula > Terminalia bellerica > Cajanus cajan > Tinospora cordifolia > Caesalpinia crista. Most of the plants showed reducing power, which alternatively proved their antioxidant property. The phenolic content of the plant extracts were found to be in the order: Terminalia chebula > Emblica officinalis > Terminalia bellerica > Cajanus cajan > Tinospora cordifolia > Caesalpinia crista. The flavonoid content of the plant extracts were found to be in the order: Emblica officinalis > Terminalia chebula > Terminalia bellerica > Tinospora cordifolia > Cajanus cajan > Caesalpinia crista
In case of ROS scavenging activities of the plant extracts were found to be in varying proportions. Based on the IC\textsubscript{50} values of the plant extracts for hydroxyl radical scavenging, the activities followed the order: \textit{Cajanus cajan} > \textit{Caesalpinia crista} > \textit{Tinospora cordifolia} > \textit{Terminalia belerica} > \textit{Terminalia chebula} > \textit{Emblica officinalis}. Similarly, for superoxide scavenging we found the order for the plants being \textit{Terminalia chebula} > \textit{Terminalia belerica} > \textit{Emblica officinalis} > \textit{Cajanus cajan} > \textit{Caesalpinia crista} > \textit{Tinospora cordifolia}. As for nitric oxide scavenging, the order of activity was found to be \textit{Terminalia chebula} > \textit{Terminalia belerica} > \textit{Emblica officinalis} > \textit{Cajanus cajan} > \textit{Caesalpinia crista} > \textit{Tinospora cordifolia}. The results for hydrogen peroxide was not found to be satisfactory, but the activity of the plants based on their IC\textsubscript{50} value followed the order \textit{Terminalia belerica} > \textit{Caesalpinia crista} > \textit{Tinospora cordifolia} > \textit{Emblica officinalis} > \textit{Terminalia chebula} > \textit{Cajanus cajan}. In case of peroxynitrite radical, \textit{Emblica officinalis} > \textit{Terminalia chebula} > \textit{Terminalia belerica} > \textit{Cajanus cajan} > \textit{Caesalpinia crista} > \textit{Tinospora cordifolia} was the order that was found for the activities of the plants in scavenging, whereas, \textit{Emblica officinalis} > \textit{Terminalia chebula} > \textit{Tinospora cordifolia} > \textit{Terminalia belerica} > \textit{Caesalpinia crista} > \textit{Cajanus cajan} was the order for the same plant extracts in scavenging singlet oxygen radical. Hypochlorous acid scavenging activity for the plant extracts followed the trend \textit{Cajanus cajan} > \textit{Terminalia belerica} > \textit{Tinospora cordifolia} > \textit{Emblica officinalis} > \textit{Terminalia chebula} > \textit{Caesalpinia crista}.

The iron chelating property of the plants is in accordance with the order of their IC\textsubscript{50} values following the trend \textit{Terminalia chebula} > \textit{Terminalia belerica} > \textit{Emblica officinalis} > \textit{Cajanus cajan} > \textit{Tinospora cordifolia} > \textit{Caesalpinia crista}. The inhibitory activity of lipid peroxidation of the plant extracts followed the order \textit{Terminalia belerica} > \textit{Emblica officinalis} > \textit{Terminalia chebula} > \textit{Cajanus cajan} > \textit{Tinospora cordifolia} > \textit{Caesalpinia crista}. All the plants showed reasonable DNA protective ability in the order \textit{Terminalia chebula} > \textit{Emblica officinalis} > \textit{Terminalia belerica} > \textit{Caesalpinia crista} > \textit{Cajanus cajan} > \textit{Tinospora cordifolia}.

It was observed from our study that none of the plants have any cytotoxic effect on normal cell. However, most of the plants showed significant cytotoxic activity on EAC cells in varying fashion with the trend: \textit{Cajanus cajan} > \textit{Caesalpinia crista} > \textit{Tinospora cordifolia} > \textit{Terminalia chebula} > \textit{Emblica officinalis} > \textit{Terminalia belerica}. The dose dependent anticancer effects by selective killing of Ehrlich’s Ascites Carcinoma (EAC) cell line in an apoptotic pathway by the extracts of \textit{Cajanus cajan}, \textit{Tinospora cordifolia} and \textit{Terminalia belerica} were found to be quite impressive to be represented. This result was found to be in consistency with that found in the cytotoxicity studies. However, the extracts of \textit{Caesalpinia crista}, \textit{Terminalia chebula} and \textit{Emblica officinalis} did not show promising anticancer activities by induction of
apoptosis, since their cytotoxicities may be due to other mechanisms of cell death, viz., necrosis, which is yet to be studied.

So, it may be drawn from our detailed study that ROS generation is an important step for the formation of cancer, and although three plant extracts out of six did not show anticancer effect by inducing apoptotic pathway, it may be for sure that all these plants are useful in prevention point of view as can be derived from their antioxidant, ROS scavenging, iron chelation and DNA protective results.

Herbal medicines have been widely used for treatment of diseases in India for thousands of years. Ayurveda is one of the oldest systems of health care dealing with both the preventive and curative aspects of health. Herbal medicines usually are prepared in traditional formulations that were used for different diseases such as healing of ulcers, anti-inflammatory, anti-allergic, anti-virus and most importantly anti-cancer. However, the pharmacological applications of herbal medicines are hampered by their quality control, authenticity and our limited understanding of their biological effects. The value of our research lies in the fact that the assays done with the plant materials will be useful to establish favourable scientific evidences behind the principles of Ayurvedic medicine.

14. Conclusions summarizing the achievements and indication of scope for future work: From our results, it is observed that most of the plant materials shown quite reasonable activities in their hydroalcoholic crude extract. The plant extracts showed their efficacies in antioxidative studies through ROS scavenging and lipid peroxidation, and in iron chelating studies along with DNA protection. The studies of the cytotoxic effect and the anticancer efficiencies of the crude extracts in the cancer cell (EAC) and normal splenocytes in vitro systems have also been found that not only the plants were found to be non toxic for normal cell (splenocyte), but also most of them showed quite positive anticancer effect. It can be said that these plants do possess medicinal importance and can be used in the purpose of pharmacognosy studies to be accepted as Ayurvedic medicines. These observations have provided the stimuli to continue with further in vivo studies with these extracts and their different formulations. The future effort regarding the in vivo studies will further corroborate the above mentioned piece of information.
### 15. Procurement/usage of Equipment:

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of Equipment</th>
<th>Make/Model</th>
<th>Cost FE/Rs</th>
<th>Date of Installation</th>
<th>Utilisation Rate %</th>
<th>Remarks regarding maintainence/breakdown</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Freeze Dryer (Lyophilizer)</td>
<td>Scanlaf Bench Top Microprocessor Controlled Multipurpose Lyophiliser Model No. Cool Safe 110-4</td>
<td>CIF Kolkata EUR 7050/ Rs. 4,97,000</td>
<td>22/04/2009</td>
<td>60</td>
<td>Nothing so far</td>
</tr>
</tbody>
</table>

### 16. Manuscript for Publication (300 words for possible publication in Council’s Bulletin):

The study evaluated the *in vitro* antioxidant, iron chelator, DNA protective and anticancer activities of hydroalcoholic (60% ethanolic) extracts of various plant parts of *Terminalia chebula*, *Terminalia belerica*, *Emblica officinalis*, *Caesalpinia crista*, *Cajanus cajan* and Guduchi (*Tinospora cordifolia*), which were well known for their use in *Ayurveda* where herbal medicines are usually prepared in traditional formulations that were used for different inflammatory, cardiovascular, degenerative diseases and cancer. The extracts were used to determine the antioxidant potential using different tests including total antioxidant activity; efficiencies for scavenging of hydroxyl, superoxide, nitric oxide, singlet oxygen radicals and hypochlorous acid. Iron chelating, inhibition of lipid peroxidation and DNA protective properties were measured along with total phenolic and flavonoid content determination. The cytotoxic effects and the anticancer activities of various doses of the plant extracts were studied *in vitro* in cultured mouse splenocyte and Ehrlich ascites carcinoma (EAC) cell line.

Briefly, results indicate that the TEAC values of the plants followed the order *Emblica officinalis* > *Terminalia chebula* > *Terminalia belerica* > *Cajanus cajan* > *Tinospora cordifolia* > *Caesalpinia crista*. The phenolic and flavonoid contents of the plant extracts were found to be in the orders: *Terminalia chebula* > *Emblica officinalis* > *Terminalia belerica* > *Cajanus cajan* > *Tinospora cordifolia* > *Caesalpinia crista* and *Emblica officinalis* > *Terminalia chebula* > *Tinospora cordifolia* > *Cajanus cajan* > *Caesalpinia crista*, respectively. Based on the IC₅₀ values of the plant extracts for hydroxyl radical scavenging, the activities followed the order: *Cajanus cajan* > *Caesalpinia crista* > *Tinospora cordifolia* > *Terminalia belerica* > *Terminalia chebula* > *Emblica officinalis*. Similarly, for superoxide scavenging we found the order for the plants being *Terminalia chebula* > *Terminalia belerica* > *Emblica officinalis* > *Cajanus cajan* > *Caesalpinia crista* > *Tinospora cordifolia*. As for nitric oxide scavenging, the order of activity was found to be *Terminalia chebula* > *Terminalia belerica* > *Caesalpinia crista* > *Cajanus cajan* > *Emblica officinalis* > *Tinospora cordifolia*. The results for hydrogen peroxide was not found to be satisfactory, but the activity of the plants based on their IC₅₀ value followed the order *Terminalia belerica* > *Caesalpinia crista* > *Tinospora cordifolia* >
In case of peroxynitrite radical, *Emblica officinalis* > *Terminalia chebula* > *Cajanus cajan* was the order that was found for the activities of the plants in scavenging, whereas, *Emblica officinalis* > *Terminalia chebula* > *Tinospora cordifolia* > *Terminalia belerica* > *Caesalpinia crista* > *Cajanus cajan* was the order for the same plant extracts in scavenging singlet oxygen radical. Hypochlorous acid scavenging activity for the plant extracts followed the trend *Cajanus cajan* > *Terminalia belerica* > *Tinospora cordifolia* > *Terminalia chebula* > *Caesalpinia crista* > *Emblica officinalis*.

The iron chelating property of the plants is in accordance with the order of their IC\textsubscript{50} values following the trend *Terminalia chebula* > *Terminalia belerica* > *Emblica officinalis* > *Cajanus cajan* > *Tinospora cordifolia* > *Caesalpinia crista*. The inhibitory activity of lipid peroxidation of the plant extracts followed the order *Terminalia belerica* > *Emblica officinalis* > *Terminalia chebula* > *Cajanus cajan* > *Tinospora cordifolia* > *Caesalpinia crista*. All the plants showed reasonable DNA protective ability in the order *Terminalia chebula* > *Emblica officinalis* > *Terminalia belerica* > *Caesalpinia crista* > *Cajanus cajan* > *Tinospora cordifolia*.

It was found to be very interesting to find that all the plant extracts did not show any cytotoxic effect on the normal mouse splenocyte cell, whereas most of them showed cytotoxic effect on cancer cell (EAC). The cytotoxic activities of the plants showed the trend: *Cajanus cajan* > *Caesalpinia crista* > *Tinospora cordifolia* > *Terminalia chebula* > *Emblica officinalis* > *Terminalia belerica*. The induction of apoptosis in EAC cell in a dose dependent manner suggested that three plants viz., *Cajanus cajan*, *Tinospora cordifolia* and *Terminalia belerica* have anticancer activity which is in consistency with that found in the cytotoxicity studies.

The above results showed the importance of the application of the studied six plants in the treatment of cancer. Our study has played a pivotal role to establish scientific facts behind the principle and authenticity of Ayurvedic medicines to treat cancer, both from the curative and preventive aspects.