IN VITRO FREE RADICAL SCAVENGING AND ANTIOXIDANT POTENTIAL OF ETHANOLIC EXTRACT OF EUPHORBIA NERIIFOLIA LINN

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ABSTRACT

As far as our literature survey could ascertain, no information was available on the in vitro antioxidant activities of the Euphorbia neriifolia Linn (Family: Euphorbiaceae). Therefore, the aim of this current investigation was to evaluate the in vitro antioxidant capacities of the ethanolic extract of Euphorbia neriifolia leaves. The antioxidant activity of EN extract was evaluated by various antioxidant assays such as TAC, FRAP, FTC, TBA and Non specific activity. All these antioxidant activities were compared with standard antioxidants. Phytochemical screening and the total phenolics, flavonols and proanthocyanidin content were also determined. A positive correlation between the antioxidant activities and phytochemical assays was observed and the highest scavenging activity of extract was noticed at concentration of 1mg/ml. Results obtained in the present investigation indicate clearly that the extract of EN possesses antioxidant properties and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

Keywords: Euphorbia neriifolia, Antioxidant, ROS, FTC, TBA

INTRODUCTION

A part of the oxygen taken into living cells is changed to several harmful reactive oxygen species (ROS). ROS, are highly reactive molecules which include free radicals such as superoxide ions (O2·−), hydroxyl radicals (OH·), nitric oxide radical (NO·), singlet molecular oxygen peroxynitrite radicals and hydrogen peroxide (H2O2)·. Superoxide anion radical (O2·−) is one of the strongest reactive oxygen species among free radicals that are generated first after oxygen is taken into living cells2. All these radicals exert oxidative stress towards the cells of human body and this leads to a number of physiological disorders such as atherosclerosis, arthritis, ischemia, reperfusion injury of many tissues, central nervous system injury, gastric, cancer and AIDS4. In treatment of these diseases, antioxidant therapy has gained an immense importance. There are some synthetic antioxidant compounds, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propylgallate (PG) and tertiary butylhydroquinone (TBHQ) are suspected to have some toxic effects such as carcinogenicity5. Therefore, research for the determination, development and utilization of more effective antioxidants of natural origin is desired.

The medicinal plants (Rasayana) are the plants whose parts (leaves, seeds, stems, roots, fruits, foliage etc.) extracts, infusion, decoctions, powders have been extensively used in the Indian traditional (Ayurveda) system of medicine for the treatment of different diseases of humans. Medicinal properties of plants have also been investigated in the light of recent scientific developments through out the world, due to their potent pharmacological activities, low toxicity and economic viability, when compared with synthetic drugs6.

Euphorbia neriifolia Linn. (Euphorbiaceae) commonly known as “Sehund or thochar” in Hindi, is found throughout the Deccan Peninsula of India and grows luxuriously around the dry, hilly, rocky areas of North, Central and South India. Ayurveda describes the plant as bitter, pungent, lacative, carminative, improves appetite useful in abdominal troubles, bronchitis, tumors, loss of consciousness, delirium, leucoderma, piles, inflammation, enlargement of spleen, anaemia, ulcers and fever7. As far as our literature survey could ascertain, no information was available on the in vitro antioxidant activities of the E. neriifolia. Therefore, the aim of this current investigation was to evaluate the in vitro antioxidant capacities of the ethanolic extract of Euphorbia neriifolia.

The antioxidant activities of Euphorbia neriifolia (EN) were measured in a concentration range of 0.1-1mg/ml (100 – 1000 µg/ml), using different antioxidant assays. Furthermore, the total phenolics, flavonols and proanthocyanidins contents were also measured and their correlation with the antioxidant activities was ascertained.

MATERIAL AND METHODS

Chemical reagents

DPPH (1, 1-diphenyl-2-picryl hydrazyl), TPTZ (2,4,6-tripryidyl-s-triazine), Ferrozine, Deoxyribose were purchased from Sigma Chemical Co. Ltd USA. Trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), L-ascorbic acid, ammonium molybdate, quercetin were purchased from HI Media, Mumbai. DMSO (Dimethyl sulfoxide) was purchased from Merck Co. (Germany), Mumbai. All other unlabelled chemicals and reagents were of analytical grade and used without further purification.

Collection of plant material

Euphorbia neriifolia leaves were collected from Pharmacological garden of Banasthali University, Banasthali, India, in the month of September 2009. The plant was identified with the help of available literature and authenticated by Botanist of Krishi Vigyan Kendra, Banasthali Vidyanipath, Banasthali, Tonk district.

Preparation of the extract

Freshly collected Euphorbia neriifolia leaves were dried in shade and coarse powder was extracted by macerating 500 g in 1.5 L of ethanol (70% v/v) for one week with occasional stirring. The macerated mixture was filtered through muslin cloth and evaporated at 40°C up to one third of initial volume. Remaining solvent was completely evaporated at 40°C, using a hot air oven (Mvtx, India) and kept in dissector for two days. The yield (20% w/w) of the powdered plant material was collected dried and stored at 5°C in airtight container.

Qualitative phytochemical screening

The ethanolic extract was qualitatively tested for the presence of various phyto constituents using the following reagents and chemicals according to the methods described by Parekh, & Chanda: Test for alkaloids performed with Dragendorffs reagent, flavonoids with the use of ammonia and concentrated H2SO4, tannins with ferric chloride and potassium dichromate solutions, phenolics with FeCl3, Saponins (frothing test), Steroids (Liebermann-Burchard test), terpenoids with Fehling’s solution, Cardiac glycosides (Keller-Kiliani test).
Quantitative physico-chemical assays

Determination of total Phenolic content

The total phenolic content of plant extract was determined using Folin-Ciocalteu reagent\(^{14}\). To 1 ml of Folin-Ciocalteu’s reagent, previously diluted (1:20) was added to 1 ml of samples (250 μg/ml) and mixed thoroughly. To the mixture, 4 ml of sodium carbonate (75 g/L) and 10 ml of distilled water were added and mixed well. The mixture was allowed to stand for 2 h at room temperature. Contents were then centrifuged at 2000 g for 5 min and the absorbance of the supernatant was taken at 760 nm. All determinations were carried out in triplicates. Total content of phenolic compounds in plant extract in gallic acid equivalents (GAE) was calculated by the following formula:

\[
C = \frac{c \times V}{m'}
\]

Where: C- Total content of phenolic compounds, mg/g plant extract (GAE),

\(c\)-The concentration of gallic acid established from the calibration curve (mg/ml), \(V\)- The volume of extract (ml),

\(m\)- The weight of pure plant ethanolic extract (g).

Determination of total flavonoids

Total flavonoids in the plant extract was estimated using the method of Kumaran, & Karunakaran\(^{11}\). To 2.0 ml of sample (standard), 2.0 ml of 2% AlCl\(_3\) ethanol and 3.0 ml (50 g/L) sodium acetate solutions were added. The absorption at 440 nm was read after 2.5 h at 20°C. Extract samples were evaluated at a final concentration of 1 mg/ml. All determinations were carried out in triplicates. Total flavonoids content was expressed as rutin equivalents (mg/g) using the following equation based on calibration curve: \(y = ax + b\), where \(x\) was the absorbance and \(y\) was the rutin equivalent (mg/g).

Evaluation of antioxidant assay

Total antioxidant capacity

The antioxidant capacity of the extract was evaluated according to the procedure reported by Sun et al.\(^{12}\). A volume of 0.5 ml of 0.1 mg/ml of extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm. Extract samples were evaluated at a final concentration of 1 mg/ml. All determinations were carried out in triplicates. Total proanthocyanidin content was expressed as rutin equivalents (mg/g).

Ferric thiocyanate (FTC) method

The antioxidant potential of EN leaves was determined according to the FTC method\(^{13}\) with slight modification. Four milligrams of each extract samples were dissolved in 4.0 ml ethanol (99.5 %) and kept in dark bottle (d = 40.0 mm, t = 75.0 mm). Each mixture was mixed with 4.1 ml linoleic acid (2.5 % in ethanol 99.5 %), 8.0 ml phosphate buffer (0.02 M, pH 7.0) and 3.9 ml distilled water to make up the volume to 20.0 ml. BHT was used as a positive control while the other bottle without sample was used as a negative control. The mixture was incubated at 40 – 45°C. After incubation, 9.7 ml ethanol (75 %) and 0.1 NH\(_4\)SCN (30%, as a colour reagent) were added to 0.1 ml of the mixture. Precisely 3 min after the addition of 0.1 ml of FeCl\(_2\) (0.002 M) in HCl 3.5 % to the reaction mixture, the absorbance of the resulting red colour was measured at 500 nm using spectrophotometer (570455, Electronic corporation of India limited).

Thiobarbituric acid (TBA) test

The TBA test was conducted according to the combined method\(^{17,18}\). A milliliter of sample from the previous FTC method was added with 2 ml of trichloroacetic acid and 2 ml of thiobarbituric acid solution. This mixture was then placed in a boiling water bath at 100°C for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min and absorbance of the supernatant was then measured at 532 nm using UV-Vis spectrophotometer (570455, Electronic corporation of India limited).

Non specific assay

Different concentrations of extract were mixed with 1 ml of reaction buffer (100 μM FeCl\(_3\), 10 μM EDTA, 1.5 mM H\(_2\)O\(_2\), 2.5 mM DMSO, 100 μM L-ascorbic acid, pH 7.4) and incubated for 1 h at 37°C. One ml of 0.5 % 2-thiobarbituric acid in 0.025 M sodium hydroxide and 1 ml of 2.8 % trichloroacetic acid was added to the mixture and heated for 30 min at 80°C. Finally the mixture was cooled and absorbance was measured at 532 nm using spectrophotometer.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. The data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by Bonferroni's multiple comparison test using the SPSS 16.0 (Statistical program for Social Sciences) program. Results with p<0.05 were regarded as statistically significant and considered p<0.001 as very significant. Pearson correlation analysis was performed between antioxidant activity and total phenolic content.

RESULTS AND DISCUSSION

Qualitative phytochemical screening

Preliminary phytochemical screening of the ethanolic extract of *Euphorbia nerifolia* leaves revealed the presence of various bioactive components of which alkaloid, saponin, tannin and cardiac glycosides were the most prominent and the result of phytochemical test has been summarized in Table 1. All these phytochemicals possess good antioxidant activities and has been reported to exhibit multiple biological effects including anti-inflammatory, antitumor activities. *Euphorbia nerifolia* is tested negative for phlobatannins. The presence of phytochemicals like flavonoids, saponins and tannins in the extract of EN act as primary antioxidants or free radical scavengers.
Table 1: Qualitative phytochemical screening of ethanolic extract of *Euphorbia neriifolia*.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th><em>Euphorbia neriifolia</em></th>
</tr>
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<tbody>
<tr>
<td>Alkaloid</td>
<td>++</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
</tr>
<tr>
<td>Tannin</td>
<td>++</td>
</tr>
<tr>
<td>Saponin</td>
<td>+++</td>
</tr>
<tr>
<td>Steroid</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic</td>
<td>-</td>
</tr>
<tr>
<td>Phlobatannins</td>
<td>+</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>++</td>
</tr>
</tbody>
</table>

(-)– absent, (+)– weak, (++) – moderate, (+++) strong

The amount of phenolic, flavonols and proanthocyanidin content

The yield of extract and the total antioxidant capacity of total phenolic, flavonols and proanthocyanidins content (mg/g of dry material) are shown in Table 2. The amount of the ethanolic extract obtained from the extraction was 20g (20% w/w yield).

Plant phenolics are the widest spread secondary metabolite in plant kingdom. These compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. Therefore, it is worthwhile to determine the total amount of phenolic content in the plant chosen for the study.

Table 2: Extraction yield and total amount of plant phenols, flavonols and proanthocyanidins of ethanolic extract of *Euphorbia neriifolia* leaves extract.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Yield %</th>
<th>Total phenols mg/g plant extract (GAE)</th>
<th>Total flavonols mg/g plant extract (RE)</th>
<th>Total Proanthocyanidins mg/g plant extract (RE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Euphorbia neriifolia</em></td>
<td>20</td>
<td>0.60±0.09*</td>
<td>1.79±0.10*</td>
<td>3.96±0.06*</td>
</tr>
</tbody>
</table>

Results are mean±SD (n=3). *P- Value <0.001 Vs standard group, Bonferri test.

The content of phenolic compounds (mg/g) in ethanolic extract (figure 1) determined from regression equation of calibration curve ($y = 0.124x + 0.214$, $R^2 = 0.26$) and expressed in gallic acid equivalent (GAE).

The concentration of flavonols, expressed in rutin equivalent, using the regression equation of calibration curve ($y = 0.560x + 0.100$, $R^2 = 0.385$) in mg/g of EN extract (figure 2).

Proanthocyanidins are a type of bioflavonoid that has been shown to have very potent antioxidant activity. Total proanthocyanidin content was expressed as rutin equivalents (mg/g) using regression equation of calibration curve ($y = 2.699x + 0.030$, $R^2 = 0.987$). Our present investigation depicts high content of proanthocyanidin in the plant extract as compared to other phenolic compounds (figure 3).

Fig. 1: The total phenolic content of ethanolic extract of *Euphorbia neriifolia*

Fig. 2: The total flavonols content of ethanolic extract of *Euphorbia neriifolia*

Fig. 3: The total proanthocyanidins content of ethanolic extract of *Euphorbia neriifolia*
Results obtained in the present study reveals that the level of all these compounds in extract were significantly higher (p < 0.001) as compared to the reference standard used for this study. Polyphenol are the major plant compounds and are commonly found in both edible and inedible plants and they have been reported to have multiple biological effects, including antioxidant activity. Their antioxidant activity is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers, and consumers. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g was daily ingested from a diet rich in fruits and vegetables.

Total antioxidant capacity (TAC)

The TAC of the plant extract is shown in Figure 4. TAC mainly concentrates on the thermodynamic conversion and measures the number of electrons or radicals donated or quenched by a given antioxidant molecule and measure the capacity of biological samples under defined conditions. The phosphor-molybdenum method was based on the reduction of M0 (VI) to M0 (V) by the antioxidant compound and the formation of green phosphate/M0 (V) complex at acidic pH with a maximal absorption at 695 nm. In this assay extract was found to have higher activity, as compared to the standard (gallic acid) used for this study. This study reveals that the antioxidant activity of the extract exhibited increasing trend with the increasing concentration of the plant extract. Thus, the extract demonstrated electron donating capacity, may act as radical chain terminators, transforming reactive free radical species into stable non reactive products. In this assay extract was found to have higher activity, as compared to the standard (gallic acid) used for this study.

![Fig. 4: Effect of ethanolic extract of Euphorbia neriifolia leaves on total antioxidant capacity (TAC)](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>TBA (absorbance at 532 nm)</th>
<th>FRAP (g mol Fe(II)/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.128</td>
<td>-</td>
</tr>
<tr>
<td>EN</td>
<td>0.066</td>
<td>149.2 ± 0.576*</td>
</tr>
<tr>
<td>BHT</td>
<td>0.082</td>
<td>333.1 ± 0.067*</td>
</tr>
<tr>
<td>EDTA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quercetin</td>
<td>-</td>
<td>4.38 ± 0.063*</td>
</tr>
</tbody>
</table>

Results are mean ± SD (n=3). *P-Value <0.001 Vs standard group, Bonferroni test.

The results obtained were not significantly different from BHT. The absorbance recorded of control sample obviously showed the highest reading (1.128). This could be due to greater amount of peroxidation at the initial stage than that in secondary stage. Secondary product such as malonaldehyde is not stable for a long period of time. It would be turned into alcohol and acid, which cannot be detected by a spectrophotometer.
Non-Specific assay

It has been observed that the extract showed excellent antioxidant activities. Concentration dependent inhibition of hydroxyl radical induced deoxyribose degradation was observed in non site-specific assay. Pro oxidant effect was not observed in case of extract. The Figure 6 shows dose dependent increase in antioxidant potential in the extract when compared to standard (rutin). The extract showed highly significant value (p<0.001) as compared to standard.

CONCLUSION

It can be concluded that the Euphorbia neriifolia possesses the significant antioxidant activity compared to other well characterized, standard antioxidant systems in vitro and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants, which might be due to the presence of alkaloids, tannins, flavonoids, proanthocyanidin and sapogenin. These finding suggest that this plant is a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of ageing and age associated oxidative stress related degenerative diseases such as cancer and various other human ailments. Further studies are warranted for the isolation and characterization of antioxidant components and also in vivo studies are needed for understanding their mechanism of action as an antioxidant better.

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REFERENCES