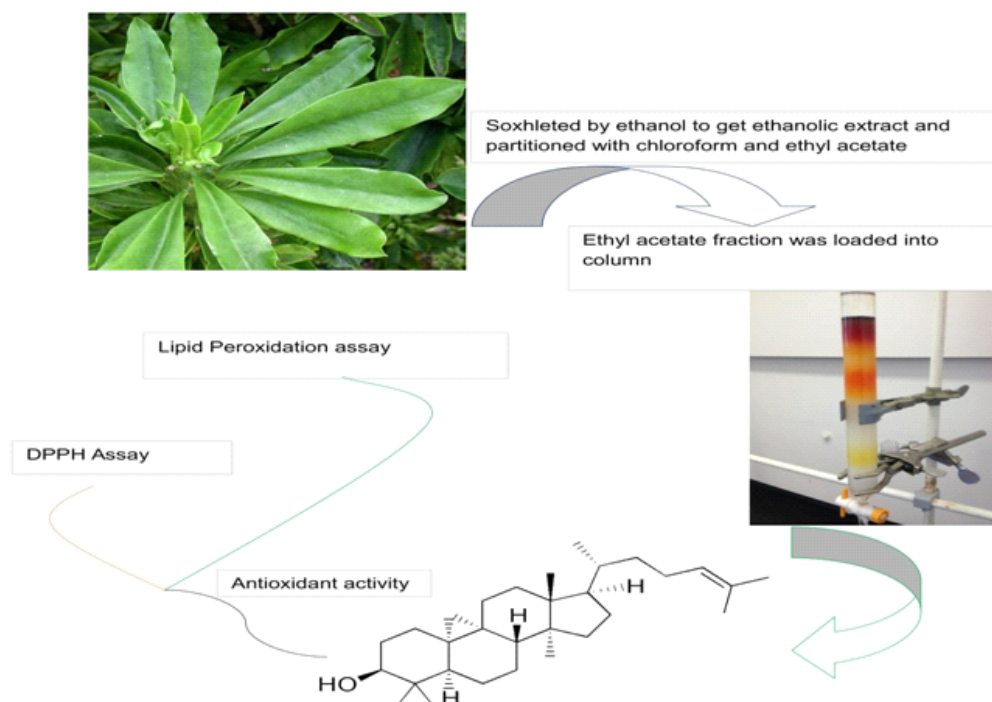


Antioxidant properties of Cycloartenol isolated from *Euphorbia Neriifolia* Leaves

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Graphical Abstract



Abstract

Background: *Euphorbia nerifolia* leaves belonging to family euphorbiaceae being used traditionally for the treatment of asthma, bronchitis, tumours, leucoderma, piles, inflammations and ulcers. Earlier studies reported various chemical constituents such as euphorbon, resin, euphorbol and euphol.

Hypothesis of work: On the basis of recent studies, it is concluded that reactive oxygen species are involved in several diseases. Hence the present study investigated to isolate the compounds from leaves of *E. nerifolia* and perform its antioxidant activity.

Methods: Column chromatography and paper chromatography was used to isolate the pure constituent from the extract and antioxidant activity was performed using free radical scavenging and inhibition of lipid peroxidation assay.

Results: Extractive value of ethanolic extract obtained was 36.52%w/w, which upon partitioned with ethyl acetate to get ethyl acetate fraction was 50.00%w/w. Column chromatography of ethyl acetate fraction (26-34) yielded cycloartenol upon repeated preparative TLC.

The phytochemical screening of ethanolic extract of *E. nerifolia* revealed the presence of sterols, glycosides, phenolic compounds, flavonoids, saponins, carbohydrates and proteins and amino acids. The ethyl acetate fraction showed positive test for presence of glycosides, steroids, triterpenoids and flavonoids.

The antioxidant activity of isolated cycloartenol was determined by measuring its 1, 1 diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity and ability to inhibit lipid peroxidation. Cycloartenol exhibited strong antioxidant activity. Concentrations of 10, 20, 40, 60, 80, and 100 µg /mL showed 34.56, 39.78, 44.68, 61.98, 66.46 and 72.87% inhibition of lipid peroxidation respectively. In addition cycloartenol had effective DPPH free radical scavenging activity at 40 µg/mL.

Conclusion: It is concluded from present study that cycloartenol isolated from *E. nerifolia* leaves exhibited significant antioxidant activity and is a source of natural antioxidants that may be used in prevention of various diseases.

Key words: Antioxidant, DPPH, cycloartenol, lipid peroxidation

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Introduction

Euphorbia neriiifolia belongs to family euphorbiaceae and occurs in dry, rocky and hilly areas of North, Central and South India. This plant is extensively grown as a hedge plant (Pandey, 1998). It is a large fleshy, glabrous, small tree, 1.8-4.5 m high Green and with sharp stipular spines (Longmann, 2005). In Ayurvedic treatment the plant has been used as laxative, carminative, improves appetite, useful in abdominal troubles, asthma, bronchitis, tumours, loss of consciousness, leucoderma, piles, inflammations, anaemia and ulcers (Nadkarni, 2007).

In Unani treatment the juice is purgative, carminative, useful in gonorrhoea, whooping cough, asthma, dropsy, leprosy, enlargement of the spleen, dyspepsia, jaundice, colic, tumours, stone in bladder (Kirtikar, 2008).

The chemical constituents reported are Euphorbon, resin, gum, triterpenoids, euphol, 24-methylenecycloartenol, euphorbol, haxacosonate, taraxerol, friedelan-3- α -ol and 3 β -ol (Khare, 2007).

Recent studies have shown that reactive oxygen species (ROS) are involved in several diseases, such as asthma, arthritis, inflammation, cancer, atherosclerosis, neurodegeneration and Parkinson's disease (Sawale, 2014).

Therefore, the objectives of the present study were to isolate phytochemicals and investigate the *in vitro* antioxidant activity of isolated phytoconstituent through free radical scavenging and inhibition of lipid peroxidation method.

Materials and methods

Plant material

Collection and Authentication

Leaves of *E. neriiifolia* belonging to family euphorbiaceae were collected in the month of August from local region of Bhopal district, Madhya Pradesh (India) and were authenticated from Dr Vinayak Naik, Senior Research Scientist, Piramal Life Sciences India Ltd. Goregaon (E), Mumbai. A voucher specimen of the plant (NPIL/PLS/10-1038) has been deposited for future reference.

Extraction of plant material

The air dried powdered leaves of *Euphorbia neriiifolia* (1000g) were extracted with ethanol (70%v/v) in Soxhlet apparatus and concentrated in rotary evaporator under reduced pressure to get ethanolic extract (365.0g). Ethanolic extract was dissolved in ethanol and water (1:2 v/v) and partitioned with chloroform and ethyl acetate in 50mL portion for several times till complete extraction takes place. Resulted in chloroform fraction (149.8g) and ethyl acetate fraction (182.0g) upon concentration under reduced pressure (Mukherjee, 2010; Harborne, 1998).

Phytochemical investigation

Ultimately, the goal in surveying plants for biologically active

useful compounds should be to isolate one or more constituents responsible for a particular activity. Plant is selected for phytochemical investigation, either on the basis of Phytopharmacological approach or through some other avenue, phytochemical screening techniques can be a valuable aid (Farnsworth, 1966).

The ethanolic extract of *E. neriiifolia* and their different fractions were subjected to preliminary phytochemical screening for the detection of various phytoconstituents such as alkaloids, glycosides, carbohydrates, tannins, flavonoids, saponins, sugars and proteins (Mukherjee, 2010).

Isolation and Characterization of Compound EN1

The obtained ethyl acetate fraction was chromatographed on silica gel column (70cmX15cm, 60-120mesh, 2kg) chromatography and preparative TLC.

Column was first eluted with hexane, then polarity of mobile phase was gradually increased by adding ethyl acetate in different concentrations (100:0, 95:5, 90:10, 85:15, 80:20 v/v). 117 fractions each of 50mL were collected and TLC was performed of each fraction individually and eluates were monitored for the presence of various constituents. Fractions were pooled on the basis of their TLC profile, pooled fractions (26-34) were selected for the isolation of constituents. Further purification was performed by preparative TLC of isolated constituents to offer EN1.

Preparation of Sample

The cycloartenol was dissolved in dimethyl sulfoxide to get different concentration and was kept in refrigerator until used

Determination of DPPH radical scavenging assay

The DPPH assay (1, 1-Diphenyl, 2-picryl hydrazyl) is based on the reaction where the purple-coloured DPPH (a stable free radical) is reduced to the yellow-coloured diphenylpicrylhydrazine

when reacting with the free radicals of the sample. DPPH solution (25mg/mL) in methanol was prepared and 2mL of this solution was added to 0.5mL of sample solution at different concentrations (20, 40, 60, 80, 100 μ g/mL). The mixture was vigorously shaken and allowed to stand at room temperature in the dark for 30 minutes and absorbance was measured at 517nm in a spectrophotometer against the corresponding blank solution. The assay was performed in triplicates. Ascorbic acid was taken as reference. Percentage inhibition of DPPH free radical was calculated based on the control reading, which contain DPPH and distilled water without any sample using the following equation:

$$\text{DPPH Scavenged (\%)} = \frac{(\text{A}_{\text{control}} - \text{A}_{\text{test}})}{\text{A}_{\text{control}}} \times 100$$

Where ' A_{cont} ' is the absorbance of the control reaction and ' A_{test} ' is the absorbance in the presence of the sample. The antioxidant activity of the sample was expressed as IC_{50} . The IC_{50} value was defined as the concentration (in $\mu\text{g/mL}$) of sample that inhibits the formation of DPPH radicals by 50% (Kori, 2009; Prajapati, 2003; Senthilkumar, 2014).

Lipid peroxidation by thiobarbituric acid assay

It is based on the reaction between thiobarbituric acid and malondialdehyde and the production of a coloured pigment. Normal male rats (250g) were used for the preparation of liver homogenate. The perfused liver was isolated, and 10% (w/v) homogenate was prepared with homogenizer at 0-4°C with 0.15M KCl. The homogenate was centrifuged at 8000 x g for 15 min and clear cell-free supernatant was used for the study with *in vitro* lipid peroxidation assay. Different concentrations (10-100 $\mu\text{g/mL}$) of sample, 1mL of 0.15M KCl and 0.5mL of rat liver homogenates were added to the test tubes. Peroxidation was initiated by adding 100 μL of 0.2 mM ferric chloride. After incubation at 37°C for 30 min, the reaction was stopped by adding 2 mL of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid, 0.38% thiobarbituric acid, and 0.5% butylated hydroxytoluene. The reaction mixtures were heated at 80°C for 60min. The samples were cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm. The percentage inhibition of lipid peroxidation was calculated by the formula: (Kashif, 2014)

Inhibition of lipid peroxidation (%) = $1 - (\text{sample OD}/\text{blank OD}) \times 100$

Chemicals

All chemicals and solvents were of analytical grade and were obtained from Central Drug House Pvt. Ltd., India. 1,1-Diphenyl, 2-picryl hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. The other chemicals were potassium ferricyanide, trichloroacetic acid, butylted hydroxytoluene, ferric chloride, ascorbic acid and potassium chloride. Adsorbent for column chromatography was silica gel G 60-120, Merck.

Detection of Phytosterols

Salkowski test

A pinch amount of isolated compound was dissolved in chloroform, to this solution few drops of concentrated sulphuric acid was added. Reddish colour produces shows the presence of phytosterols.

Liebermann-Burchard test

A pinch amount of isolated compound was dissolved in chloroform, to this solution few drops of concentrated sulphuric acid followed by few drops of diluted acetic acid, 3mL of acetic anhydride. A bluish green colour formation indicates the presence of phytosterols (Harborne, 1998).

Analytical methods

TLC was performed on silica gel GF₂₅₄ precoated (Merck) plates. IR spectrum was recorded with FTIR (Shimadzu), ¹H and ¹³C spectra recorded on Bruker (300MHz and 75.4MHz) in CDCl₃ used TMS as internal standard. ESIMS were measured using a Q-TOF micro mass spectrometer (Waters, USA).

Results and Discussion

The phytochemical screening of ethanolic extract of *E. nerifolia* revealed the presence of sterols, glycosides, phenolic compounds, flavonoids, saponins, carbohydrates and proteins and amino acids. Its chloroform fraction showed presence of sterols, phenolic compounds and flavonoids. The ethyl acetate fraction of showed positive test for presence of glycosides, phenolic compounds and flavonoids.

Therefore ethyl acetate fraction was loaded in column chromatography to isolate various compounds, which yielded fractions (26-34), gave cycloartenol (EN1) (16mg) of pure compound by repeated preparative TLC. Upon qualitative test performed on pure compound, indicated its triterpenoidal nature.

Cycloartenol (EN1): It was yellow amorphous powder, soluble in chloroform. Mass spectrum of isolated compound showed molecular ion m/z 540.9[M+1] corresponding to the molecular formula C₃₀H₅₁O.

IR (KBr, in cm⁻¹): An intently broad band at 3437 shows OH stretching and 1736 shows carbonyl stretch.

¹H NMR (400 MHz, DMSO, δ , TMS=0): δ 4.57ppm(1H,t, H-24), 3.12(1H,m,H-3), 1.58(3H,s,H-27), 1.51(3H, s, H-26), 1.07(1H,s, H-29), 0.93(1H,s, H-28), 0.92(1H,s,H-30).

¹³C NMR (150 Hz, DMSO, δ , TMS=0): δ 32.54 (C-1), 30.17 (C-2), 58.93 (C-3), 40.73 (C-4), 47.51 (C-5), 20.77 (C-6), 28.24 (C-7), 48.67 (C-8), 20.77 (C-9), 25.97 (C-10), 25.47 (C-11), 25.97 (C-12), 46.57 (C-13), 48.67 (C-14), 32.54 (C-15), 27.01 (C-16), 55.17 (C-17), 18.31 (C-18), 29.79 (C-19), 36.86 (C-20), 19.20 (C-21), 37.06 (C-22), 24.92 (C-23), 121.93 (C-24), 128.17 (C-25), 19.13 (C-26), 25.16 (C-27), 33.37 (C-28), 14.22 (C-29), 25.16 (C-30).

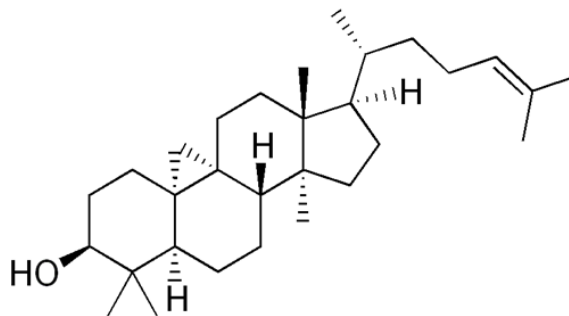
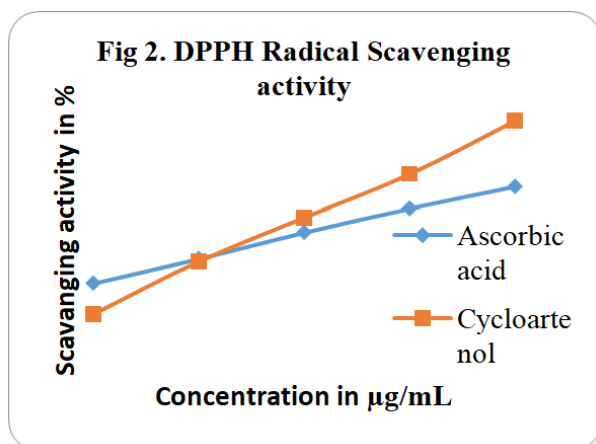


Figure 1. Chemical structure of cycloartenol

The structure of cycloartenol was elucidated and compared with literature to confirm the structure (Tin, 2016).

Antioxidant activity of Cycloartenol

In order to understand biological role of cycloartenol, it has become important to study its antioxidant property. Therefore *in vitro* antioxidant activity based on the ability of cycloartenol to scavenge the various free radicals is determined and results are represented in figure 2 to 3.

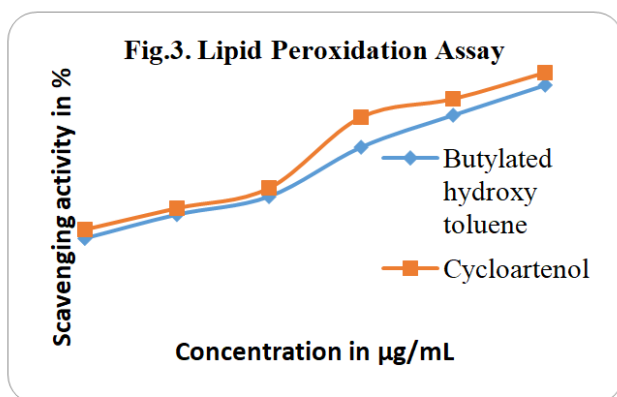


DPPH radical scavenging assay

Cycloartenol exhibited significant dose dependent inhibition of DPPH activity. The IC_{50} value of cycloartenol was found to be $40\mu\text{g/mL}$.

Lipid peroxidation assay

Fig 3 depicts inhibition of lipid peroxidation by cycloartenol in a dose dependent manner. The IC_{50} value of cycloartenol was found to be $44.68\mu\text{g/mL}$.



Discussion

DPPH assay

Figure 2 illustrates decrease in the concentration of DPPH radicals due to the scavenging ability of cycloartenol and ascorbic acid. A $40\mu\text{g/mL}$ of cycloartenol and ascorbic acid (std.) exhibited 50.03% and 51.04% inhibition respectively.

The results indicated that cycloartenol reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles.

Lipid peroxidation assay

Fig 3 shows that the cycloartenol inhibited ferric chloride induced lipid peroxidation in a dose dependent manner. The inhibition could be caused by scavenging the $\cdot\text{OH}$ radical or the superoxide radicals or by changing the $\text{Fe}^{3+}/\text{Fe}^{2+}$ or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself.

Conclusion

It is concluded that cycloartenol isolated from *E. neriifolia* leaves which exhibited the significant antioxidant activity through the scavenging of free radicals which participate in various pathophysiology of diseases including ageing. Overall, the cycloartenol is a source of natural antioxidants that may be used in prevention of various diseases.

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