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Studies on total antioxidant activity of the extract of *Nyctasnthes arbortristis* flower extract by DPPH radical-scavenging activity and superoxide anion scavenging activity assay

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Abstract
The fee radicals (oxidants) are species with very short half-life, high reactivity and damaging activity towards macromolecules like proteins, DNA and lipids. In general, the reactive oxygen species (ROS) circulating in the body tend to react with the electron of other molecules in the body and these also effect various enzyme systems and cause damage which may further contribute to conditions such as cancer, ischemia, aging, adult respiratory distress syndromes, rheumatoid arthritis etc. Some plants are considered as important source of nutrition and as a result of that they are recommended for their therapeutic values. Treatment with medicinal plants is considered very safe as there is no or minimal side effects. These remedies are in synchronization with nature, which is the biggest advantage. The medicinal value of the chosen plant *Nyctanthes arbortristis* flower extract has not been extensively worked out. Therefore, the present study was to investigate the *in vitro* antioxidants activity of *Nyctanthes arbortristis* flower extract.
The ethanolic extract was screened for *in vitro* antioxidant activity by oxygen radical scavenging such as DPPH, total antioxidant assay and superoxide metal chelation activity at different concentrations. Throughout the studies flower extract showed marked antioxidant activity. The antioxidant activity was found to be concentration dependent and may be attributed to the presence of bioflavonoids content in the flowers of *Nyctanthes arbortristis*.

Keywords: Antioxidant, *Nyctanthes arbortristis*, ethanolic extract, DPPH, total antioxidant assay

Introduction
A free radical is an atom, ion, or molecule possessing an unpaired electron in an outer orbit. It becomes harmful because in looking for a pairing electron, the free radical takes one electron from a nearby stable molecule, which in turn converts the stable one into a free radical; thus, the propagation of chain reaction can injure tissues and impair their functions. Most common radical derivatives of oxygen such as superoxide free radical anion ($O_2^{\bullet}$), hydroxy free radical ($^{\bullet}OH$), lipid peroxyl ($LO^{\bullet}$), lipid alkoxyl ($LOO^{\bullet}$), and lipid peroxide (LOOH), as well as non-radical derivatives such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2^{\bullet}$), are collectisvely known as reactive oxygen species (ROS) (Tandon *et al*., 2005) [14]. The nitrogen-derived free radicals are nitric oxide ($NO^{\bullet}$) and peroxidynitrite anion ($ONOO^{\bullet}$) (Koppenol *et al*., 1992) [3].
The unavoidable generation of superoxides can play a bigger role in tissue damage. The superoxide ($O_2^{\bullet}$) radical is formed by adding one electron to the oxygen molecule, and then, it becomes superoxide due to "accidents of chemistry", in that many molecules in the body react directly with oxygen to form superoxide, for example catecholamine and some constituents of mitochondria electron transport chains. In addition, some superoxides are formed deliberately. For example, activated phagocytes generate large amounts of superoxide as part of the mechanism by which foreign organisms are killed (Tandon *et al*., 2005) [14].
Hydrogen peroxide (H_2O_2) is the most stable reactive oxygen metabolites. Divalent reduction of O_2 may generate H_2O_2 directly or indirectly by univalent reduction of $O_2^{\bullet}$. Hydrogen peroxide is the primary product of the reduction of O_2 by numerous oxidases. Redox-active metal complexes are very sensitive to decomposition of H_2O_2, of which catalase and peroxidase are the most effective exponents.
The highly reactive nature of hydroxyl radical is attributable to radiation, which can split water

in the body to produce the hydroxyl radical. Hydroxyl radicals have very short lifespan *in vivo* because they react at the site of formation, usually leaving behind a legacy in the form of a propagating free-radical chain reaction (Sohal *et al*., 1995) [13].

Singlet oxygen ($^1O_2$) is not a free radical, but it has a greater potential to damage the tissues. The half-life of singlet oxygen is very short, which is formed as a result of spin reversal of electron in the outer orbital of oxygen molecule (Langseth, 1996) [5].

Nitric oxide ($NO^{\bullet}$) is another physiological free radical produced by the vascular endothelium as a relaxing factor and also by phagocytes in brain. Nitric oxide has many physiological functions, but excess nitric oxide can be toxic (Halliwell, 1994).

Antioxidants

Exposure of aerobic organism to oxidative stress is an unavoidable consequence. Although cells are equipped with an impressive repertoire of antioxidant enzymes as well as small antioxidant molecules, these agents may not be sufficient enough to normalize the redox status during oxidative stress (Seifried *et al*., 2007). A broad class of protective agents termed antioxidants remove the harmful effect of ROS by reacting with free radicals before any other molecules can become targets. Antioxidants are probably now regarded as the new generation 'superheroes' to maintain the health (Aruoma, 1994). Every living organism has antioxidant defense to cope with the ROS. Enzymatic antioxidants such as SOD, CAT, and GPx and non-enzymatic antioxidants such as vitamin C, vitamin E, ceruloplasmin, albumin, and reduced glutathione play an important role in the protection of cells against free radical–mediated damage (Halliwell, 1994). Oxidative stress is an imbalance condition between ROS production and antioxidant defense. Any natural or synthetic compound with antioxidant properties might contribute toward the partial or total alleviation of this type of damage.

The recent abundant evidence suggesting the involvement of oxidative stress in the pathogenesis of various disorders and diseases has attracted much attention of the scientists and general public to the role of natural antioxidants in the maintenance of human health and prevention and treatment of diseases (Niki, 2010). Plant and its products are rich sources of a phytochemicals and have been found to possess a variety of biological activities including antioxidant potential (Velavan *et al*., 2007) [16]. The majority of the active antioxidant constituents are flavonoids, isoflavones, flavones, anthrocyanins, coumarins, lignans, catechins and isocatechins. In addition to the above compounds found in natural foods, vitamins C and E, beta-carotene and tocopherol are known to possess antioxidant potential (Prior, 2003) [10]. With this background and abundant source of unique active components harbored in plants.

The use of plants for treating diseases is as old as the human species. Popular observations on the use and efficacy of medicinal plants significantly contribute to the disclosure of their therapeutic properties, so that they are frequently prescribed, even if their chemical constituents are not always completely known. All over the globe, especially in Asian countries, the use of medicinal plants has significantly supported primary health care. Liver plays a vital role in metabolism and detoxification. Impairment due to injury or infection leads to deterioration of function may imparts many implications on one's health. Till date treatment for liver diseases by modern medicine is a challenge. Only phytoconstituents are known remedies for the liver management while allopathic medicine has little to offer for the hepatic ailments.

Antioxidants are the compounds of exogenous or endogenous in nature which either prevent the generation of toxic oxidants or intercept any that are generated and inactivate them and thereby block the propagation of chain reaction produced by these oxidants. Therefore the uses of antioxidants, both natural and synthetic are gaining wide importance in the prevention of oxidative stress. Many synthetic antioxidant compounds have shown toxic and/or mutagenic effects, which have stimulated the interest of many investigators to search natural antioxidant. Currently there has been an increased interest globally to identify of plant origin with free-radical scavenging properties could have great importance as therapeutic agents in several diseases caused due to oxidative stress. Plants produces large amount of antioxidants which are pharmacologically potent and have low or no side effects for therapeutic use. Here we study the phytochemical screening and antioxidant hepatoprotective activity of *Nyctanthes arbortristis* flower extract.

Materials and Methods

Preparation of plant extract

Different concentrations of *Nyctanthes arbortristis* flower extract (20, 40, 60 and 80 µg/ml) was chosen for *in vitro* antioxidant activity. L-Ascorbic acid was used as the standard.

DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined by the method of Shimada, *et al.,* (1992) [12].

Reagents

- DPPH : 25 µg/ml in methanol
- Methanol

Procedure:

Briefly, a 2 ml aliquot of DPPH methanol solution (25µg/ml) was added to 0.5 ml sample solution at different concentrations. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517nm in a spectrophotometer. Lower absorbance of the reaction mixture indicated higher free-radical scavenging activity.

$$\text{Radical scavenging activity (\%)} = 100 - \left(\frac{Ac - As}{Ac} \right) X\ 100$$

Where A_C = control is the absorbance and A_S = sample is the absorbance of reaction mixture (in the presence of sample).

Determination of Total Antioxidant Capacity

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al*., (1999) [9].

Reagents:

- Sulfuric acid : 0.6M
- Sodium phosphate : 28mM
- Ammonium molybdate : 4mM

Procedure

The assay is based on the reduction of Mo(VI)–Mo(V) by the

extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. 0.3 ml extract was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid. The scavenging activity was calculated according to the following equation: % Inhibition

$$\% \text{ of Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Superoxide anion scavenging activity assay

The superoxide anion radicals scavenging activity was measured by the method of Liu *et al*., (1997) [6].

Reagents

1. Tris-HCl buffer : pH 7.4
2. Nitroblue tetrazolium (NBT) : 300 µM
3. Nicotinamide adenine dinucleotide (NADH) : 936 µM
4. Phenazine methosulfate (PMS) : 120 µM

Procedure

In these experiments the superoxide anion was generated in 3 ml of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 µM) solution and 0.3 ml of different concentrations of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

% Inhibition = $((A_0-A_1) / A_0 \times 100)$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Statistical analysis

Tests were carried out in triplicate for 3–5 separate experiments. The amount of extract needed to inhibit free radicals concentration by 50 %, IC_{50}, was graphically estimated using a nonlinear regression algorithm.

Results

DPPH radical scavenging activity of *Nyctanthes arbortristis* flowers

DPPH radical scavenging activity of *Nyctanthes arbortristis* and extract standard as ascorbic acid are presented in Fig 1. The half inhibition concentration (IC_{50}) of *Nyctanthes arbortristis* extract and ascorbic acid were 46.62µg/ml^{-1} and 35.03µg/ml^{-1} respectively**.** The plant extract exhibited a significant dose dependent inhibition of DPPH activity (Table 1).The potential of L-ascorbic acid to scavenge DPPH radical is directly proportional to the concentration. The DPPH assay activity is near to standard as ascorbic acid.

Table 1: DPPH radical scavenging activity of *Nyctanthes arbortristis*

S. No.	Concentration (µg/ml)	*Nyctanthes arbortristis*	Standard (Ascorbic acid)
1	20	20.91 ± 1.46	25.6±2.04
2	40	36.82 ± 2.57	61.26±4.90
3	60	67.73 ± 4.74	88.98±7.11
4	80	90.91 ± 6.36	99.34±7.94
IC_{50}		46.62	35.03

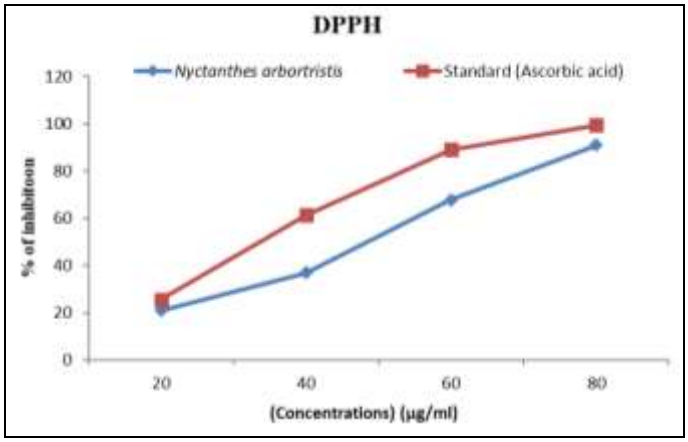

Fig 1: DPPH radical scavenging activity of *Nyctanthes arbortristis*

Superoxide scavenging activity

The superoxide anion radical scavenging activities of the extract from *Nyctanthes arbortristis* assayed by the PMS-NADH system were shown in Fig 2. The superoxide scavenging activity of *Nyctanthes arbortristis* was increased markedly with the increase of concentrations (Table 2). The half inhibition concentration (IC_{50}) of *Nyctanthes arbortristis* was 50.73 µg/ml^{-1} and ascorbic acid was 46.44 µg/ml^{-1} respectively.

Table 2: Superoxide radical scavenging activity of *Nyctanthes arbortristis*

S. No.	Concentration (µg/ml)	*Nyctanthes arbortristis*	Standard (Ascorbic acid)
1	20	18.42 ± 1.49	22.16 ±1.55
2	40	30.57± 2.69	40.25 ± 2.81
3	60	62.42 ± 4.64	64.23 ± 4.49
4	80	85.21 ± 6.17	89.54 ± 6.26
IC_{50}		50.73	46.44

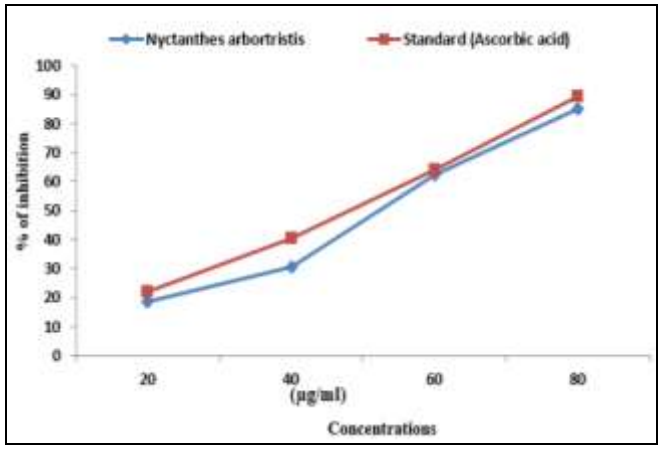

Fig 2: Superoxide radical scavenging activity of *Nyctanthes arbortristis*

Total antioxidant activity

The yield of the ethanol extract of the plant and its total antioxidant capacity are given in Fig. 3. The study reveals that the antioxidant activity of the *Nyctanthes arbortristis* extract is in the increasing trend with the increasing concentration of the plant extract (Table 3). The half inhibition concentration (IC_{50}) of *Nyctanthes arbortristis* extract and ascorbic acid were 45.46 µg/ml^{-1} and 48.78µg/ml^{-1} respectively**.**

Table 3: Total antioxidant activity of *Nyctanthes arbortristis*

S. No.	Concentration (µg/ml)	*Nyctanthes arbortristis*	Standard (Ascorbic acid)
1	20	23.12 ± 1.61	20.70 ±1.50
2	40	38.43± 2.69	34.23 ± 2.24
3	60	68.43 ± 4.79	62.32 ± 4.44
4	80	91.25 ± 6.38	90.25 ± 6.56
IC_{50}		45.46	48.78

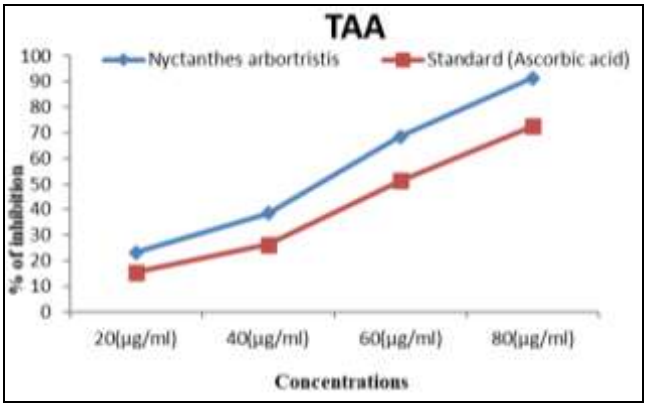

Fig 3: Total antioxidant activity of *Nyctanthes arbortristis*

Discussion

In Vitro* Antioxidant Activity of *Nyctanthes Arbortristis

DPPH assay

1,1- Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical. DPPH is gained its stability as free radical molecules due to the delocalization of odd electron throughout the molecules. This more stabilized DPPH produce intense violet colour in ethanol solution. The antioxidant present in the extracts reacts with DPPH free radical solution and converts them into reduced form either by donating hydrogen atom or transferring electron followed by proton. This oxidation reaction is accompanied with loss of violet colour which can be measured quantitatively at 517 nm (Nuutila *et al.*, 2003) [7]. The plant extract exhibited a significant dose dependent inhibition of DPPH activity. The potential of L-ascorbic acid to scavenge DPPH radical is directly proportional to the concentration. The DPPH assay activity of plant extract is near to standard as ascorbic acid.

Total antioxidant activity

Total antioxidant capacity of *Nyctanthes arbortristis* is expressed as the number of equivalents of ascorbic acid. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/ Mo (V) complex with a maximal absorption at 695 nm. The assay is successfully used to quantify vitamin E in seeds and being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extract (Prieto *et al.*, 1999) [9]. Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The study reveals that the antioxidant activity of the extract is in the increasing trend with the increasing concentration of the plant extract.

Superoxide anion radical scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system (Korycka-Dahl and Richardson, 1978). The superoxide anion radical scavenging activities of the extract from *Nyctanthes arbortristis* assayed by the PMS-NADH system. The superoxide scavenging activity of *Nyctanthes arbortristis* was increased markedly with the increase of concentrations. These results suggested that *Nyctanthes arbortristis* had notably superior superoxide radical scavenging effects.

Summary and Conclusion

Medicinal plants are considered as rich resources of ingredients which can be used in drug development either pharmacopoeial, non- pharmacopoeial or synthetic drugs. A part from that, these plants play a critical role in the development of human cultures around the whole world. Moreover, some plants are considered as important source of nutrition and as a result of that they are recommended for their therapeutic values. Treatment with medicinal plants is considered very safe as there is no or minimal side effects. These remedies are in synchronization with nature, which is the biggest advantage. The golden fact is that, use of herbal treatments is independent of any age groups and the sexes. The medicinal value of the chosen plant *Nyctanthes arbortristis* flower extract has not been extensively worked out. Therefore, the present study was to investigate the phytochemical screening and antioxidants activity of *Nyctanthes arbortristis flower extract*.

In vitro antioxidant activity of *Nyctanthes arbortristis* flower extract was studied. The ethanolic extract was screened for *in vitro* antioxidant activity by oxygen radical scavenging such as DPPH, total antioxidant assay and superoxide metal chelation activity at different concentrations. Throughout the studies flower extract showed marked antioxidant activity. The antioxidant activity was found to be concentration dependent and may be attributed to the presence of bioflavonoids content in the flowers of *Nyctanthes arbortristis.* Over all, the flower extract as a source of natural antioxidants and important in health preservation.

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