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Research Article

Anti–inflammatory, Antioxidant and Free Radical Scavenging Activities of *Nyctanthes Arbor-Tristis Linn*. Seed Extract Under *In Vitro*

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Abstract: Anti–inflammatory and Antioxidant properties of *Nyctanthes arbor-tristis L*. (Oleaceae) seeds extracted with Hexane, ethyl acetate and methanol revealed that ethyl acetate (Ny-El) extract showed potent effect than hexane and methanol extracts. The ethyl acetate extract exhibits 50% inhibition of anti–inflammatory activity at 7.19 ± 1.71 mg/mL. The total phenolic content of Ny-El extract was 206.81 ± 1.11 mgof catechol equivalents/g extract. Ny-El extract showed great scavenging activity on 2, 2-diphenyl-picrylhydrazyl (DPPH) (IC_{50} 459.91±1.40 µg/mL), hydroxyl (IC_{50} 363.32±1.58 µg/mL), nitric oxide (IC_{50} 545.03 ± 1.69 µg/mL) andsuperoxide (IC_{50} 338.82 ±1.72 µg/mL) radicals, as well as had high reducing power. Ny-El extract also showed a strong suppressive effect on lipid peroxidation. The metal chelating ability of Ny-El extract also showed strong inhibition effect when compared to the reference standard.
Keywords: Anti–inflammatory, *Nyctanthes arbor-tristis L*, 2, 2-diphenyl-picrylhydrazyl, total phenolic content

INTRODUCTION

Oxygen consumption inherent in cell growth leads to the generation of a series of reactive oxygen species (ROS) [1]. They are continuously produced by the body's normal use of oxygen such as respiration and some cell-mediated immune functions. ROS include free radicals such as superoxide (O_2), hydroxyl radical (OH), peroxyl radical (RO_2) as well as non-radical species such as hydrogen peroxide (H_2O_2) [2]. Free radicals are known to be the source of major causes of various chronic and degenerative diseases including aging, coronary heart disease, stroke, diabetes mellitus, cancer and inflammation [3, 4]. Inflammation is a reaction of living tissues towards injury, and it comprises systemic and local responses [5]. The main action of anti-inflammatory agents is the inhibition of Cyclooxegenase enzymes which responsible for conversion of Arachidonic acid to prostaglandins. The human red blood cell (HRBC) membranes are similar to these lysosomal membrane components, the prevention of hypotonicity induced HRBC membrane lysis was taken as a measure in estimating the anti-inflammatory property. Thus, HRBC method has been used in estimating the anti-inflammatory [6, 7]. Recently, natural foods and food derived antioxidants such as vitamins and phenolic phytochemicals have received growing attention, because they are known to function as chemo-preventive agents against oxidative damage. Many synthetic antioxidant components have shown toxic and/or mutagenic effects. Hence, attention has been given to naturally occurring antioxidants. Numerous plant constituents have shown free radical scavenging or antioxidant activity [8, 9].

Nyctanthes arbor-tristis Linn. (Oleaceae) is a traditional Indian medicinal plant, commonly called as Night Jasmine (*Pavalamalli* in Tamil) is a small tree growing up to 10 m tall, with flaky grey bark which is widely cultivated throughout India as a garden plant and found wild in the forests of South Indian regions. It is a traditional plant used in the treatment of chronic fevers and rheumatism. It is also used as a liver and nerve tonic. The indigenous people of India use *Nyctanthes arbor-tristis* to cure various ailments along with its use in Ayurveda, Siddha and Unani systems of medicines [10]. The indigenous people of Chittoor district in Andhra Pradesh (India) widely use whole plant for the treatment of cancer, root for fever, sciatica, anorexia; bark as expectorant [11, 12]. The seeds of *N. arbor-tristis* are used to treat cancer [13] and as shown antiallergic [14, 15], immunomodulatory [16, 17], inflammation [10, 18], antiviral [14], anthelmintics, alopecia, antibilious properties [19]. Earlier, researchers have reported the isolation of polysaccharide, nyctanthoside, nyctanthic acid, β-sitosterol, 6β-hydroxy loganin, glycerides of linoleic oleic, stearic, palmitic, myristic acids, and arbortristoside –A and arbortristoside-B from the plant parts [20-23]. Arbortristoside – A and Arbortristoside – B have been reported to possess leismanicidal [24], antipalsmodial [23] and anti-inflammatory [10] properties. The medicinal properties of *N. arbor-tristis* seeds might be attributed to its ROS-scavenging ability. The antioxidant and anti-inflammatory properties of different extracts of the seeds were investigated in present study.

MATERIALS AND METHODS

Chemicals and reagents

DPPH(1,1-diphenyl, 2-picryl hydrazyl), NBT(Nitro blue tetrazolium), NADH (Nicotinamide adenine dinucleotide phosphate reduced), PMS(Phenazine metho sulphate), TCA (Trichloroacetic acid), ferric chloride and BHT(Butylated hydroxyl toluene) were obtained from the Sigma Chemical Co., USA. Ascorbic acid obtained from SD the Fine chemicals. Ltd., Biosar, India. All the other chemicals were of analytical grade.

Plant material

Two Kilogram of *Nyctanthes arbor-tristis* Linn. dried seeds were collected from the Chinamandali village, Thiruvallur District, South India in May, 2012. The taxonomical identity of the plant was confirmed by Dr. D. Narashiman, Department of Botany, Madras Christian College (Tambaram, Chennai, India), and the voucher specimen (PCHC - 400) was preserved in Pachaiyappa's College Herbarium, Chennai for further reference

Method of extraction

The collected *N. arbor-tristis* Linn seeds were dried and powdered. One Kg of powder was subjected for three successive extractions by cold percolation method with 3000 mL of hexane, ethyl acetate and methanol, at room temperature for 72 h. The filtrates were concentrated under reduced pressure at 40°C and stored in a refrigerator at 2–8 °C for the subsequent experiments. The percentage yield of hexane, ethyl acetate and methanol extracts were 0.76, 2.72 and 5.751% (w/w), respectively.

Determination of *in vitro* Anti-inflammatory activity and antioxidant assays

In vitro Anti-inflammatory activity

Human red blood cell (HRBC) method was used for the estimation of anti-inflammatory activity *in vitro* [6, 7]. Blood was collected from healthy volunteers and mixed with equal volume of sterilized Alsevers solution. The sample was centrifuged at 3,000 rpm and the packed cells were separated. The packed cells were washed three times with isosaline solution and 10% v/v suspension was made with isosaline. This HRBC suspension was used for the estimation of anti-inflammatory property. Different concentrations of extract, reference sample and control were separately mixed with 1 mL of phosphate buffer, 2 mL of hyposaline and 0.5 mL of HRBC suspension. All the assay mixtures were incubated at 37°C for 30 minutes and centrifuged at 3,000 rpm. The supernatant liquid was decanted and the hemoglobin content was estimated by a spectrophotometer at 560 nm. The percentage hemolysis was estimated by assuming the hemolysis produced in the control as 100%.

Percentage protection= 100 - (OD sample/ OD control)*100……. (i)

Determination of total phenolic content

Total phenolic content of *N. arbor-tristis* hexane, ethyl acetate and methanol extracts were assessed according to the Folin–Ciocalteau method [25] with some modifications. Briefly, 0.1 mL of extracts (200–1000 µg/mL), 1.9 mL distilled water and 1 mL of Folin–Ciocalteau's reagent were added in a test tube, and then 1 mL of 100 g/L Na_2CO_3 was added. The reaction mixture was incubated at 25°C for 2 h and the absorbance of the mixture was read at 765 nm. The sample was tested in triplicate and a calibration curve with six data points for catechol was obtained. The results were compared to a catechol calibration curve and the total phenolic content of *N. arbor-tristis* was expressed as mg/g of catechol equivalents per gram of extract.

Reducing ability assay of *N. arbor-tristis*

The reducing power of *N. arbor-tristis* hexane, ethyl acetate and methanol extracts were evaluated according to the method of [26]. Different amounts of the extracts viz., 200–1000 µg/mL were suspended in glass distilled water and mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6), and 2.5 mL of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min; 2.5 mL of 10% TCA was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with 2.5 mL glass distilled water and added $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated the ability of reducing power. Butylated hydroxy toluene (BHT) was used as standard.

DPPH radical scavenging assay of *N. arbor-tristis*

DPPH quenching ability of *N. arbor-tristis* hexane, ethyl acetate and methanol extracts were measured according to Hanado et al. [27]. The methanol DPPH solution (0.15%) was mixed with serial dilutions viz., 200–1000 µg/mL of the extracts and after 10 min, the absorbance was read at 515 nm. The antiradical activity was expressed as IC_{50} (µg/mL), (the antiradical dose required to cause a 50% inhibition). Vitamin C was used to prepare standard.

The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = (A0 - A1) / A0× 100 ……. (ii)

Where A0 is the absorbance of the control at 30 min, and A1 is the absorbance of the sample at 30 min. All samples were analyzed in triplicate.

Hydroxyl radical scavenging assay of *N. arbor-tristis*

The assay was performed as described by the method of Elizabeth and Rao [28] with minor changes. All solutions were prepared freshly. One milliliter of the reaction mixture contained 100 µl of 28 mM 2-deoxy-2-ribose (dissolved in phosphate buffer, pH 7.4), 500 µl

solution of various concentrations of *N. arbor-tristis* hexane, ethyl acetate and methanol extracts (200–1000 µg/mL), 200 µl of 200 µM $FeCl_3$ and 1.04 mM EDTA (1:1 v/v), 100 µl H_2O_2 (1 mM) and 100 µl ascorbic acid (1 mM). After an incubation period of 1 h at 37° C, the extent of deoxyribose degradation was measured by the TBA reaction. The absorbance was read at 532 nm against the blank solution. Vitamin C was used as a positive control. The scavenging activity was calculated using formula (ii).

Nitric oxide radical inhibition assay of *N. arbor-tristis*

Sodium nitroprusside in an aqueous solution at physiological pH spontaneously generates nitric oxide; it interacts with oxygen to produce nitrite ions, which can be estimated by the use of GriessIllosvoy reaction [29]. In the present investigation, GriessIllosvoy reagent was Modified using naphthyl ethylenediamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). The reaction mixture (3 mL) containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (0.5 mL) and different concentration of *N. arbor-tristis* hexane, ethyl acetate and methanol extracts (200–1000 µg/mL) or standard solution (0.5 mL) were incubated at 25°C for 150 min. After incubation, 0.5 mL of the reaction mixture containing nitrite was pipetted and mixed with 1 mL of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 mL of naphthyl ethylenediamine dihydrochloride (1%) was added, mixed and allowed to stand for 30 min. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank. Vitamin C was used as positive control. The scavenging activity was calculated using the formula (ii).

Superoxide scavenging activity of *N. arbor-tristis*

Superoxide scavenging activities of *N. arbor-tristis* hexane, ethyl acetate and methanol extracts were determined by monitoring the competition of those with NBT for the superoxide anion generated by the PMS–NADH system [30]. Superoxide radicals were generated in 1 mL of 20 mM Tris–HCl buffer pH 8.0 containing 0.05 mM nitro bluete trazolium (NBT), 0.01 mM phenazine methosulphate (PMS) and different concentration of extracts (200–1000 µg/mL) were preincubated for 2 min. The reaction was initiated by the addition of 0.078 mM NADH. Blue chromogen, formed due to NBT reduction was read at 560 nm. Results were expressed as percentage of inhibition of superoxide radicals. Vitamin C was used as a positive control. The scavenging activity was calculated using the formula (ii).

Inhibition of lipid peroxidation in rat liver homogenate by *N. arbor-tristis*

To carry out the assay rat liver samples were obtained from healthy animals from CAS in Botany, Animal House. The inhibition effect of *N. arbor-tristis* hexane, ethyl acetate and methanol extracts on lipid peroxidation was determined according to the thiobarbituric acid method. $FeCl_2$–H_2O_2 was used to induce liver homogenate peroxidation [31]. In this method, 0.2 mL of different concentration of extracts (200–1000 µg/mL) was mixed with 1 mL of 1% liverhomogenate (each 100 mL homogenate solution contains 1 g rat liver); then 50 µl of $FeCl_2$ (0.5 mM) and H_2O_2 (0.5 mM) was added. The mixture was incubated at 37°C for 60 min; then 1 mL of trichloroacetic acid (15%) with thiobarbituric acid (0.67%) was added and the mixture was heated in boiling water for 15 min. The absorbance was recorded at 532 nm. Vitamin C was used as positive control. The percentage of inhibition was calculated using the formula. (ii).

Metal chelating activity of *N. arbor-tristis*

The chelating of ferrous ions by *N. arbor-tristis* hexane, ethyl acetate and methanol extracts were estimated by the method of [32]. The different concentrations of extract (200 - 1000 µg/mL) were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. EDTA was used as a positive control. The percentage inhibition of ferrozine Fe^{2+} complex formation was calculated as using the formula (ii).

Preliminary phytochemical analysis of active extract

Preliminary phytochemical screening of *N. arbor-tristis* Ethyl acetate extract (Ny-El extract) was carried out to detect the phytoconstituents using standard conventional protocols [33].

Statistical analysis

The data for biochemical and physiological parameters were analyzed and expressed as mean ± SD. The IC_{50} values were calculated from linear regression analysis. Results were processed by computer program, Microsoft Excel (2010).

RESULT

In vitro* Anti-inflammatory activity and antioxidant assays of *N. arbor-tristis

Anti-inflammatory activity

The results for Anti-inflammatory activity of Different extracts and Diclofenac were shown in Table 1. The Ny-El extract and Diclofenac were found to be an effective protection respectively. The hexane and methanol extracts showed less compared protection to ethyl acetate extract.

Table 1: Anti–inflammatory activity of different extracts of *Nyctanthes arbor-tristis* seeds

	Percentage of Protection			
Concentration (mg/mL)	**Diclofenac Sodium**	**Hexane Extract**	**Ethyl Acetate Extract**	**Methanol Extract**
2	13.79 ± 0.54	10.52 ± 0.60	10.98 ± 0.86	10.86 ± 0.31
4	26.22 ± 0.41	20.93 ± 0.93	24.06 ± 0.69	21.72 ± 0.56
6	40.13 ± 0.54	31.69 ± 1.61	38.08 ± 1.59	34.09 ± 1.04
8	54.21 ± 1.59	41.51 ± 0.88	52.63 ± 1.04	43.85 ± 0.56
10	66.10 ± 0.39	52.06 ± 1.40	62.08 ± 1.86	56.20 ± 1.75

Total phenolic content

The total phenolic content of hexane, ethyl acetate and methanol extracts were found to be 77.80 ± 0.46, 256.16 ± 0.77 and 186.81 ± 1.22 mg catechol equivalent/gram extract, respectively.1. The concentration for 50% inhibition of Ny-El extract and acarbose were found to be 492.20 ± 1.11

Reducing power

Fig.1 (a) shows the reductive capabilities of hexane, ethyl acetate and methanol extracts of compared to butylatedhydroxy toluene. The reducing power of Ny-El extract was very potent than hexane and methanol extracts and the power of the extract was increased with quantity of sample. The seed extract could reduce the most Fe^{3+} ions, which had a lesser reductive activity than the standard of butylatedhydroxy toluene.

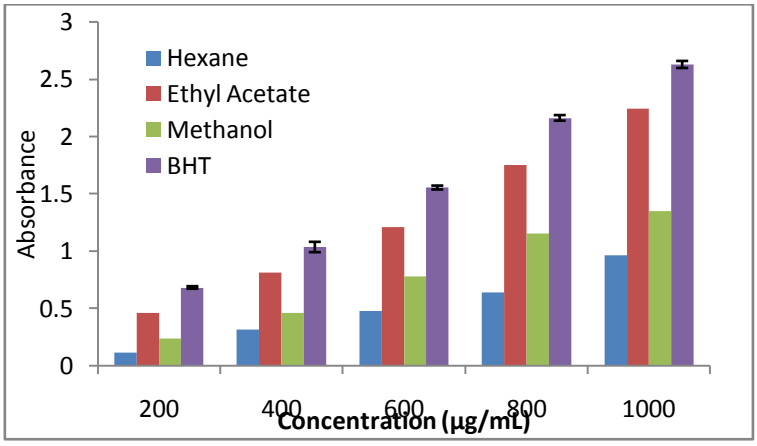

Fig. 1a: Reductive ability of different concentrations (200–1000 µg/mL) of Nyctanthes arbor-tristis hexane, ethyl acetate, methanol extracts and BHT. Each value represents the mean ± SEM of triplicate experiments

DPPH radical scavenging activity

The extract of Ny-El exhibited a significant dose dependent inhibition of DPPH activity when compared to hexane and methanol extracts, with a 50% inhibition (IC_{50}) at a concentration of 459.91±1.40 µg/mL. The results are presented in Fig. 1(b). The IC_{50} value of vitamin C was 338.57±2.21 µg/mL.

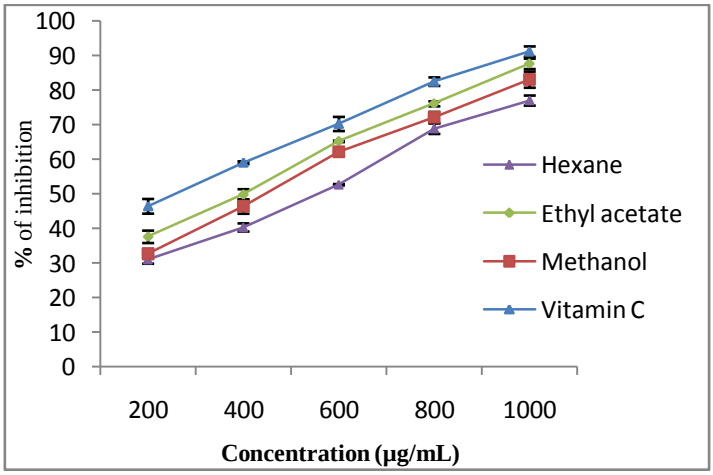

Fig. 1b: DPPH scavenging effect of different concentrations (200–1000 µg/mL) of Nyctanthes arbor-tristis hexane, ethyl acetate, methanol extracts and vitamin C. Each value represents the mean ± SEM triplicate experiments

Hydroxyl radical scavenging assay

To attack the substrate deoxyribose hydroxyl radicals were generated by reaction of Ferric-EDTA together with H_2O_2 and ascorbic acid. When the plant extracts were incubated with the above reaction mixture, it could prevent the damage against sugar. The results for hydroxyl scavenging assay are shown in Fig. 1(c). The concentrations for 50% inhibition were found to be 363.32±1.58 and 345.11±1.43 µg/mL for the ethyl acetate extract and vitamin C respectively. Hexane and methanol extracts showed less effect.

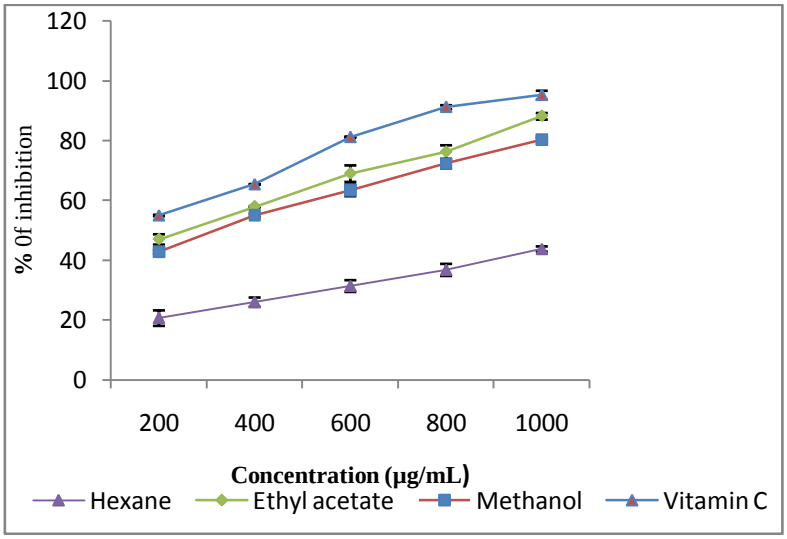

Fig. 1c: Hydroxyl radical scavenging effect of different concentrations (200– 1000 µg/mL) of Nyctanthes arbor-tristis hexane, ethyl acetate, methanol extracts and vitamin C. Each value represents the mean ± SEM of triplicate experiments

Nitric oxide radical inhibition assay

The scavenging of nitric oxide by Ny-El extract was increased in a dose-dependent manner as illustrated in Fig. 1(d). At concentration of 545.03 ± 1.69 µg/mL of extract 50% of nitric oxide generated by incubation was scavenged. The IC_{50} value of vitamin C was 340.11 ± 0.98 µg/mL.

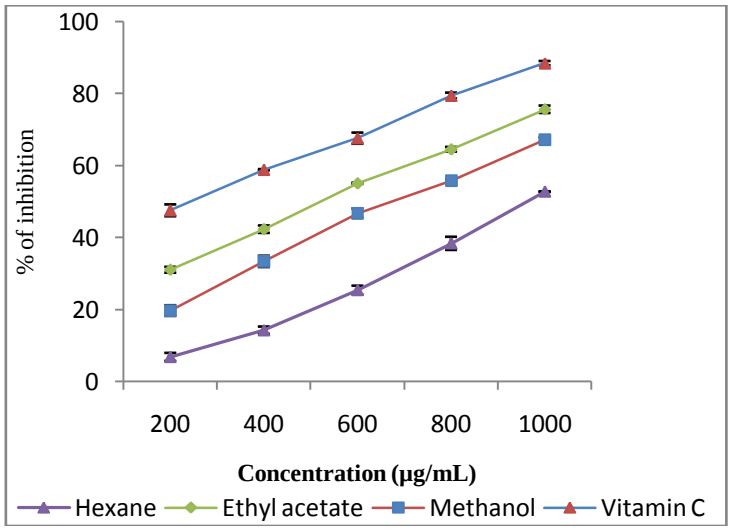

Fig. 1d: Nitric oxide scavenging effect of different concentrations (200–1000 µg/ ml) of Nyctanthes arbor-tristis hexane, ethyl acetate, methanol extracts and vitamin C. Each value represents the mean ± SEM of triplicate experiments

Superoxide scavenging activity

The superoxide anion derived from dissolved oxygen by Phenazinemethosulphate/NADH coupling reaction reduces nitro blue tetrazolium. The decrease the absorbance at 560 nm with the plant extract thus indicates the consumption of superoxide anion in the reaction mixture. Asmentioned in Fig. 1(e), the Ethyl acetate extract as well as vitamin C showed the scavenging activity; IC50 values, 338.82 ±1.72 µg/mL and 299.22± 1.34 µg/mL, respectively.

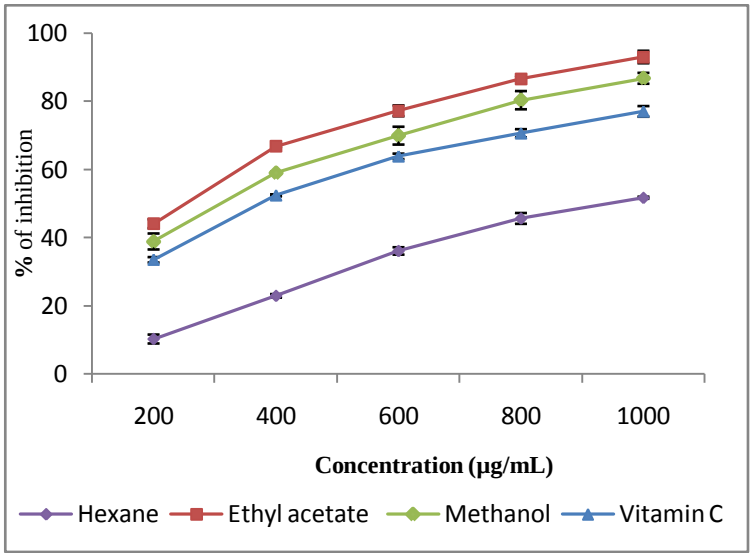

Fig. 1e: Superoxide scavenging effect of different concentrations (200–1000 µg/mL) of Nyctanthes arbor-tristis hexane, ethyl acetate, methanol extracts and vitamin C. Each value represents the mean ± SEM of triplicate experiments

Lipid peroxidation assay

Activity of extracts on lipid peroxidation is shown in Fig. 1(f). Addition of Fe_2+ / ascorbate to the liver microsomes cause increase in lipid peroxidation. Ny-El extract showed inhibition of peroxidation effect in all concentrations compared to hexane and methanol, which showed 50% inhibition effect at 399.09 ± 1.56 µg/mL. The IC_{50} value of vitamin C was 309.29 ± 2.77 µg/mL.

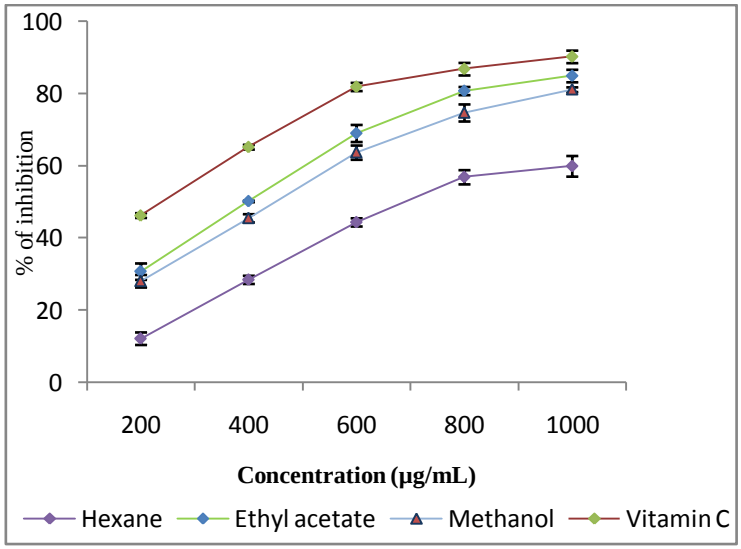

Fig. 1f: Anti-lipid peroxidation effect of different concentrations (200–1000 µg/mL) of Nyctanthes arbor-tristis hexane, ethyl acetate, methanol extracts and vitamin C. Each value represents the mean ± SEM of triplicate experiments

Metal chelating

Activity of extracts on chelating the ferrous ions is shown in Fig. 1(h). Ny-El extract showed 50% of chelate ions generation at the concentration of 550.50 ± 2.68 µg/mL. The IC_{50} value of EDTA was 340.20 ± 2.63 µg/mL.

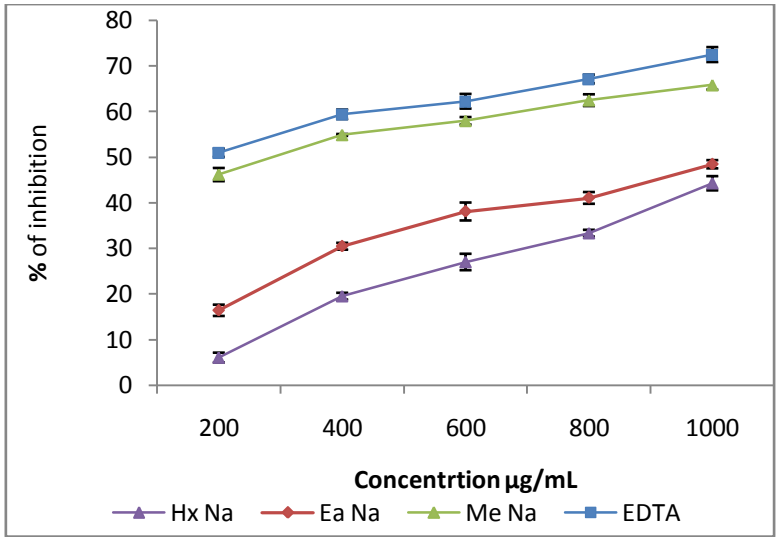

Fig. 1h: Metal chelating effects of different concentrations (200–1000 µg/mL) of Nyctanthes arbor-tristis hexane, ethyl acetate, methanol extracts and EDTA. Each value represents the mean ± SEM of triplicate experiments

Preliminary phytochemical analysis

The preliminary phytochemical evaluation of Ny-El extract showed the presence of steroids, triterpenoids and phenolic compounds.

DISCUSSION

Inflammation is a common phenomenon and it is a reaction of living tissues towards injury. Steroidal anti-inflammatory agents will lyse and possibly induce the redistribution of lymphocytes, which cause rapid and transient decrease in peripheral blood lymphocyte counts to affect longer term response. Ny-El extract showed a potential in-vitro anti–inflammatory activity on HRBC cells. *Nytanthes arbor-tristis.* Studies of similar kind [34-37] also reported antioxidant activity from *N. arbor-tritis.* For the measurements of the reductive ability, we studied the Fe^{3+} to Fe^{2+} transformation in the presence of *N. arbor-tristis* extracts, using the method of Oyaizu [26]. The reducing power increased with increasing concentration of the extract. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [38]. DPPH test is usually used as the substrate to evaluate antioxidative activity of antioxidants [26]. This method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction [39]. Ny-El extract has the ability to reduce the stable radical DPPH to the yellow-colored diphenyl picryl hydrazine. The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells [40]. Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity [41]. Ny-El extract inhibited free radical mediated deoxyribose damage remarkably. Nitric oxide plays an important role in various types of inflammatory processes in the animal body. Nitric oxide radical inhibition study showed that the extract was a potent scavenger of nitric oxide. The extract inhibited nitrite formation by competing with oxygen to react with nitric oxide directly and also to inhibit its synthesis. Scavengers of nitric oxide competed with oxygen leading to reduced production of nitric oxide [42]. In the PMS–NADH–NBT system, superoxide anion derived from the dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease in the absorbance at 560 nm with antioxidants thus indicates the consumption of the generated superoxide anion in the reaction. Superoxide, the one-electron reduced form of molecular oxygen, is a precursor of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen that have the potential of reacting with biological macromolecules and thereby inducing tissue damages [43]. These results evidently indicated that Ny-El extract is a compelling scavenger of superoxide radicals in a dose-dependent manner. Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates a number of degradation products. Malondialdehyde (MDA), one of the products of lipid peroxidation, has been studied widely as an index of lipid peroxidation and as a marker of oxidative stress [44]. The chelating of ferrous ions by the extract was estimated by the method of Dinis et al. [32]. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red colour of the complexes decreases. The rate of colour reduction Measurement allows estimation of the chelating activity of the coexisting chelator [45]. In the study Ny-El extract reveals effective capacity for iron binding, suggesting that action as an antioxidant may be related to its iron binding capacity. This is a very first study reporting antioxidant properties from seeds of *N. arbor-tristis.* Previous reports revealed that the antioxidant activities from leaves, calyx, flowers, petals [34-37, 46-47].

CONCLUSION

This study is known to be superlative report where antioxidant activities of Ny-El seeds extract were exploited. Further research on the isolation and characterization of bio-active compound that response to antioxidant and free radical scavenging.

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