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# Antioxidant Activity and Estimation of Quercetin from Methanolic Extract of *Euphorbia nerifolia* Leaves by RP-HPLC

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The present study was designed to investigate the antioxidant activity of methanolic extract of *Euphorbia nerifolia* leaves and its column fractions followed by identification and quantification of the quercetin by using RP-HPLC technique in the effective fraction. The phenolic and flavonoid contents in the crude methanolic extract and its fractions are in the order of fraction 2 (F2) > methanolic extract (ME) > fraction 3 (F3) > fraction 1 (F1). The fraction 2 of methanolic extract of *Euphorbia nerifolia* leaves showed moderate free radical scavenging activity as evidenced by $IC_{50}$ values in DPPH (65.4 µg/mL) and ABTS (64 µg/mL) scavenging assays. The chromatogram of fraction 2 was found to be almost the same peak eluted at 3 min same as that of the standard quercetin. In the present study, a rapid RP-HPLC method was suitable for the standardization of herbal extracts for the estimation of quercetin and the results of antioxidant study may have implication in the use of quercetin as a therapeutic agent in the prevention of oxidative stress related diseases.

**Keywords: *Euphorbia nerifolia*, Flavonoids, Radical scavenging assay, Oxidative stress, RP-HPLC.**

## INTRODUCTION

The free radicals are mostly generated by exogenous and endogenous factors in the human body. The common reactive oxygen species (ROS) includes superoxide anion ($O^{2-}$), hydrogen peroxide ($H_2O_2$), peroxyl radicals ($ROO^{\bullet}$) and nitric oxide (NO)[1]. The body immune system requires free radicals but it has been shown to be harmful as they react with important cellular components such as proteins, DNA and cell membrane linked to certain chronic diseases of liver, heart, some form of cancers, aging, neuro-degenerative diseases, atherosclerosis and rheumatoid arthritis[2,3]. Antioxidant compounds could be either synthetic (BHA and BHT *etc.*) or plant secondary metabolites such as polyphenols and flavonoids. Natural antioxidants have gained importance in recent years due to their ability to delay the oxidation process, inhibiting the polymerization chain initiated by free radicals and other subsequent oxidizing reactions[4]. Therefore, in recent decade, there has been an upsurge of interest in finding naturally occurring antioxidants in foods or medicinal plants to replace synthetic antioxidants, which are being restricted due to their side effects such as inflammation and carcinogenicity *etc*. The evidences from the reported research articles suggesting the antioxidant activity of plants might be due to the presence of phenolic compounds, flavonoids and proanthrocyanidines[5].

The plant *Euphorbia nerifolia* (*E. nerifolia* ) belonging to family Euphorbiaceae is a shrub or tree, which is used in Indian System of Medicine for the treatment of several type of diseases such as gastropathy, bronchitis, asthma, splenomegaly, jaundice, leprosy, rheumatism and ulcer[6,7]. The milky juice is acrid, purgative, rubifacient and useful in otalgia. This plant is known to possess immense pharmacological wound healing, aphrodisiac, anticancer[8] activities. The plant yielded in the isolation of several classes of secondary metabolites, many of which expressed biological activities such as triterpenes (nerrifolione), flavonoids and steroidal saponins[9,10].

Several triterpenoids like 24-methylenecycloartenol, euphorbol hexacosonate, glut-5-en-3-ol, glut-5(10)-en-1-one, Glut-5-en-3β-yet-acetate, taraxerol, friede-lan-3α-ol and -3β-ol and amyrin were isolated from the powdered plant, stem and leaves of *E. nerifolia*[11]. This plant is rich in many pharmaceutical active ingredients like sugars, several terpenes, flavonoids, alkaloids, anthocyanins, triterpenoidal saponin like glut-5-en-3-ol, glut-5(10)-en-1-one, teraxerol, euphol and amyrin[12].

Many previous methods for the estimation of phytoconstituents include polarographic, iodometric, spectrophotometric and colorimetric methods are found as the non-specific methods. Nevertheless, UV spectrophotometry is still used by the manufacturers as it is rapid and low cost. However,

there are circumstances in which a more specific determination of phytoconstituents is of value and thus the chromatographic method, principally reversed phase high performance liquid chromatography (RP-HPLC) was applied. The literature survey revealed that so far no scientific studies carried out on antioxidant activity and quantification of flavonoids in methanolic extract of *E. nerifolia* leaves by RP-HPLC. Hence, in the present study, we have focused to evaluate *in vitro* antioxidant activity and quantification of flavonoids of *E. nerifolia* leaves. In addition, the total phenolic and flavonoid contents were determined using gallic acid and rutin as reference standards.

## EXPERIMENTAL

Butylated hydroxytoluene (BHT), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), gallic acid, rutin, quercetin were purchased from HI-Media. Sodium nitrite, sodium hydroxide, ferrous chloride, trichloroacetic acid (TCA), aluminum trichloride, Folin-Ciocalteu reagent were procured from Merck. Acetonitrile, methanol and water of HPLC-grade were obtained from Merck (Mumbai, India). All chemicals used including the solvents were of analytical grade.

The leaves of *E. neriifolia* were collected during month of December 2011 from Balasore district, Odisha, India. The plant material was taxonomically identified and authenticated by Dr. Arun K. Mohanty. A voucher specimen (no. SPS-05) has been preserved in the herbarium of the Department of Pharmacognosy, School of Pharmaceutical Sciences, Siksha O Anusandhan University, India. The leaves were picked and washed with water to remove dust particles and shade dried (25 ± 2 °C for 14 days).

**Preparation of plant extract and fractions:** The dried leaves (500 g) were grounded to a coarse powder in a mechanical blender and macerated with methanol (2L) at room temperature for 3 days. The methanolic extract was concentrated under vacuum to get a dark brown residue (130 g). A part of the residue (25 g) was dissolved in methanol and then silica gel (40 g) was added to make the extract to get adsorbed in the silica gel. The solvent was then evaporated slowly in water bath to get the dried sample and the prepared sample was charged over the packed column with silica gel (60-120 mesh) eluted with *n*-hexane (100 %) followed by *n*-hexane-methanol mixture to yield fraction F1, F2 and F3 at the ratio of 95:5, 90:10 and 80:20 respectively.

### Evaluation of antioxidant activities

**Determination of total phenolic content:** The total phenolic compounds (TPC) of the methanolic extract and fractions were determined by the Folin-Ciocalteu method using gallic acid as reference standard[13]. To prepare each of the sample solutions, in a glass cuvette, 3.16 mL of distilled water, 40 µL of a sample or standard solution and 200 µL of Folin-Ciocalteu reagent was added and the solutions were mixed thoroughly. To this, 600 µL of sodium carbonate (7 %) solution was mixed after incubation of 8 min. The glass cuvettes containing the samples were incubated at 40 °C for 0.5 h and absorbance of the mixture was observed against a blank at 765 nm. Total phenolic content were expressed as mg of gallic acid equivalent (GAE)/g of each sample.

**Determination of total flavonoid content:** Total flavonoid content (TFC) of the methanolic extract and fraction was estimated using reported method with little modification[14]. In a glass vial, 3.4 mL of 30 % aqueous methanol was taken and 300 µL of plant sample or standard was added and mixed followed by the addition of 150 µL of $NaNO_2$ (0.5 M). After an interval of 5 min, 150 µL of $AlCl_3$ (0.3 M) solution and after another 5 min, 1 mL of NaOH (1 M) solution was added and thoroughly mixed. The absorbance of the mixture was then measured at 506 nm. Total flavonoid content was calculated as mg/g rutin equivalent.

**ABTS radical scavenging assay:** The effect of methanolic extract and fraction on ABTS radical was estimated using reported method[15]. The stock solution containing equal volume of 7 mM ABTS salt and 2.4 mM potassium persulfate was allowed to stand in dark for 16 h at room temperature. The resultant ABTS solution was diluted methanol until absorbance of about 0.70 ± 0.01 at 734 nm was reached. Varying concentrations of the plant extracts (1 mL) was reacted with 1 mL of the ABTS solution and the absorbance was taken at 734 nm between 3-7 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of standard BHT and the percentage of inhibition was calculated:

$$\text{ABTS radical scavenging activity (\%)} = [(Abs_{control} - Abs_{sample})/(Abs_{control})] \times 100$$

where $Abs_{control}$ is the absorbance of ABTS radical + methanol; $Abs_{sample}$ is the absorbance of ABTS radical + sample extract/standard.

**DPPH radical scavenging activity:** The free radical scavenging activity of methanolic extract and fraction was estimated using the method of Liyana-Pathirana and Shahidi[16]. A solution of DPPH (0.135 mM) in methanol was prepared and 1 mL of this solution was mixed with 1 mL of varying concentration of the plant extracts. The reaction mixture was vortex thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm using BHT as reference standard. The ability to scavenge DPPH radical was calculated:

$$\text{(\%) of DPPH scavenging activity} = [(Abs_{control} - Abs_{sample})/(Abs_{control})] \times 100$$

where $Abs_{control}$ is the absorbance of DPPH radical + methanol; $Abs_{sample}$ is the absorbance of DPPH radical + sample extract/standard.

**Determination of ferric reducing power:** The ferric reducing potential of the extract and fractions were assayed as described previously reported method[17]. The ferric reducing potential of extracts and the standard, BHT (0.02-0.10 mg/mL, 1 mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1 % w/v). The mixture was incubated at 50 ºC for 20 min. 2.5 mL of trichloroacetic acid (10 % w/v) was mixed with 2.5 mL distilled water and 0.5 mL 0.1 % w/v $FeCl_3$. The absorbance was measured at 700 nm in a spectrophotometer. The ferric reducing potential of the extract and fractions were depicted in the Fig. 3.

**TLC study:** The co-TLC was performed for the column fraction F2 of the methanolic extract and the standard, quercetin. 10 µL of sample solution was spotted on the TLC plate along with standard, quercetin solution. Stationary phase

consisted of TLC aluminum sheets pre-coated with silica gel GF 254, thickness 0.2 mm, (20 × 20 cm) (E Merck, Germany). The solvent system, *n*-butanol-ethyl acetate-acetic acid (6:3:1) was selected and one yellow coloured spot under UV light (254 nm) was observed, which corresponded to the RF value of standard quercetin. On the basis of TLC study, an HPLC method was developed for the quantification of quercetin in the fraction F2 of the methanolic extract of *E. nerifolia*.

**Determination of quercetin by RP-HPLC:** Chromatographic methods were applied for the quality control of medicinal plants due to their many advantages such as high efficiency, speed and the possibility of their utilization in automatized systems. Quantitative analysis of flavonoid quercetin in the column fraction F2 was performed using HPLC system. HPLC analysis was performed using Jasco MD-2010 plus, JAPAN equipment consisting of a PU 2089 PLUS pump with PDA detector. Screening of flavonoids quercetin in the sample was carried out by methodology using a 4.6 × 1500 mm (XTERRA) $C_{18}$ column. Separations were done in the isocratic mode, using acetonitrile: methanol: water in a ratio of 70: 20: 10, at a flow rate of 1 mL $min^{-1}$; with an injection volume of 10 µL. The UV detection for identification and quantification was done at 256 nm. The mobile phase was filtered through 0.45 µm nylon filter prior to use.

**Preparation of standard solution:** About 1 mg of standard quercetin sample, was accurately weighed and taken into 10 mL volumetric flasks and dissolved in 10 mL mobile phase to prepare a concentration of 100 µg/mL. The solution was sonicated for 10 min and then filtered with 0.45 µm membrane filter paper prior to injection.

**Preparation of sample solution:** About 10 mg of the fraction F2 of the methanolic extract of *Euphorbia nerifolia* of the leaves sample was accurately weighed and taken into 10 mL volumetric flask and dissolved in 10 mL mobile phase to prepare a concentration of 1000 µg/mL. Further from this stock solution, working solution of 50 and 100 µg/mL solutions were prepared. The solutions were sonicated for 10 min and then filtered with 0.45 µm membrane filter paper prior to injection.

**Preparation of calibration curve:** To prepare a calibration curve, a secondary stock solution (10 µg/mL) was prepared from the stock solution 100 µg/mL, from which 0.1, 0.25, 0.4, 0.5, 0.65, 0.75, 0.8, 0.9, 1 and 1.25 mL were pipetted out, adjusted volume up to 10 mL with methanol for analyzing a series of standard, quercetin at the concentration range from 0.1 to 1.25 µg/mL. Peak area ratios (y) of the standard quercetin was measured and plotted against the concentration (x).

**Method validation:** The developed analytical method was validated as per ICH guidelines for precision, accuracy and robustness[18]. Precision of the method was ensured by analyzing six replicates of quercetin at three different concentrations (0.5, 0.65 and 0.7 µg/mL). The precision values were determined to confirm reproducibility of method expressed in terms of % RSD. Robustness of the method was analyzed by evaluating effect of variation in mobile phase composition and flow rate on the peak areas and retention time of quercetin at three different concentrations each (0.5, 0.65 and 0.7 µg/mL). Sensitivity of the method was assessed by calculating limit of detection (LOD) and limit of quantitation (LOQ).

**Statistical analysis:** The experimental results were expressed as mean ± SD of three replicates.

## RESULTS AND DISCUSSION

**Total phenolic and flavonoid contents:** Total phenolic contents of the methanolic extract and fractions of the leaves of *E. nerifolia* was determined in terms of mg of gallic acid equivalent per gram of the dried mass of the extract (mg GAE/g). The TPC of the ME, F1, F2 and F3 of *E. nerifolia* were 9.22 ± 0.25, 7.56 ± 0.293, 18.64 ± 0.58 and 8.57 ± 0.474 respectively. As the results showed, there is a notable difference in TPC in various extracts obtained from sequential extraction process. The content of total phenolics is expressed as mg gallic acid/g of extract (GAE/g) (The standard curve equation: $y = 0.0158 + 0.0133$, $R^2 = 0.9943$). The plant has much lower TPC content in the ME, F1 and F3, however appreciable quantity was remain present in the column fraction F2 of the methanolic extract.

Total phenolic contents of the different extracts of leaves of *E. nerifolia* was determined in term of mg of rutin equivalents per g of the dried mass of the extract (mg RE/g). The TFC of the ME, F1, F2 and F3 of *E. nerifolia* were found to be 28.49, 13.08, 77.52 and 21.76, respectively. (The standard curve equation of standard rutin: $y = 0.227x-0.0286$, $R^2 = 0.9931$). Significantly, highest results are found in the fraction F2 as compared to ME, F1 and F3. The content of phenolics and flavonoids in the crude extract and fractions are in the order of F2 > ME > F3 > F1.

Medicinal plants and their bioactive phenolic contents play important role in scavenging of free radicals. Phenolic compounds found in the extracts are considered to be the major contributors to the antioxidant activity of medicinal plants[19]. Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen and various free radicals implicated in several diseases[20]. So comparing with these findings from the literature of plant products, our results suggested that flavonoids found in methanolic extract and fractions of *E. nerifolia* may be the major contributors for the antioxidant activity. Since *E. nerifolia* is rich in polyphenolic compounds, these compounds may effectively act as antioxidant agents.

**Free radical scavenging by ABTS assay:** The results obtained in ABTS assay are shown in Fig. 1. The scavenging activity values on ABTS of extract and the fractions of *E. nerifolia* decrease than that of BHT in the order of BHT (96 %) > F2 (85.63 %) > ME (53.45 %) > F3 (47.34 %) > F1 (38 %) at the concentration of 250 µg/mL. Among all the extract and fractions, the F2 fraction exhibited highest scavenging effects against ABTS with an $IC_{50}$ value of 64 µg/mL, whereas $IC_{50}$ value of standard BHT is 48.5 µg/mL.

The reaction of $ABTS^+$ with free radical scavengers present in the test sample occurs rapidly and is assessed by following the decrease in sample absorbance at 734 nm. A concentration dependant activity was observed in this experiment. Higher concentrations of the extract were more effective in quenching free radical system.

ABTS radical scavenging assay involves a method that generates a blue/green $ABTS^+$ chromophore *via* the reaction

of ABTS and potassium persulfate. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate. The hydrogen-donating antioxidants property is measured spectrophotometrically at 734 nm. All the fractions of methanol extract showed strong ABTS scavenging activity and these observations are supported by previous researchers[21].

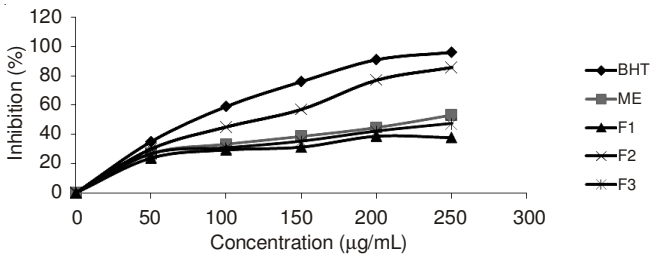


Fig. 1. ABTS radical scavenging activity of methanol extract/fractions of *E. nerifolia* and standard drug, BHT. Values represent mean ± SD of three replicates

**Free radical scavenging by DPPH assay:** The effects of methanolic extract and fractions in the DPPH assay method are depicted in Fig. 2. The fraction F2 showed significant DPPH radical inhibiting activity at a concentration of 250 µg/mL. The $IC_{50}$ values of ME, F1, F2, F3 and standard BHT were 93.22, 149.3, 65.4, 125.78 and 50.5 µg/mL, respectively. At 250 µg/mL, the percentage inhibition of the ME, F1, F2 and F3 fractions of *E. nerifolia* were 56.4, 39, 78.34 and 45.3 % whereas, at the same concentration the standard BHT showed 92.5 % inhibition. The DPPH activity of extract and fractions of *E. nerifolia* was found to be increasing on dose dependent manner.

The DPPH scavenging assay is used to assess the cell membrane stabilizing capacities of plant constituents, which may be the possible way by which phytomedicines could help to reduce oxidative stress induced diseases[22]. DPPH is a nitrogen-centered radical with a characteristic absorption at 517 nm and converted into 1,1-diphenyl-2-picryl hydrazine, due to its hydrogen donating ability at a rapid rate[23]. The low $IC_{50}$ value of F2 fraction of methanol extract of the plant is due to the presence of high polyphenolic and flavonoid compounds.

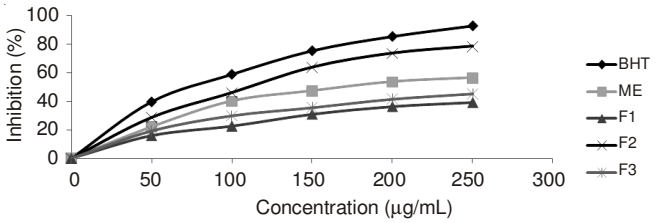


Fig. 2. DPPH radical scavenging activity of methanol extract/fractions of *E. nerifolia* and standard drug, BHT. Values represent mean ± SD of three replicates

**Reducing power assay by iron (III):** The absorbance of extract and fractions of the plant in reducing power assay are shown in Fig. 3. The reducing power of the extract, fractions and BHT were increased with increasing concentration in the following order, BHT > F2 > ME > F3 > F1.

The presence of antioxidant substances in the fraction F2 of the extract has the ability to reduce free radical chain reactors and causes reduction $Fe^{3+}$ to $Fe^{2+}$ form[24].

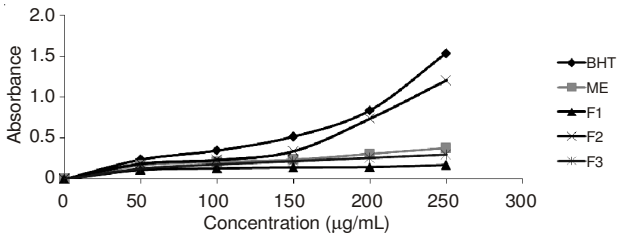


Fig. 3. Total ferric reductive potential of methanol extract/fractions of *E. nerifolia* and standard drug, BHT. Values represent mean ± SD of three replicates

**TLC study:** The solvent system, *n*-butanol-ethyl acetate-acetic acid (6:3:1) was selected and one yellow colored spot under UV light (254 nm) was observed, which corresponded to the $R_f$ value of standard quercetin. On the basis of TLC study, an HPLC method was developed for the quantification of quercetin in the fraction F2 of the methanolic extract of *E. nerifolia*.

**Quantification of quercetin by RP-HPLC analysis:** The qualitative detection of the quercetin has been studied and the acceptable results were obtained at 1 mL $min^{-1}$ flow rate at 256 nm. Typical HPLC chromatograms for standard quercetin and fraction F2 of the methanolic extract at the concentration of 50 and 100 µg/mL were shown in Fig. 4. The chromatogram of F2 was found to be almost the same peak eluted at 3 min same as that of the standard quercetin indicating the suitability of the procedure. Hence the developed method proposed for the extraction of quercetin and its HPLC estimation can be used routinely in the quality control laboratories.

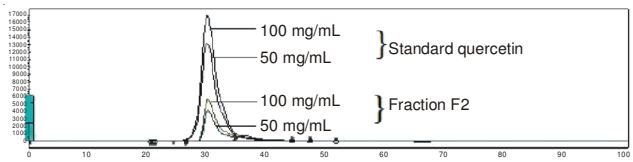


Fig. 4. Overlain chromatograms of standard quercetin and fraction F2 of the methanolic extract at 256 nm

**Calibration curve:** The calibration equation and correlation coefficients were calculated by least square linear regression analysis of peak area and concentrations and the line equation was found to be Y = 4567x-200 with a regression of 0.995. The calibration curve was linear over the range of 0.1 to 1.25 µg/mL.

## Validation

**Precision:** The developed method was found to be precise since the relative standard deviation (% RSD) values for intra and interday precision was within the limit for selected concentrations (n = 6). The results are shown in Table-1.

**Accuracy:** The accuracy of the method was evaluated by investigating the relative standard deviation (% RSD) of quercetin at three different concentration levels of 0.5, 0.65 and 0.7 µg/mL (n = 6) and was found to be within the limit for quercetin (Table-1).

**Robustness of the method:** Results from the change in the chromatographic conditions were observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed is robust.

TABLE-1
STATISTICAL RESULTS OF ACCURACY AND PRECISION OF THE METHOD (n = 3)

| Conc. (µg/mL) | Statistical values | | | | | |
|---|---|---|---|---|---|---|
| | Inter-day precision | | Intra-day precision | | Accuracy | |
| | % Mean ± SD | % RSD | % Mean ± SD | % RSD | % Mean ± SD | % RSD |
| 0.50 | 103.41 ± 1.550 | 1.50 | 101.55 ± 2.91 | 2.86 | 101.979 ± 1.839 | 1.79 |
| 0.65 | 100.14 ± 2.060 | 2.08 | 100.70 ± 0.34 | 2.03 | 104.870 ± 2.090 | 2.01 |
| 0.70 | 101.08 ± 2.014 | 2.01 | 101.86 ± 2.06 | 2.03 | 99.590 ± 1.090 | 1.09 |

**Selectivity and sensitivity:** No interference was observed at the retention time when chromatograms of blank and spiked quercetin samples (n = 6) were monitored. The LOD and LOQ were calculated by using slope and the residual standard deviation of a regression line. The LOD and LOQ values were found to be 0.13 and 0.47 µg/mL, respectively.

Several changes in mobile phase composition were tried and finally, acetonitrile: methanol: water in a ratio of 70: 20: 10 improves baseline resolution and reduces the peak tailing (tailing factor peak < 1.2) along with elution time (10 min). The acceptable results were obtained at 1 mL $min^{-1}$ flow rate and with a satisfactory faster elution. The correlation coefficient was indicative of high significance. The high percentage recovery indicates that the proposed method is highly accurate. The % RSD was found to be within the limit in the developed method, indicating high degree of precision. The changes in flow rate and composition of mobile phase did not affect the percentage assay of the drug, confirming the robustness of the method[25]. No such appreciable interfering peaks were found in the chromatogram indicating that the purity of the fraction from the extract of *E. nerifolia* leaves. Proposed RP-HPLC method indicates that the method is specific and can be used for estimation of quercetin in quantitative analysis.

## Conclusion

The finding of this study suggests that *E. nerifolia* leaves could be a potential source of natural antioxidant that could have great importance in preventing oxidative stress related diseases. In the present study a simple, accurate, precise and rapid RP-HPLC method was developed and validated for simultaneous analysis of quercetin. The method was found to be suitable for the standardization of herbal extracts for the estimation of quercetin. The simplicity of the procedure, combined with excellent sensitivity, resolution and short analysis time, makes this method a useful tool for identification of quercetin from herbal medicines.

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