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PHYTOCHEMICAL AND *INVITRO* ANTIOXIDANT ACTIVITY OF AQUEOUS EXTRACT OF *NYCTANTHES ARBOR-TRISTIS* (L) FLOWER

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ABSTRACT

Objective: The objective of present work is to study the phytochemical analysis and *in vitro* antioxidant activity of aqueous extract of *Nyctanthes arbor-tristis*(L) flower and to find out the method having highest antioxidant activity. **Materials and methods**: Phytochemical analysis and the in vitro anti-oxidant activity of the aqueous extract were studied using 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, hydrogen peroxide scavenging activity and total antioxidant activity. **Result**: The preliminary phytochemical analysis was done to find out the presence of various bioactive compounds such as flavonoids, glycosides, tannins, terpenoids, saponins, resins, carbohydrate, phlobatannins, antheroquinones. Besides it is also possess strong antioxidant activity. Aqueous extract of flowers was studied for its *invitro* antioxidant activity with different methods viz DPPH, hydrogen peroxide and total antioxidant activity. **Conclusion:** It was concluded that *Nyctanthes arbor-tristis* (L) flower possessed a wide range of pharmacologically important phytoconstituents which exhibited strong antioxidant activity in the method of DPPH radical scavenging activity.

KEYWORDS: Phytochemical, Antioxidant, Aqueous extract.

INTRODUCTION

Natural antioxidants present in the plants scavenge harmful free radicals from our body. Free radical is any species capable of independent existence that contains one or more unpaired electrons which reacts with other molecule by taking or giving electrons, and involved in many pathological conditions.[1] Antioxidants are radical scavenger which protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neurodegeneration, Parkinson's diseases, mongolism, ageing process and perhaps dementias.[2]

Oxidative damage to cellular biomolecules such as lipids, proteins and DNA is thought to play a crucial role in the incidence of several chronic diseases.[3,4] Flavonoids are a group of polyphenolic compounds found abundantly in the plant kingdom. Interest in the possible health benefits of flavonoids and other polyphenolic compounds has increased in recent years owing to their potent antioxidant and free-radical scavenging activities.[5]

Most living species have an efficient defense system to protect themselves against the oxidative stress induced by Reactive Oxygen Species (ROS).[6] Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases including cancer, atherosclerosis and the aging process.[4,7] The antioxidants can interfere with the oxidation process by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers.[8]

MATERIALS AND METHODS

Collection and processing of plant material

The flower of the plant *Nyctanthes arbor-tristis* (L) flower were collected from in and around Mayiladuthurai, Nagai district, Tamil Nadu, India. The sample were washed thoroughly in running tap water to remove soil particles and adhered debris and finally washed with sterile distilled water. The flowers were cut, shade dried, ground into fine powder and stored in air tight polythene bags until use.

Preparation of plant extract

The powdered materials were stored in air tight container until the time of use. 25 grams of this powdered material were soaked in 100 ml of aqueous separately and kept at room temperature for 48 hours and kept at shaker for 3 hours. The sample were filtered and used for phytochemical screening and excess filtrate was filtered

through a single layer of muslin cloth and then final filtrate was collected by passing it through a Whattman grade 1 filter paper in a Buchner funnel under vaccum. The filtrate was evaporated to dryness. The crude extract of *Nyctanthes arbor-tristis*(L) flower was obtained.

PHYTOCHEMICAL SCREENING

The aqueous extract of this plant flower was screened qualitatively for the presence of various phytochemical constituents such as alkaloids, flavonoids, phlobatannins, anthroquinones, Steroids, tannins, phenols, terpenoids, saponins, resins, carbohydrate, protein and amino acids by standard procedure.[9]

Test for alkaloids

0.5 to 0.6 ml of aqueous extract was mixed with 8 ml of 1% HCL,warmed and filtered.2 ml of the filtrate was treated separately with both reagent (Mayer's and Dragendorff's), after which it was observed whether the alkaloids were present in the turbidity or precipitate formation.

Test for flavonoids

0.5ml of aqueous extract was shaken with petroleum ether to remove the fatty materials (lipid layer). The defatted residue was dissolved in 20 ml of 80% ethanol and filtered. The filtrate was used for the following test:3ml of the filtrate was mixed with 4ml of 1% potassium hydroxide in a test tube and the colour was observed. A dark yellow colour indicated the presence of flovonoids.

Test for glycosides

5ml aqueous extract was hydrolysed with 5ml of Conc.HCl and boiled for few hours on a water bath and hydrolysates were subjected to the following test:A small amount of alcoholic extract of sample was dissolved in 1ml water and then aqueous 10% sodium hydroxide was added. Formation of a yellow colour indicated the presence of glycosides.

Test for steroids

0.5ml of the aqueous extract mixed with 2ml of acetic anhydride followed by 2ml of sulphuric acid. The colour changed from violet to blue or green in sample indicated the presence of steroids.

Test for tannins

2.5ml of aqueous extract was dissolved in 10 ml distilled water and filtrate.1% aqueous ferric chloride ($FeCI_3$) solution was added to the filtrate. The appearance of intense green, purple, blue or blank colour indicated the presence of tannin.

Test for phenols

To 1ml of aqueous extract of sample, 2ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution was added. Formation of blue or green colour indicated the presence of phenols.

Test for terpenoids

5 ml of aqueous extract was mixed with 2 ml of chloroform followed by the careful addition of 3 ml Conc.H_2SO_4. A layer of the reddish brown colouration was formed at the interface thus indicating a positive result for the presence of terpenoids.

Test for saponins

To 1ml of aqueous extract was diluted to 5ml of water and the tubes were shaken vigorously, formation of 1 cm layer of foam indicate the presence of saponins.

Test for resins

To 1ml of aqueous extract was treated with few drops of acetic anhydride solution followed by 1ml of concentrated sulphuric acid. Resins give colouration ranging from orange to yellow.

Test for carbohydrate

Aqueous extract (1 ml) was added to 1 ml of water and 20 drops of boiling Fehling's solution (A and B) in a test tube was added. The formation of a precipitate red-brick in the bottom of the tube indicates the presence of carbohydrate.

Test for protein and amino acid

To 1 ml of the aqueous extract was treated with few drops of ninhydrin reagent. Appearance of purple colour shows the presence of amino acids.

Test for phlobatannins

When aqueous extract was boiled with 2% aqueous HC1.The deposition of a red precipitate was taken as avidence for the presence of phlobatannins.

Test for anthroquinones

5 ml of aqueous extract solution was hydrolysed with diluted Conc.H_2SO_4 extracted with benzene. 1 ml of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones. Acetone and ethanolic extracts also screened in the same procedure.

***In-vitro* anti-oxidant activity**

DPPH radical scavenging activity

The ability of the plant extract to scavenge 1,1-diphenyl-2-picryhydrazyl (DPPH) free radicals was assessed by the standard method.[10] The stock solution of extracts were prepared in aqueous to achieve the concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 50, 100, 150, 200 µg/ml. Diluted solutions (1 ml each) were mixed with 3 ml of ethanolic solution of DPPH (DPPH, 0.004%). After 30 min of incubation at room temperature the reduction of the DPPH free radical was measured by reading the absorbance at 517nm using UV-

Visible Spectrophotometer. Initially, absorption of blank sample containing the same amount of ethanol and DPPH solution was prepared and measured as control. Ascorbic acid was used as standard. The experiment was

carried out in triplicate. Percentage inhibition was calculated using equation (1), whilst IC50 values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data were presented as mean values ± standard deviation (n = 3).

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{Absorbance of control}} \times 100 \quad \text{equation (1)}$$

Hydrogen peroxide scavenging activity

Scavenging activity of Hydrogen peroxide (H_2O_2) by the plant extract was determined by the method.[11] Plant extract (4 ml) prepared in distilled water at various concentration(50, 100, 150, 200 µg/ml) was mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm. Ascorbic acid was used as a positive control compound. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples using Eq. (1). IC50 values were estimated from the percentage of inhibition versus concentration plot, using a non-linear regression algorithm.

$$\% \text{ inhibition} = \left(1 - \frac{(\text{Absorbance of the presence of the sample of extract and standard})}{\text{Absorbance of the control}}\right) \times 100$$

Total antioxidant activity

For total antioxidant activity assay[12] various concentrations of the substrate dissolved in water were combined in an eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as the standard and the total antioxidant activity is expressed as equivalents of ascorbic acid.

$$A = (c \times V)/m$$

A=Total content of antioxidant compounds, mg/g plant extract, in ascorbic acid equivalent,C=The concentration of Ascorbic acid established from the calibration curve,mg/ml, V=The volume of extract (ml), and m=the weight of crude plant extract(g).

RESULTS AND DISCUSSION

Recent years have seen an exponential increase in research antioxidant properties of medicinal plants. If it is accepted that higher intakes of plant phenolics are associated with long-term health benefits, then the results presented in this paper offer possible avenues toward health promotion by identifying those compounds. The identification and investigation on antioxidants from medicinal plants is a fast expanding field of research and several antioxidants have been investigated such as flavonoids and other phenolic compounds. As given in the introduction, the link between degenerative diseases and antioxidants of plant origin has been recognized, leading to the production of natural antioxidants/dietary supplements. Antioxidant quality is measure of the effectiveness of the antioxidant(s) present as a pure compound or a mixture.[13]

TABLE 1: Phytochemical analysis of aqueous extract of *Nycthathes arbor-tristis*(L) flower

Phytochemical test	Aqueous extract	
	Observation	**Inference**
Carbohydrate	Red color	+++
Tannins test	Greenish black color	+++
Saponin test	Presence of foam	+++
Flavonoid test	Yellow color	+++
Glycosides	Yellow color	+++
Terpenoids test	Red brown color	++
Phenols	Green Color	+++
Alkaloids	White turbidity	++
Resins	Orange to yellow color	+
Antheroquinones	Rose pink color	+++
Phlobatanoins	Precipitate formed	++

(+)=Indicate presences, (-)=Indicate absences

PHYTOCHEMICAL COMPOUNDS

The aqueous extract of *Nyctanthes arbor-tristis*(L) flower was screened for the presence of various bioactive phytochemical compounds. Specific qualitative tests were performed to identify bioactive compounds of pharmacological importance through standard methods. The analysis revealed the presence of carbohydrates, tannins, saponin, flavonoid, glycosides, antheroquinones, in most prominent amount while phenols, terpenoids, phlobatanoins, alkaloids and resins in less amount in aqueous extract of *Nyctanthes aebor-tristis*(L) flower.[14]

Tannins

Tannins and phenols, which together constitute the polyphenolic group, are known to have antioxidant, anticancer and antimicrobial activities. [15] The secondary metabolites present in *Nyctanthes arbor-tristis*(L) flower are known to be biologically active. Tannins have found to form irreversible complexes with proline rich proteins, resulting in the inhibition of the cell protein synthesis. This activity was exhibited against test organisms with all the plant extract. Tannins have important roles such

as stable and potent antioxidant[16], astringent and for treating diarrhea and dysentery.

Saponins

Saponins have been covered to show hypercholesterolemia and tumor inhibiting activity in experimental animals.[17] The presence of saponins in plants have been reported to be responsible for the tonic and stimulating activities observed in Chinese and Japanese medical herbs.[18] The studied bioactive compounds have a broad range of biological activities. For example, phytochemicals such as saponins have anti-inflammatory effects[19], hemolytic activity, and cholesterol binding properties.[20]

Flavonoids

Flavonoids have been demonstrated to have antibacterial, anti-inflammatory, anti-allergic, anti-viral activity.[21] Flavonoids are phenolic structures containing one carbonyl group complexes with extra-cellular and soluble protein and with bacterial cell wall. Thus, exhibits antibacterial activity.[22]

Earlier reports revealed that plant phenolic compounds including flavonoids are potent antioxidants with reported antimutageneic and anticarcinogenic effects.[23]

Flavonoids and tannins are phenolic compounds and plantphenolics are a major group of compounds that act as primary antioxidants or free radical scavengers. Since these compounds were found to be present in the extracts, it might be responsible for the potent antioxidant capacity of pomegranate. The secondary metabolites (phytochemicals) and other chemical constituents of medicinal plants account for their medicinal value. Flavonoids exhibited a wide range of biological activities such as antioxidant, anti-inflammatory, antimicrobial, anti-angionic, anticancer and anti-alergic.[24]

Glycosides

Glycosides are known to lower blood pressure[25] and tannins exhibit antioxidant, antimicrobial and antiviral effects.[26]

Glycosides are naturally cardioactive drugs used in the treatment of congestive heart failure and cardiac arrhythmia.[27] Glycosides have been known to lower blood pressure, although some workers have attributed the cardiac action of these oils to the presence of the alkaloid, carpaine[21] had earlier shown aqueous extracts from *A. cissampeloides* to have antihypertensive effect on blood pressure and serum analyses of hypertensive patients. This effect could be attributed to the presence of steroidal nucleus and deoxy-sugar both of which are present in glycosides.

Terpenoids

The presence of higher terpenoids that have carboxylic acid groups could also be responsible for the activity of the organic extracts.

Phenols

Phenolic compound are one of the most important groups of secondary metabolites. Structural chemistry of phenolics is quint essential for free radical scavenging as it possesses at least one aromatic ring (C6) bearing one or more hydroxyl groups. In general phenolic compounds act as potential metal chelators as well as inhibit lipid per-oxidation by quenching free radicals via forming resonance-stabilized phenoxyl radicals. Among the multifarious phenolic compounds, flavonoids are probably the most important class. Phenolic acids are the most commonly occurring natural products noted for allelopathic activities.[28,29]

Alkaloids

Alkaloids often have pharmacological effects and are used as medication and recreational drugs.[30] The alkaloids the largest groups of chemical produced by plants have many biological activities. Several workers have reported on the analgesic properties of alkaloids.[31] The alkaloids contained in plants are used in medicine as anaesthetic agents.[32] Alkaloids that have been reported to exert analgesic, antispasmodic and antibacterial activities.

Steroids

The plant extracts were also revealed to contain steroids, which are known to produce an inhibitory effect on inflammation.[33] The presence of some of these compounds have been confirmed to have antimicrobial activity[34], hence it could be inferred that the plant extracts could be a source for the industrial manufacture of drugs useful in the chemotherapy of some microbial infections.[35]

TABLE 2: *Invitro* antioxidant activity of *Nyctanthes arbor-tristis*(L) flower

S.no	Concentration	Ascorbic acid (control)	Aqueous extract of *Nyctanthes arbor-tristis*(L) flower by DPPH assay.	Aqueous extract of *Nyctanthes arbor-tristis*(L) flower by total antioxidant assay.	Aqueous extract of *Nyctanthes arbor-tristis*(L) flower by hydrogen peroxide activity.
1.	50	16.4±0.2	24.5±2.0	19.6±0.9	17.6±0.5
2.	100	17.4±1.0	29.2±1.5	22.6±0.5	18.4±1.1
3.	150	22.3±0.3	33.6±0.6	26.5±0.9	23.6±0.5
4.	200	26.6±2.3	37.6±0.62	34.1±3.2	27.3±0.8

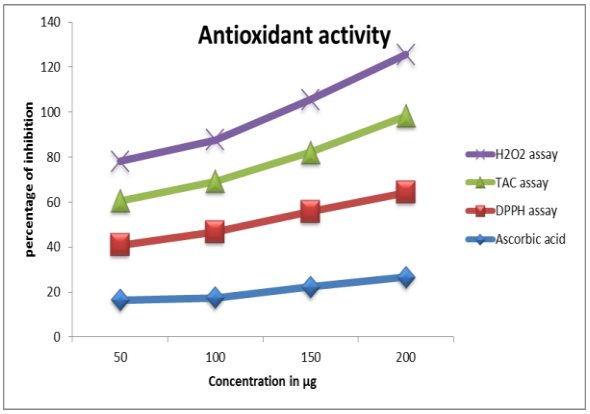

Figure1: *Invitro* Antioxidant Activity Of *Nyctanthes arbor-tristis*(L) flower

***Invitro* antioxidant activity**

In-vitro antioxidant studies are widely carried to screen various plant containing phenolic and flavanoids constituents. Plant derived antioxidant compounds, flavonoids and phenolics have received considerable attention because of their physiological effect like antioxidant, anti-inflammatory, antitumor activities and low toxicity compared with those of synthetic phenolics antioxidant such as BHA (Butylated Hydroxyanisole), BHT (Butylated Hydroxytoluene) and Propyl Gallate(PG).[36]

There was a close correlation between the antioxidant activity and the amount of polyphenols, flavonoids, and flavonols present in the plant. Total polyphenols play a vital role in anti-oxidization as well as in the biological functions of the plant.[37] Other studies have also indicated that the anti-oxidative properties of polyphenols in edible plants and plant products may help prevent diseases.[38]

The phenolic and flavanoids are widely distributed secondary metabolites in plants having anti-oxidant activity and have wide range of biological activities as anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities. Recent studies have shown that many dietary polyphenolic constituents derived from plants are more effective antioxidants *in-vitro* than vitamins E or C, and thus might contribute significantly to the protective effects *in vivo*.[39]

DPPH free radical scavenging activity

The aqueous extract of *Nyctanthes arbor-tristis*(L) flower of showed maximum activity of 33.6±0.6 and 37.6±0.62 respectively at 150 and 200 µg/ml were as ascorbic acid at the same concentration exhibited 22.3±0.3 and 26.6±2.3 inhibition respectively. This result indicated that extract has a noticeable effect on scavenging the free radicals. However, a maximum inhibition was achieved at a higher concentration of 200 µg/ml compared to 200 µg/ml for both of aqueous extract of *Nyctanthes arbor-tristis*(L) flower and ascorbic acid.

DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts. The *E.indica* root extract demonstrated H-donor activity. The DPPH radical scavenging activity 61.15% was detected and compared with ascorbic acid 55.67%.

DPPH is long-lived nitrogen radical. Antioxidants react quickly with DPPH and tend to decrease its oxidation ability. The natural antioxidants might directly react with or quench the stable cation radical, which is reflected as their antioxidant activity. The excellent reducing power of the sample may be due to the hydrogen donating abilities of the active constituents. More antioxidant activity in herbs comes from the ingredients other than antioxidant vitamins, indicating the presence of other potentially important antioxidants. The phenolic compounds are dominant antioxidants distributed widely in the plant kingdom that exhibit scavenging efficiency on free radicals and reactive oxygen species.[40]

In free radical scavenging activity, DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become stable diamagnetic molecule. The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517 nm, which is induced by different antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants because of the reaction between antioxidant molecules and radical progress which results in the scavenging of the radical by hydrogen donation.[41]

Antioxidant behavior can be assessed either by activity in foods or bioactivity in humans. Antioxidant activity cannot be measured directly but rather by the effects of the antioxidant in controlling the extent of oxidation. DPPH radical bleaching is one of the methods used to evaluate the antioxidant properties of phytoconstituents and is based on the capacity of herbal extract to bleach the DPPH radical, a nitrogen-centred free radical.[42]

DPPH is a purple colored stable free radical; when reduced it becomes the yellow-colored diphenyl-picryl hydrazine. DPPH radicals react with suitable reducing agents and then electrons become paired-off and the solution loses colour stoichimetrically with the number of electrons taken up.[43] Such reactivity has been widely used to test the ability of compounds/plant extracts to act as free radical scavengers.[44]

Chloroform extract of *Citrullus lanatus* and ethanol extract of *Citrullus lanatus* seeds were detected and compared with ascorbic acid. The IC_{50} values for DPPH assay of for hexane extract was found maximum followed by ethanol extract and for chloroform extract was minimum. Though the extracts showed good DPPH scavenging activity but it was less effective than standard ascorbic acid. The difference of activity is due to

presence of phenolic components in different extracts. Thus, choosing the appropriate solvent is one of the most important factors for obtaining extracts with a high content of bioactive compounds and antioxidant activity.[45]

The DPPH assay constitutes a quick and low cost method, which has frequently been used for the evaluation of the antioxidative potential of various natural products.[46]

Hydrogen peroxide scavenging activity

Scavenging of hydrogen peroxide of different concentration of aqueous extract *Nyctanthes arbor-tristis*(L) flower is presented in figure:1. The percentage of H_2O_2 scavenging activity of aqueous extract was found to be 27.3±0.8 which is highest among the concentration of 200 µg/ml compared to antioxidant activity of strandard ascorbic acid.

Hydrogen peroxide, although not a radical species play a role to contribute oxidative stress. The generation of even low levels of H_2O_2 in biological systems may be important. Naturally-occurring iron complexes inside the cell believed to react with H_2O_2 *in vivo* to generate highly reactive hydroxyl radicals and this may be the origin of many of its toxic effects.[47]

Hydrogen peroxide itself is not very reactive, but can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells.[48] Thus, removal of H_2O_2 is very important for protection of food systems. Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner.[49]

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzyme directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly, and inside the cell, H_2O_2 probably reacts with $Fe^{2+,}$ and Cu^{2+} ions to form hydroxyl radical which may be the origin of many of its toxic effects.[50]

It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. *N. arbor*(L) flowers scavenged H_2O_2 and this may be attributed to the presence of phenols, which could donate electrons there by neutralizing it into water. It was observed that methanol extracts of *N. arbor*(L) flowers exhibit slight high inhibition than ethanol and aqueous extracts ranged from 60.75 to 50.69% inhibition and also seen that fresh and dry flowers do not show much significant difference in inhibition of H_2O_2. All tested extracts can inhibit H_2O_2 but lesser when compared to reference standards ascorbic acid and BHT. Similar results were reported in *Carissa carandas* and *Pergularia daemia* root extracts.[51]

The measurement of H_2O_2 scavenging activity is one of the useful methods of determining the ability of antioxidants to decrease the level of pro-oxidants such as H_2O_2.[52] It can cross membranes and may slowly oxidize a number of compounds. Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of rise in the hydroxyl radicals in the cells.[53]

Total antioxidant activity

The total antioxidant activity of the aqueous extract of *N. arbor*(L) flowers is given in table-2. The results of total antioxidant activity are expressed as equivalents of ascorbic acid. Antioxidant activity of aqueous extract of *Nyctanthes arbor-tristis*(L) flower was performed at different concentration ranging from 50-200 µg/ml. The percentage of H_2O_2 scavenging activity of aqueous extract was found to be 34.1±3.2 which is highest among the concentration of 200 µg/ml compared to antioxidant activity of strandard ascorbic acid.

CONCLUSION

We can conclude that the selected aqueous extract was showing many secondary metabolites are present. The screening of phytochemical constituents of *Nyctanthes arbor- tristis*(L) flower extract indicated the presence of flovonoids, glycosides, tannins, terpenoids, saponins, resins, carbohydrate, phlobatanoins, antheroquinones.

Based on the results described, it may be concluded that the aqueous extract shown high significant activity in DPPH radical scavencing assay compared with hydrogen peroxide activity and total antioxidant activity. The anti-oxidant potential of aqueous extract may be attributed to the presence of saponins and flavonoids.

The result of this study show that aqueous extract of *Nyctanthes arbor-tristis*(L) flower can be used as easily accessible source of natural antioxidant and as a possible food supplement or in pharmaceutical industry.

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