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Research Article

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

**PRACHETA, VEENA SHARMA*, RITU PALIWAL, SADHANA SHARMA**

**Department of Bioscience and Biotechnology, Banasthali University, Banasthali-304022, Rajasthan, India**
**Email: drvshs@gmail.com**

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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 physiochemical 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 ($O_2^-$), hydroxyl radicals ($OH^-$), nitric oxide radical (NO), singlet molecular oxygen peroxynitrite radicals and hydrogen peroxide ($H_2O_2$)[1, 2]. Superoxide anion radical ($O_2^-$) is one of the strongest reactive oxygen species among free radicals that are generated first after oxygen is taken into living cells[2]. All these radicals exert oxidative stress towards the cells of human body and this leads to a number of physiological disorders[3] such as atherosclerosis, arthritis, ischemia, reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS[4]. 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 carcinogenicity[5]. 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 drugs[6].

*Euphorbia neriifolia* Linn (Euphorbiaceae) commonly known as "Sehund or thohar" 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, laxative, carminative, improves appetite useful in abdominal troubles, bronchitis, tumors, loss of consciousness, delirium, leucoderma, piles, inflammation, enlargement of spleen, anaemia, ulcers and fever[7, 8]. 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-1,2-picryl hydrazyl), TPTZ (2,4,6,-tripyridyl-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 Vidyapith, 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 (Mvtex, 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 air tight 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[9]: Test for alkaloids performed with Dragendorffs reagent, flavonoids with the use of ammonia and concentrated $H_2SO_4$, tannins with ferric chloride and potassium dichromate solutions, phenolics with $FeCl_3$, Saponins (frothing test), Steroids (Liebermann-Burchard test), terpenoids with Fehling's solution, Cardiac glycosides (Keller-Kinliani test).

**Quantitative physico-chemical assays**

**Determination of total Phenolic content**

The total phenolic content of plant extract was determined using Folin-Ciocalteu reagent[10]. To 1 ml of Folin-Ciocalteu's reagent, previously diluted (1:20) was added to 1 ml of samples (250µg/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. A standard curve was obtained using various concentrations of gallic acid. Samples of extract were evaluated at a final concentration of 1mg/ml. Total content of phenolic compounds in plant extract in gallic acid equivalents (GAE) was calculated by the following formula:

$C = c.\ V/m'$

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 flavonols**

Total flavonols 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 1mg/ml. All determinations were carried out in triplicates. Total flavonols 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).

**Determination of total proanthocyanidins**

Proanthocyanidin content was determined 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 1mg/ml. All determinations were carried out in triplicates. Total proanthocyanidin content was expressed as rutin equivalents (mg/g).

**Evaluation of antioxidant assay**

**Total antioxidant capacity**

The antioxidant capacity of the extract was evaluated according to the procedure of Shirwaikar *et al.*[13]. About 0.1ml of extract was combined in eppendorf tube with 1ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in thermal block at 95°C for 90 minutes. After cooling to room temperature; the absorbance of the aqueous solution of each was measured at 695 nm against blank.

**Total antioxidant activity (FRAP assay)**

A modified method[14] was adopted for the FRAP assay. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2{\cdot}3H_2O$ and 16 ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3{\cdot}6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3{\cdot}6H_2O$. The temperature of the solution was raised to 37°C prior to use. Plant extract (150 µL) was allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µM $FeSO_4$. Results were expressed in µM Fe (II)/g dry mass and compared with that of BHT and quercetin.

**Ferric thiocyanate (FTC) method**

The antioxidant potential of EN leaves was determined according to the FTC method[15] 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 another 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 $NH_4SCN$ (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 ) every 24 h until a day after the absorbance of the control reached maximum value (day seven). The percentage inhibition of lipid peroxidation was calculated as follows:

% Inhibition = 100 – [($A_1/A_o$) X 100]

Where $A_o$ is the absorbance of the control reaction and $A_1$ is the absorbance in the presence of the sample extract[16].

**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 $H_2O_2$, 2.5 mM Deoxyribose and 100 µM L- ascorbic acid, pH 7.4) and incubated for 1 h at 37º 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 80º 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 neriifolia* 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 neriifolia* 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.

**Table1: Qualitative phytochemical screening of ethanolic extract of *Euphorbia neriifolia*.**

| Phytochemicals | *Euphorbia neriifolia* |
|---|---|
| Alkaloid | ++ |
| Terpenoids | + |
| Tannin | ++ |
| Saponin | +++ |
| Steroid | + |
| Flavonoid | ++ |
| Phenolic | + |
| Phlobatannins | - |
| Cardiac glycosides | ++ |

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

**The amount of phenolic, flavanols and proanthocyanidin content**

The yield of extract and the total antioxidant capacity of total phenolic, flavonols and proanthocyanidins content (mg/g of dry meterial) 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.**

| Plant | Yield % | Total phenols mg/g plant extract (GAE) | Total flavonols mg/g plant extract (RE) | Total Proanthocyanidins mg/g plant extract (RE) |
|---|---|---|---|---|
| *Euphorbia neriifolia* | 20 | 0.60±0.09* | 1.79±0.10* | 3.96±0.06* |

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

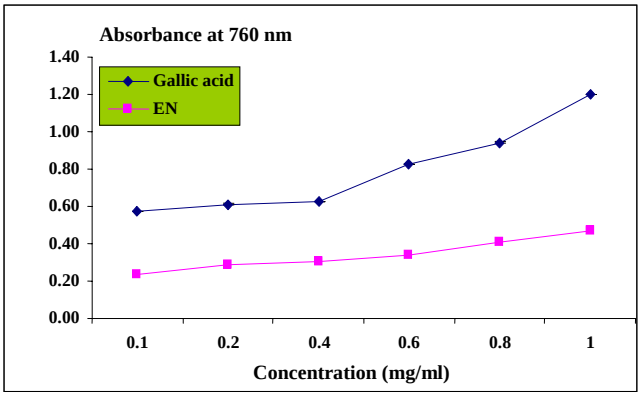


**Fig. 1: The total phenolic content of ethanolic extract of *Euphorbia neriifolia***

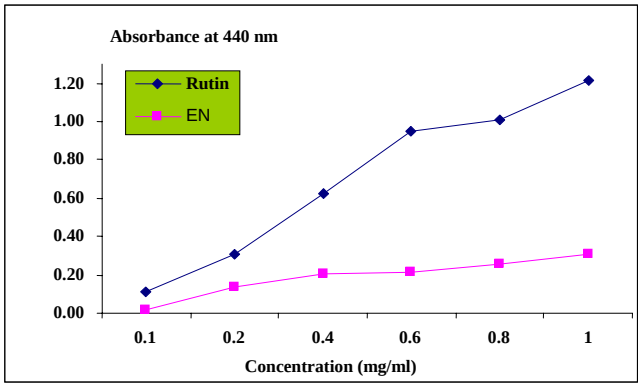


**Fig. 2: The total flavonols content of ethanolic extract of *Euphorbia neriifolia***

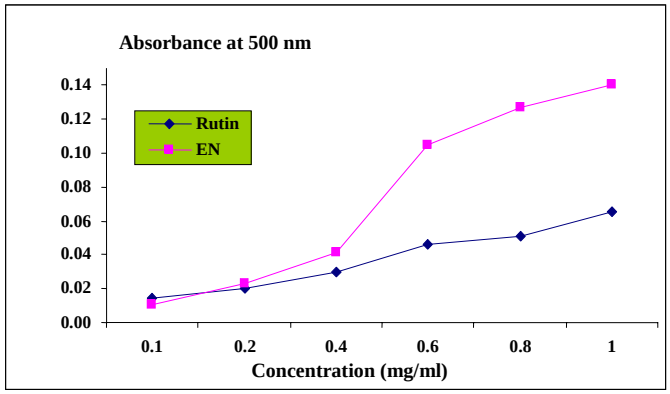


**Fig. 3: The total proanthocyanidins content of ethanolic extract of *Euphorbia neriifolia***

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[11, 19-21]. 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[22].

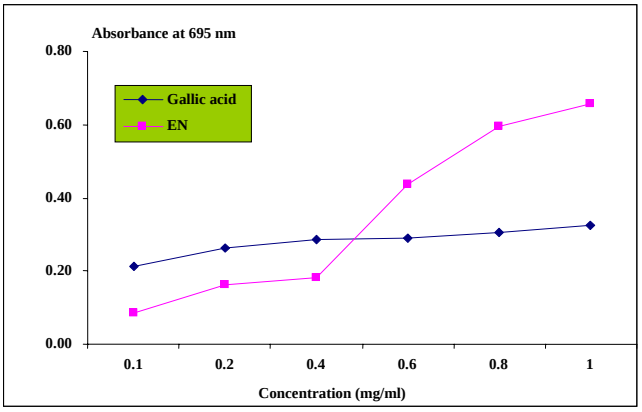


**Fig. 4: Effect of ethanolic extract of *Euphorbia neriifolia* leaves on total antioxidant capacity (TAC)**

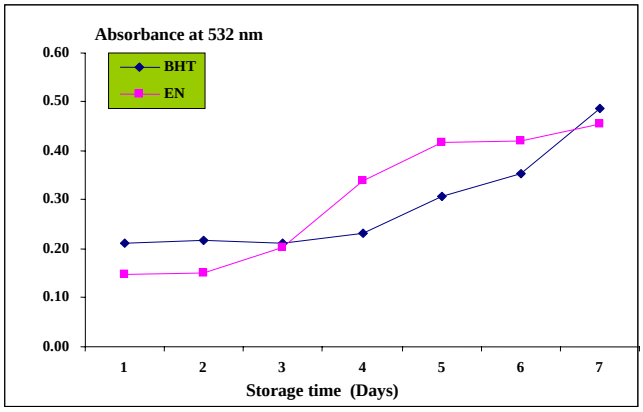


**Fig. 5: Total Antioxidant properties of ethanolic extract of *Euphorbia neriifolia* leaves by FTC method**

**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 MO (VI) to MO (V) by the antioxidant compound and the formation of green phosphate/ MO (V) complex at acidic pH with a maximal absorption at 695 nm[23]. 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, transformating reactive free radical species into stable non reactive products[24, 25]. In this assay extract was found to have higher activity, as compared to the standard (gallic acid) used for this study.

**Total antioxidant activity (FRAP)**

The ability of plant extract to reduce ferric ions was determined in FRAP assay. The change in absorbance at 593 nm owing to the formation of blue colored $Fe^{+2}$- tripyridyltriiazine (TPTZ) compound from the colourless oxidized $Fe^{+3}$ form by the action of electron donating antioxidants[26]. The FRAP values of extract was found to be significantly higher as compared to the standards i.e. quercetin and BHT (1mg/ml) is given in Table 3. Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidant present, thus it can be reported that extract of EN may act as free radical scavenger, capable of transforming reactive free radical species into stable non radical products.

The antioxidant potentials of the extract of the leaves of EN were estimated from their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II) at 593 nm and its antioxidant activity increased proportionally with the polyphenol content.

**Ferric thiocyanate (FTC)**

The FTC method measures the amount of peroxide value produced during the initial stage of lipid peroxidation, where ferric ion was formed upon reaction of peroxide with ferrous chloride. The ferric ion will then unite with ammonium thiocyanate producing ferric thiocyanate, a red-colored substance. The darker the color, the higher will be the absorbance. Result shows that the sample had been oxidized when stored for seven days at 40-45°C. Initially, the absorbance of EN was the lowest (0.148). After seven days storage, extract exhibited good effect in inhibiting linoleic acid oxidation compared to control (BHT). The percentage of inhibition of linoleic acid of EN was 84.96% respectively, with no significant difference compared to BHT[27, 28]. Figure 5 also depicts that antioxidant activities also increased with increasing concentration of the EN extract. The correlation between total flavonols content and antioxidant capacity in linoleic acid emulsion in this study was found positive ($r = 0.953$). These phenolic compounds may donate hydrogen and can terminate the free radical reaction chain by changing it to the stabile compounds[29].

**Thiobarbituric acid (TBA) test**

FTC is used to measure the production of peroxide compound at the initial stage of oxidation while TBA test is used to measure the secondary product of oxidation such as aldehyde and ketone[30]. The TBA analysis of extract of EN after seven days storage is shown in Table 3.

**Table3: Effect of ethanolic extract of the leaves of *Euphorbia neriifolia* on TBA and total antioxidant (FRAP) assay**

| Sample tested | TBA (absorbance at 532 nm) at 7 days storage | FRAP (µmolFe(II)/g |
|---|---|---|
| Control | 1.128 | – |
| EN | 0.066 | 149.2±0.05* |
| BHT | 0.082 | 333.1±0.01* |
| EDTA | – | – |
| Quercetin | – | 43.8±0.06* |

Results are mean ± SD (n=3). *P- Value <0.001 Vs standard group, Bonferrni 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[31].

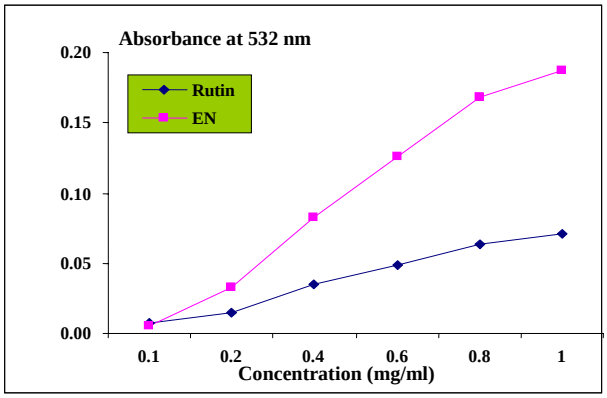


**Fig. 6: Effect of ethanolic extract of the leaves of *Euphorbia neriifolia* on Non-specific assay**

**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, proanthocynidin 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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