# CHAPTER 4

## *Protective function of Phlogacanthus tubiflorus and Nyctanthes arbor-tristis against Oxidative stress induced by $H_2O_2$*

## 4.1 Background

Oxidative stress causes the production of free radicals and reactive oxygen species (ROS) in the biological system. [222] The excessive production of such reactive species results in imbalance in between oxidants and antioxidants that leads to tissue injuries and contribute to the progression of the several degenerative diseases in humans, such as coronary heart disease, cataracts, muscle degeneration, ageing and cancer. [223-227]

Hydrogen peroxide ($H_2O_2$) is weak oxidizing agent that is produced as a bye product during normal oxidative metabolism and low level is necessary for cellular maintenance and survivability. [228,229] Under certain pathophysiological circumstance, the level increases and generates hydroxyl radicals ($OH^*$) which subsequently cause damage to the cells by interacting with micro and macro molecules and alter intracellular redox states. [230] Increased $H_2O_2$ levels also change the homeostasis of ions such as calcium and iron and change the mitochondrial membrane potential, which may result in cytochrome C release from the mitochondria into the cytosol. [231-234] Hydroxyl radicals are also known to induce apoptosis and cell death. [228]

The maintenance of optimum levels of $H_2O_2$ is mandatory in order to manage a healthy cellular environment. Several naturally occurring compounds including curcumin, ascorbic acid as well as synthetic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) have been tested for their $H_2O_2$ scavenging activity. However, the optimum level for redox maintenance and subsequent cellular protection remains a challenge. [235-237] Therefore, in the present study attempt has been made to investigate the protective function of medicinal plants selected on the basis of traditional and indigenous knowledge from the north eastern region of India. The selected medicinal plants *viz. Nyctanthes arbor-tristis* Linn. and *Phlogacanthus tubiflorus* Nees. are well known for their use in traditional system of medicine. Here, protective function of the extract of the selected plants were evaluated against oxidative stress induced by $H_2O_2$ using isolated chicken lymphocytes as model.

## 4.2 Material and methods

### 4.2.1 Chemicals and reagents

Histopaque 1077, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), tricholoroacetic acid (TCA), 2-thiobarbituric acid (TBA), reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide (NADH), 1-chloro-2,4-dinitrobenzene (CDNB), sodium pyruvate and epicatechin were obtained from Sigma Chemical Co. (St Louis, MO, USA). RPMI-1640, fetal bovine serum (FBS), sulfanilamide, *N*-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD), etc. were purchased from HiMedia Laboratories (Mumbai, India). The rest of the chemicals like Dimethylsulfoxide (DMSO), bovine serum albumin (BSA), folin-ciocalteu reagent (FCR) etc. were of analytical grade, obtained from local firms of India.

### 4.2.2 Preparation of extract

The freshly collected roots of *Phlogacanthus tubiflorus* Nees. and flowers of *Nyctanthes arbor-tristis* Linn. were washed with running tape water repeatedly, and finally with distilled water to remove impurities and blotted dry. The plant materials were dried in shade, ground into powdered form and macerated thrice with hydroalcohol (80% ethanol) in a shaking condition for 7 days at room temperature. The roots of *Phlogacanthus tubiflorus* (REPT) and flower extract of *Nyctanthes arbor-tristis* (FENA) thus obtained was filtered, dried to remove the solvents and finally powder form of the extracts was stored at -20°C.

### 4.2.3 Evaluation of protective function of REPT and FENA

The REPT and FENA were evaluated for their protective function against oxidative stress induced by hydrogen peroxide ($H_2O_2$) using isolated chicken lymphocytes, cultured *in vitro*. The protocol was approved by Tezpur University Ethical Committee (TUEC). [Annexure III] The protective function from oxidative stress was measured in terms of cell viability, lactate dehydrogenase (LDH) activity and lipid peroxidation (LP) levels. Also level of cellular reduced glutathione (GSH) and free radical nitric oxide (NO) and activity of Glutathione-*S*-transferase (GST) were assayed to correlate the protective functions.

#### 4.2.3.1 Isolation of lymphocytes

The lymphocytes were isolated from chicken blood by using Histopaque (1.077 g/ml) according to manufacturer protocol. Anticoagulated blood collected from butcher shop was diluted 1:1 with phosphate buffer saline (PBS) (pH 7.4) and layered 6 ml into 6 ml Histopaque, centrifuged for 30 minutes at 400 g and middle buffy layers containing the lymphocytes were collected. The isolated lymphocytes were washed with PBS and RPMI separately through centrifugation at 250 g for 10 minutes. Finally cell pellets were suspended in RPMI-1640 and cell viability was checked by trypan blue exclusion method using haemocytometer. Only the batches of isolation that have viable cells more than 90 % were used for subsequent studies.

#### 4.2.3.2 Lymphocyte culture, treatment and preparation of cell lysate

Isolated lymphocytes (100 or 200 µl) were seeded in 96 well culture plates in RPMI supplemented with 10% heat inactivated fetal bovine serum. The lymphocytes were treated with REPT/FENA dissolved in DMSO or $H_2O_2$ or REPT/FENA + $H_2O_2$ as per experimental requirements and maintained at 37°C and 5% $CO_2$ in an incubator. Lymphocytes were treated for 4 hours in case of only $H_2O_2$ and REPT/FENA treatment while in other cases pretreated with REPT/FENA for 1 hour and then treated with $H_2O_2$ for 4 hours.

After incubation, lymphocytes were centrifuged and washed with 1X PBS, homogenized in PBS by vigorous syringing. Supernatant of the cell lysate was used for assaying GST, GSH, protein while LP was measured in the cell pellet. Cell free culture media was used for assaying LDH. For MTT assay, lymphocytes were treated separately in 96 well culture plates.

#### 4.2.3.3 MTT based cell viability assay

Cell viability was assayed by the MTT based method as described by method of Denizot and Lang. [238] Briefly, after treatments, cells were treated with 10% of MTT for 2 hours and formazan crystals formed were dissolved in solvent as per manufacturer protocol and absorbance was measured at 570 nm. The backgroundabsorbance was measured at 690 nm. The absorbance of control cells was set as 100% viable and the values of treated cells were calculated as percentage of control.

#### 4.2.3.4 Estimation of Lactate dehydrogenase (EC 1.1.1.27)

The specific activity of Lactate dehydrogenase (LDH) was assayed by measuring the rate of oxidation of NADH at 340 nm according to the method of Bergmeyer and Bernt (1974). [239] The reaction was started at $25^0$C by adding 0.1 mM NADH to the reaction mixture consist of 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM sodium pyruvate and cell free media. The oxidation of NADH was monitored for 3 minutes and the activity was calculated using extinction coefficient 6.22 $mM^{-1}cm^{-1}$. One unit of enzyme activity is defined as that which causes the oxidation of one mmole of NADH per minute.

#### 4.2.3.5 Estimation of Lipid peroxidation

The Lipid peroxidation (LP) level was measured in the pelleted fraction of cell lysate by thiobarbituric acid reactive substances (TBARS) method, as described by Varshney and Kale (1990) and is expressed in terms of malondialdehyde (MDA) formed per mg protein. [240] In brief, pelleted cell lysate was mixed with 0.15 M Tris KCl buffer to which 30% TCA was added. Then 52 mM TBA was added and placed in a water bath for 45 minutes at 80°C, cooled in ice and centrifuged at room temperature or 10 min at 3,000 rpm. The absorbance of the clear supernatant was measured against blank of distilled water at 538.1 nm in spectrophotometer.

#### 4.2.3.6 Estimation of Glutathione-*S*-transferase (GST) (EC 2.5.1.18)

The activity of Glutathione-*S*-transferase (GST) was determined in the cytosolic fraction of cell lyasate spectrophotometricaly at 37°C according to the procedure of Habig *et al.*, (1974). [241] The reaction mixture consist of 100 mM phosphate buffer (pH 6.5), 1 mM CDNB and 1 mM of GSH, was pre-incubated at 37°C for 2 minutes and then reaction was started by addition of diluted cytosol and the absorbance was measured for 3 minutes at 340 nm. The specific activity of glutathione-S-transferase is expressed as µmoles of GSH-CDNB conjugate formed/min/mg protein using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

#### 4.2.3.7 Estimation of reduced glutathione

The level of reduced glutathione (GSH) was estimated as an acid soluble non-protein sulfhydryl (-SH) group by the method of Moron *et al.*, (1979). [242] Homogenates of cells were immediately precipitated with TCA (5%) and the precipitate was removed after centrifugation. Free -SH groups were assayed by reacting the supernatant with 0.6 mM DTNB prepared in 0.2 M sodium phosphate buffer (pH 8.0) and absorbance was read at 412 nm. The level of commercially available reduced glutathione was used as a standard to calculate nmole of -SH content/mg protein.

#### 4.2.3.8 Estimation of Nitric oxide (NO)

The level of Nitric oxide (NO) was estimated in the cell lysate by the method of Griess (1879) with some modifications. [243] Briefly, equal volume of cytosol and Griess reagent (1% sulfanilamide and 0.1% NEDD in 5% phosphoric acid) were added and after 5 minutes, absorbance was read at 550 nm. The nitrite content was calculated from the standard curve made with sodium nitrite and expressed as µM/mg protein.

#### 4.2.3.9 Estimation of protein

Cytosolic and pellet protein content was determined following the method of Lowry *et al.*, (1951) using Bovine serum albumin (BSA) as standard, at 660nm. [244]

#### 4.2.3.10 Preliminary phytochemical screening

The extract was subjected to phytochemical screening for the detection of polyphenols and flavonoids according to the standard procedure. [245,246]

#### 4.2.3.11 Statistical analysis

All the data are expressed as means ± sem, n=3. The significance differences between the experimental and the control groups were analyzed by student's t test and three levels of significance were set as $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

### 4.3 Results

The protective function of REPT and FENA has been evaluated against oxidative stress induced by $H_2O_2$. The findings has been represented in Figure 15 and 16.

### 4.3.1 Effects of oxidative stress induced by $H_2O_2$

Exposure of lymphocytes to increasing concentration of $H_2O_2$ (0.1 and 1%) for 4 hours caused significant dose dependent decrease in the cell viability upto 64% ($p \leq 0.05$–$0.001$), and increase in the level of LDH activity upto 281% ($p \leq 0.01$–$0.001$). For 1.0% of $H_2O_2$ treatment cell viability declined below 50% ($p \leq 0.001$). There were also evidence of significant increase in the level of LP (upto 121-158%, $p \leq 0.05$–$0.001$) and NO level (upto 111-165%, $p \leq 0.001$) and activity of GST (143%, ($p \leq 0.01$). Treatment of lymphocytes with 1.0% $H_2O_2$ exhibit significant decrease in the level of GSH by 54% ($p \leq 0.001$) as compared to control [Figure 14].

### 4.3.2 Modulatory effect of REPT

Lymphocytes treated with different concentration of REPT for 4 hours exhibited increase in the viability of cells and for 200 µg/ml of REPT treatment cell viability increased significantly to 121% ($p \leq 0.01$). The treatment of lymphocytes with REPT resulted in dose dependent decrease in the activity of LDH and level of LP by 9-37% and 1-33% respectively. The decrease in the activity of LDH was significant in 100 and 200 µg/ml of REPT treatment ($p \leq 0.001$), while the level of LP at 200 µg/ml of REPT treatment ($p \leq 0.01$). For the similar treatment condition, the activity of GST was observed to be decrease and significant decline was observed in 100 and 200 µg/ml of REPT treatment condition by19-27% ($p \leq 0.05$-$0.01$). In contrast to the activity of GST, the level of GSH was found to be significantly increased in dose dependent manner upto 20-40% ($p \leq 0.05$-$0.01$) [Figure 14].

### 4.3.3 Protective function of REPT

Pre-treatment of lymphocytes with REPT for 1 hour restored the viability of cells against 0.1% and 1% $H_2O_2$ treatment. The viability of cells significantly increased to 108-116% ($p \leq 0.05$-$0.01$) and 62-105% ($p \leq 0.05$) respectively as compared to only 0.1 (92%, $p \leq 0.05$) and 1% $H_2O_2$ (36%, $p \leq 0.001$) treatment. The activity of LDH was found to be significantly restored by 52-103% ($p \leq 0.01$-$0.001$) and 119-158% ($p \leq 0.001$) as compared to only 0.1 and 1% $H_2O_2$ respectively. Pre-treatment of lymphocytes with 100 and 200 µg/ml REPT significantly restored the level of LP upto 67-97% ($p \leq 0.01$-$0.001$) and 197-213% ($p \leq 0.01$) when compared to only 0.1 (121%, $p \leq 0.05$) and 1%

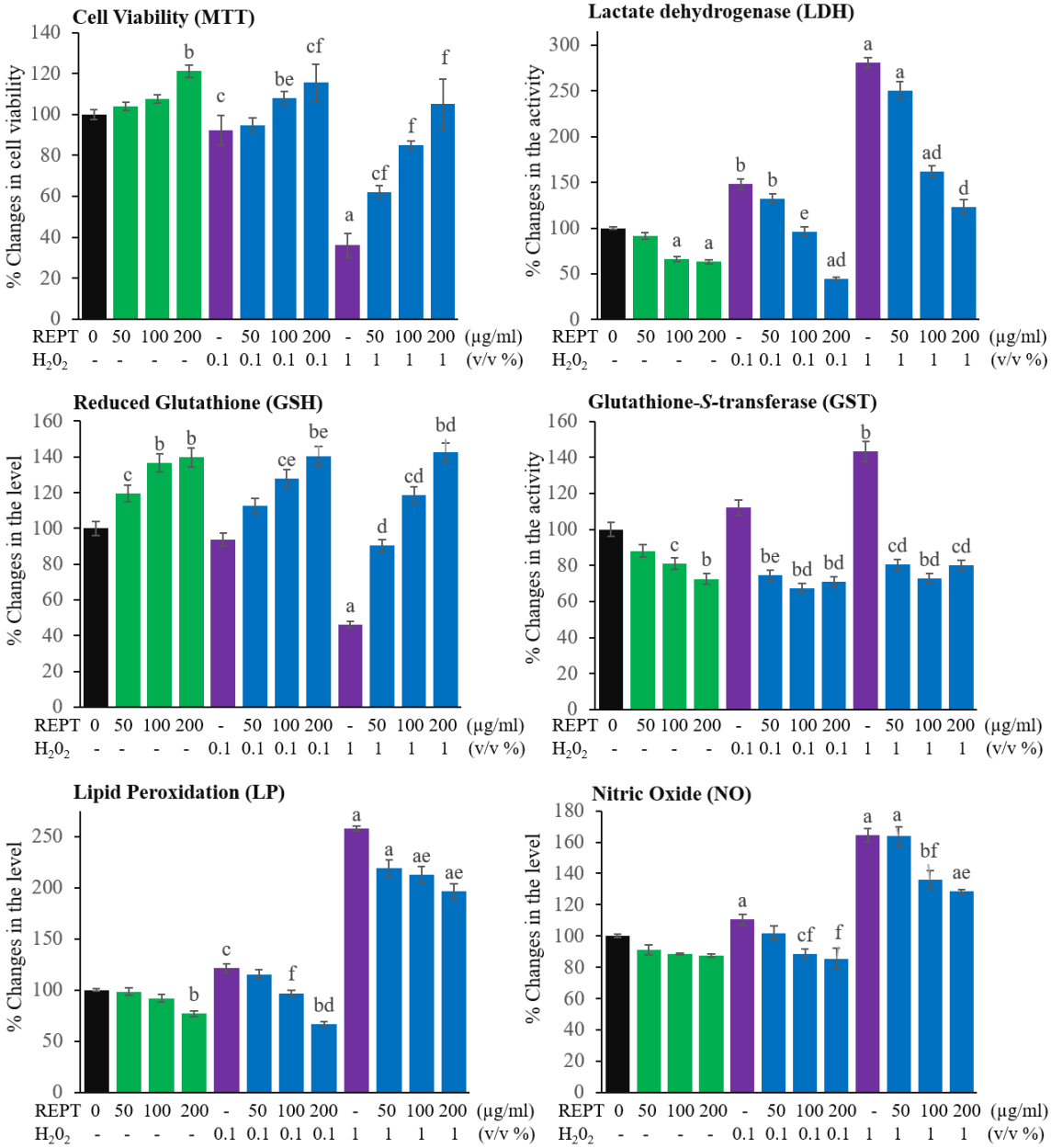


**Figure 14: Protective effect of the REPT against oxidative stress induced by $H_2O_2$ :** Lymphocytes were treated with the indicated concentrations of REPT (4 hours) / $H_2O_2$ (4 hours) / REPT (1 hour) + $H_2O_2$ (4 hours) and the activity of GST, LDH and level of GSH, NO, LP along with viability of lymphocytes were measured by spectrophotometric method as described in 'Material and methods'. The activities/levels are expressed as % changes as compared to control. Values are represented as mean ± sem; n=3; $^{a}p\leq0.001$ compared to control cells; $^{b}p\leq0.01$ compared to control cells; $^{c}p\leq0.05$ compared to control cells; $^{d}p\leq0.001$ compared to cells treated with only $H_2O_2$; $^{e}p\leq0.01$ compared to cells treated with only $H_2O_2$; $^{f}p\leq0.05$ compared to cells treated with only $H_2O_2$.

$H_2O_2$ (257%, p≤0.001) respectively. For the similar treatment condition, significant decline in the level of NO by 22-25% (p≤0.05) and 28-36% (p≤0.05-0.01) were also observed as compared to only 0.1 and 1% $H_2O_2$ respectively. The level of GSH was observed to be significantly increased when lymphocytes were pretreated with REPT for 1 hour and the increased level reached 128-141% (p≤0.05) and 90-143% (p≤0.001) as compared to only 0.1 (94%) and 1% $H_2O_2$ (46%, p≤0.001) treatment respectively. There were also evidence of significant decline in the activity of GST by 37-45% (p≤0.01-0.001) and 63-71% (p≤0.001) as compared to only 0.1 and 1% $H_2O_2$ treatment respectively [Figure 14].

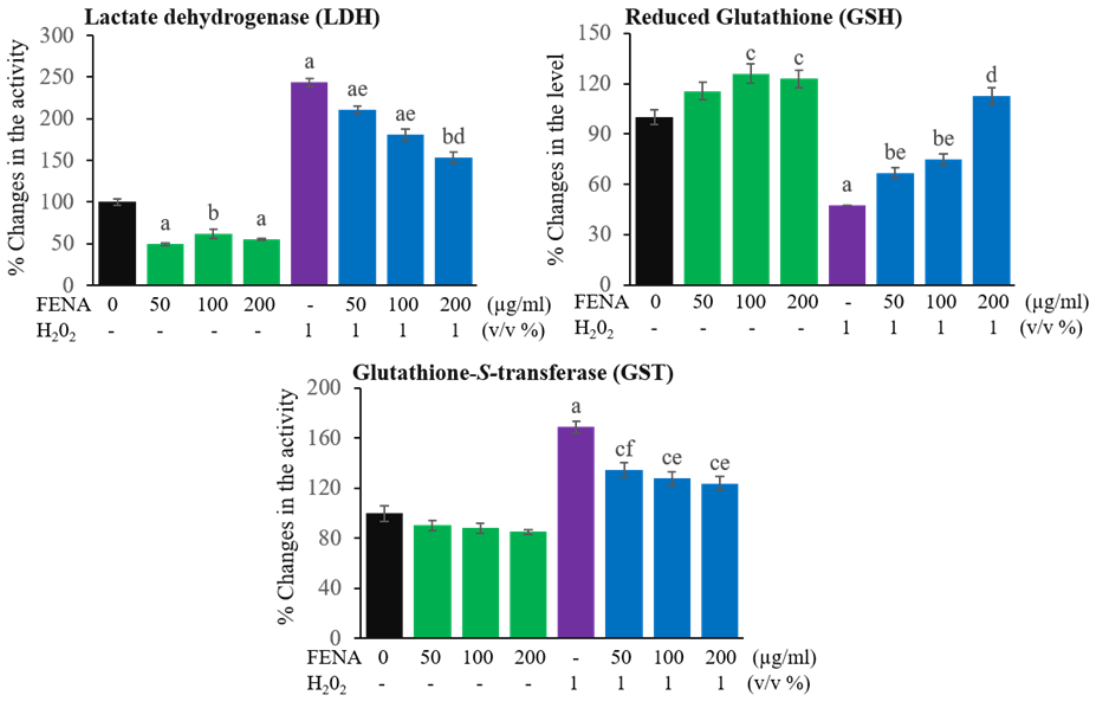


**Figure 15: Protective effect of the FENA against oxidative stress induced by $H_2O_2$:** Lymphocytes were treated with the indicated concentrations of FENA (4 hours) / $H_20_2$ (4 hours) / FENA (1 hour) + $H_20_2$ (4 hours) and the activity of GST, LDH and level of GSH were measured by spectrophotometric method as described in 'Material and methods'. The activities/levels are expressed as % changes as compared to control. Values are represented as mean ± sem; n=3; [a]p≤0.001 compared to control cells; [b]p≤0.01 compared to control cells; [c]p≤0.05 compared to control cells; [d]p≤0.001 compared to cells treated with only $H_2O_2$; [e]p≤0.01 compared to cells treated with only $H_2O_2$; [f]p≤0.05 compared to cells treated with only $H_2O_2$.

### 4.3.4 Modulatory effect of FENA

In the present study, the treatment of lymphocytes with FENA (50, 100 and 200 µg/ml) for 4 hours caused significant decrease in the activity of LDH upto 49-62% ($p \leq 0.01$-0.001). For 50 µg/ml of FENA treatment, the activity of LDH declined below 50% ($p \leq 0.001$).The level of GSH was observed to be increased upto 123-126% ($p \leq 0.05$) as compared to untreated lymphocytes. In contrast to GSH, the specific activity of GST declined by 10-15%; however, the decline was non-significant [Figure 15].

### 4.3.5 Protective function of FENA

Pre-treatment of lymphocytes with FENA (50, 100, and 200 µg/ml) for 1 hour significantly restored the increased activity of LDH to 153-211% ($p \leq 0.01$-0.001) as compared to only $H_20_2$ (244%, $p \leq 0.001$) treated lymphocytes. However, the activity of LDH at all the FENA treated condition lies above the untreated lymphocytes. Significant dose dependent restoration in the level of GSH upto 67-113% ($p \leq 0.01$-0.001). As expected, the specific activity of GST decreased significantly by 34-45% ($p \leq 0.05$-0.01) as compared to the cells treated with only $H_2O_2$ (169%, $p \leq 0.001$). The restored activity of GST at all the FENA pretreated condition was found to be above the untreated lymphocytes [Figure 15].

### 4.3.6 Preliminary phytochemical screening

Qualitative screening of the plant extract revel the presence of polyphenols and flavonoids in the root and flower extract used in this study.

## 4.4 Discussion and conclusion

The present study explored the protective functions of selected traditional medicinal plants against oxidative stress induced by $H_2O_2$ using isolated lymphocytes culture *in vitro*. Here, in the present study, dose dependent decrease in the viability of cell was observed in lymphocytes treated with increasing concentration of $H_2O_2$, with increasing level of NO and activity of GST and decreasing GSH level. Nitric oxide (NO), besides acting as intercellular messenger, also functions as a free radical. Oxidative stress induced by $H_2O_2$, increases the level of cellular NO. The increased level of NO reacts with inorganic and organic molecules causing peroxidation of

membrane lipids and the formation of several harmful products and also induces apoptosis. [247-249]. The level of MDA, indicator of lipid peroxidation also increased in the lymphocytes treated with $H_2O_2$. To counteract the increased level of lipid peroxidation and to detoxify unsaturated aldehydes, cell increases the activity of GST. [250] Cellular antioxidant GSH is co-factor of GST and is responsible for the redox status of cell. The decline in the levels of GSH is due to increase in the activity of GST. The depleted level of GSH results into elevation of reactive oxygen species, which affects the normal functioning and integrity of cell and organelle membranes. [251] The loss of integrity of cellular membrane cause increase leakage of LDH to the exterior of the cell. [252,253]

Treatment of lymphocytes with REPT/FENA increases the level of cellular antioxidant GSH and decreases the level of NO and MDA formation. As GST is involved in the detoxification of lipid peroxides, significant fall in the level of MDA formation might have led to decline in the activity of GST. As expected, the viability of lymphocytes treated with REPT/FENA increased significantly and resulted in decrease in the activity of LDH suggesting non-toxic effect of the extract on the cellular system; rather it might have decreased the endogenous cellular injury (as a part of normal cellular metabolism). [254,255] This present findings suggest the antioxidant property of REPT/FENA as their treatment has led to the dose dependent decrease in the lipid peroxide levels and significant modulation of GSH levels and GST activity which might have resulted in the significant increase in the cell viability. [254-256]

Increased level of cellular antioxidants is known to provide protection against oxidative stress. [257] Here, in this study, pre-treatment of lymphocytes with the REPT/FENA for 1 hour significantly restored the increased level of NO and depleted GSH level suggesting antioxidative potentials of the REPT/FENA. As expected, the activity of GST decreased significantly due to decreased level of lipid peroxidation. For, the similar treatment condition, significant increase in the cell viability was observed. The decline in the activity of LDH suggests reduced level of oxidative stress experience by lymphocytes when pretreated with REPT/FENA. [206,258]

In conclusion, the results of the present study demonstrate that the hydroalcoholic root extract of *Phlogacanthus tubiflorus* and flower extract of *Nyctanthes arbor-tristis* exhibit strong protective functions against oxidative stress induced by $H_2O_2$. The protection of lymphocytes from $H_2O_2$ induced cellular damage by the extract may be due to an increase of cellular antioxidant or via decrease in free radical levels. The observed protective function is possibly conferred by different phytochemical in particular polyphenols and flavonoids present the hydroalcoholic extract. Further, hydro-alcoholic extract needs an extensive study in suitable model to identify and characterize the active principle responsible for the observed activity.

# CHAPTER 5

## *Antioxidative and free radical scavenging activity of Nyctanthes arbor-tristis and Phlogacanthus thyrsiflorus*

## 5.1 Background

Reactive oxygen species (ROS) are generated as a metabolic by product in biological system during normal metabolism of oxygen and plays vital role in cell signaling homeostasis for maintaining normal functioning of cells. [222] In the stress conditions, either intrinsic or extrinsic, ROS levels increase dramatically, resulting in an imbalance in between oxidants and antioxidants that leads to various forms of damage of micro and macromolecules and finally contributes in the manifestation of diseases such as sickle cell anemia, atherosclerosis, Parkinson's disease, heart failure, myocardial infarction, Alzheimer's disease, schizophrenia, cancer etc. [259-261]

Biological systems inherently have antioxidant system to scavenge and/or neutralize ROS generated under oxidative stress. Cellular antioxidant system (AOEs) consisting of mainly superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx), reduced reductase (GR) etc. [262] These biological AOEs function in a cascade manner to neutralize or eliminate the ROS and failure of which contributes disease manifestation. For effective management of reactive species, antioxidants have been exogenously supplemented, and several botanicals and synthetic compounds such as BHT, BHA have been studied for potent source of antioxidants. However, in real, biological state of radical scavenging and subsequent reduction of disease manifestation is still a challenging area. [263]

The importance of exogenously supplemented antioxidants have been realized soon after the discovery of ascorbic acid from plants and thereafter, plants have been gaining great deal of attention as a potent source of antioxidants to counteract the undesirable effects of oxidative stress in order to prevent the development and progression of several oxidative stress related life threatening diseases. [264] Several, plants and/or their products have been tested for their antioxidative and free radical scavenging activity, but, none of them are able to fulfil the desired level of efficacy with no or negligible side effects, at low cost. Therefore, in the present study, antioxidative and free radical scavenging activity of two selected medicinal plants *viz. Nyctanthes arbor-tristis* Linn., and *Phlogacanthus thyriflorus* Nees. were evaluated using cell free chemical based direct reactions.

## 5.2 Material and methods

### 5.2.1 Chemicals and reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), nitroblue tetrazolium chloride (NBT), 2- deoxyribose, ferrozine, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), tricholoroacetic acid (TCA) 2-thiobarbituric acid (TBA), ascorbic acid, gallic acid etc. were purchased from HiMedia Laboratories (Mumbai, India). The rest of the chemicals like DMSO, folin–ciocalteu reagent (FCR) etc. were of analytical grade, obtained from local firms of India.

### 5.2.2 Preparation of extract

The hydroalcoholic leaf extract of *Phlogacanthus thyrsiflorus* Nees. (LEPT) was prepared as described in section 4.2.2.

### 5.2.3 Evaluation of antioxidative and free radical scavenging activity

The FENA and LEPT were evaluated for their antioxidative and free radical scavenging activity using cell free *in vitro* chemical based reactions as described below:

#### 5.2.3.1 ABTS radical scavenging activity

Total antioxidant activity of the FENA/LEPT was determined by ABTS radical scavenging assay following the method of Arnao *et al.*, (2001) with some modifications. [265] The working solution was prepared by reacting the stock ABTS (7.4 mM) with potassium persulfate (2.6 mM) for 12 hours at room temperature in dark. The working solution was then diluted with methanol to an OD equivalent to 1.100 ± 0.02 at 734 nm and mixed with various concentration of FENA/LEPT or BHA. After 2 hours of incubation in dark, the absorbance was measured at 734 nm using the spectrophotometer and total antioxidant activity was calculated in terms of % scavenging of $ABTS^{*+}$ radical using the following equation:

$$\% \text{ ABTS radical scavenging activity: } \frac{\text{Abs control} - \text{Abs test sample}}{\text{Abs control}} X\ 100$$

### 5.2.3.2 DPPH radical scavenging activity

DPPH radical scavenging activity of the FENA and LEPT was determined according to the method described by Kitts *et al.*, (2000) and Shahidi *et al.*, (2007) with slight modification. [266,267] Briefly, DPPH solution of 0.135 mM in methanol was mixed with various concentrations of extract or standard (BHA) in methanol in a total volume of 1ml and vortexed thoroughly. The absorbance of the reaction mixtures was recorded after 30 minutes incubation at dark condition at 517nm using a spectrophotometer against blank consists of methanol. The scavenging activity was calculated using the following equation:

$$\% \text{ DPPH radical scavenging activity: } \frac{\text{Abs control} - \text{Abs test sample}}{\text{Abs control}} X\ 100$$

### 5.2.3.3 Superoxide radical scavenging activity

The superoxide radical scavenging activity was measured by alkaline DMSO method as described by Srinivasan *et al.*, (2007) and Kumara *et al.*, (2012). [268,269] NBT solution of 0.1 ml (1 mg/ml) was added to the mixture which contains 1 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 mL water) and 0.3 mL of the different concentrations of FENA/LEPT or ascorbic acid in DMSO. The absorbance was measured at 560 nm against reagent blank consist of DMSO. The scavenging activity was calculated using the following equation:

$$\% \text{ Superoxide radical scavenging activity: } \frac{\text{Abs control} - \text{Abs test sample}}{\text{Abs control}} X\ 100$$

### 5.2.3.4 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was evaluated based on quantification of the degradation product of 2-deoxyribose by condensation with TBA as originally described by Halliwell B and Gutteridge. [270] In the reaction system, hydroxyl radical was generated by the Fenton reaction ($Fe^{3+}$-ascorbate-EDTA-$H_2O_2$ system). The reaction mixture (1ml) contained 20 mM $FeCl_3$, 0.1 mM EDTA, 0.28 mM 2-deoxyribose, 0.2 mM $H_2O_2$, 0.3 mM ascorbic acid and various concentration of FENA/LEPT or gallic acid. After 1 hour incubation at $37^0$ C, 1 ml TBA (1 %, prepared in 50 mM NaOH) and 1 ml TCA (2.8 %, prepared in MilliQ water) was added and

heated for 15 minutes on boiling water bath at $80^0$ C. After cooling, absorbance was read at 532 nm against reagent blank and the percentage inhibition was calculated using the following equation:

$$\% \text{ Hydroxyl radical scavenging activity: } \frac{\text{Abs control} - \text{Abs test sample}}{\text{Abs control}} X\ 100$$

#### 5.2.3.5 $Fe^{2+}$ chelation inhibitory activity

The $Fe^{2+}$ chelation inhibitory activity of the extract was determined according to the method described by Dinis *et al.*, (1994) with slight modifications. [271] The Different concentrations of the FENA/LEPT or EDTA were added to a solution of 1 mM $FeCl_2$ (0.05 ml) and the reaction was started by adding 1 mM ferrozine (0.1 ml). The volume of the reactions was adjusted to 1 ml with methanol, shaken vigorously and left standing at room temperature for 10 minutes. After the mixture had reached equilibrium, the absorbance of the solution was measured at 562 nm using spectrophotometer against blank consists of methanol and MilliQ water. The inhibition of chromogen formation was calculated from the following equation:

$$\% \text{ Inhibition of } Fe^{2+}: \frac{\text{Abs control} - \text{Abs test sample}}{\text{Abs control}} X\ 100$$

#### 5.2.3.6 Reducing power assay

The reducing power of plant extracts was determined according to the method of Zhu *et al.*, (2011) with slight modification. [272] To the reaction mixture containing equal volume of Potassium buffer (0.2 M, pH 6.6) and various concentrations of FENA/LEPT or ascorbic acid, 0.5 ml of potassium ferricyanide (1%) was added and incubated for 20 minutes at $50^0$C. After incubation, 0.1 ml TCA (10%) was added and centrifuged at 3000 rpm for 10 minutes. The supernatant was mixed with 0.5 ml MilliQ water and 0.5 ml Ferric Chloride (0.1%) and thoroughly mixed. The absorbance was measured spectrophotometrically at 700 nm against blanks consisting of all reagents except extract or standard.

#### 5.2.3.7 Detection of characteristic peaks of phytochemicals

The characteristic spectral analysis of FENA and LEPT was performed to detect the characteristic peaks of phytochemical present in the crude extract. The FENA and LEPT was dissolved in ethanol (1 mg/ml) and scanned in the wavelength ranging from

200 to 800 nm using spectrophotometer. For FTIR analysis lyophilized powder of plant material and extract were used. The analysis was performed on a potassium bromide disc using FTIR spectrophotometer (Spectrum-100, Perkin Elmer, Singapore) in the spectral range 4000-400 $cm^{-1}$.

#### 5.2.3.8 Estimation of total phenolic and total flavonoid content

The content of total phenolic was determined using Folin-Ciocalteu (FC) reagent as described by Singlet and Rosi (1965) with minor modification. [273] Briefly 400 µl of $Na_2CO_3$ solution was added to a mixture of FENA/LEPT and 500 µl of FC reagent (10 fold diluted) and incubated at 22°C for 2 hours. The absorbance was measured at 725 nm. The phenolic content was calculated from gallic acid standard curve and finally expressed as µg gallic acid equivalent (GAE) per mg extract.

The content of total flavonoid content was esimated with aluminium chloride ($AlCl_3$) as described by Zhishen *et al.*, (1999) with slight modification. [274] Briefly, the extract was mixed with 1.5% of $NaNO_2$ and incubated for 5 minutes at 25°C. Thereafter, 200 mM NaOH was added and the absorbance was measured at 510 nm. The phenolic content was calculated from Epicatechin standard curve and finally expressed as µg epicatechin equivalent (ECE) per mg extract.

#### 5.2.3.9 Statistical analysis

All the data are expressed as means ± sem, n=3. The significance differences between the experimental and the control groups were analyzed by student's t test and three levels of significance were set as $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

### 5.3 Results:

#### 5.3.1 Antioxidative and free radical activity of FENA and LEPT

The antioxidative and free radical scavenging activity of FENA and LEPT was investigated using chemical based *in vitro* reactions. The findings of the study reveal that FENA scavenged ABTS, DPPH radical, superoxide radical, hydroxyl radical and inhibited $Fe^{2+}$ chelation with $IC_{50}$ value 15.46 ± 0.28, 82.92 ± 4.40, 17.48 ± 0.48, 271.84 ± 23.65 and 168.96 ± 4.28 µg/ml respectively. The LEPT scavenged ABTS, DPPH radical, superoxide radical, hydroxyl radical and inhibited $Fe^{2+}$ chelation with

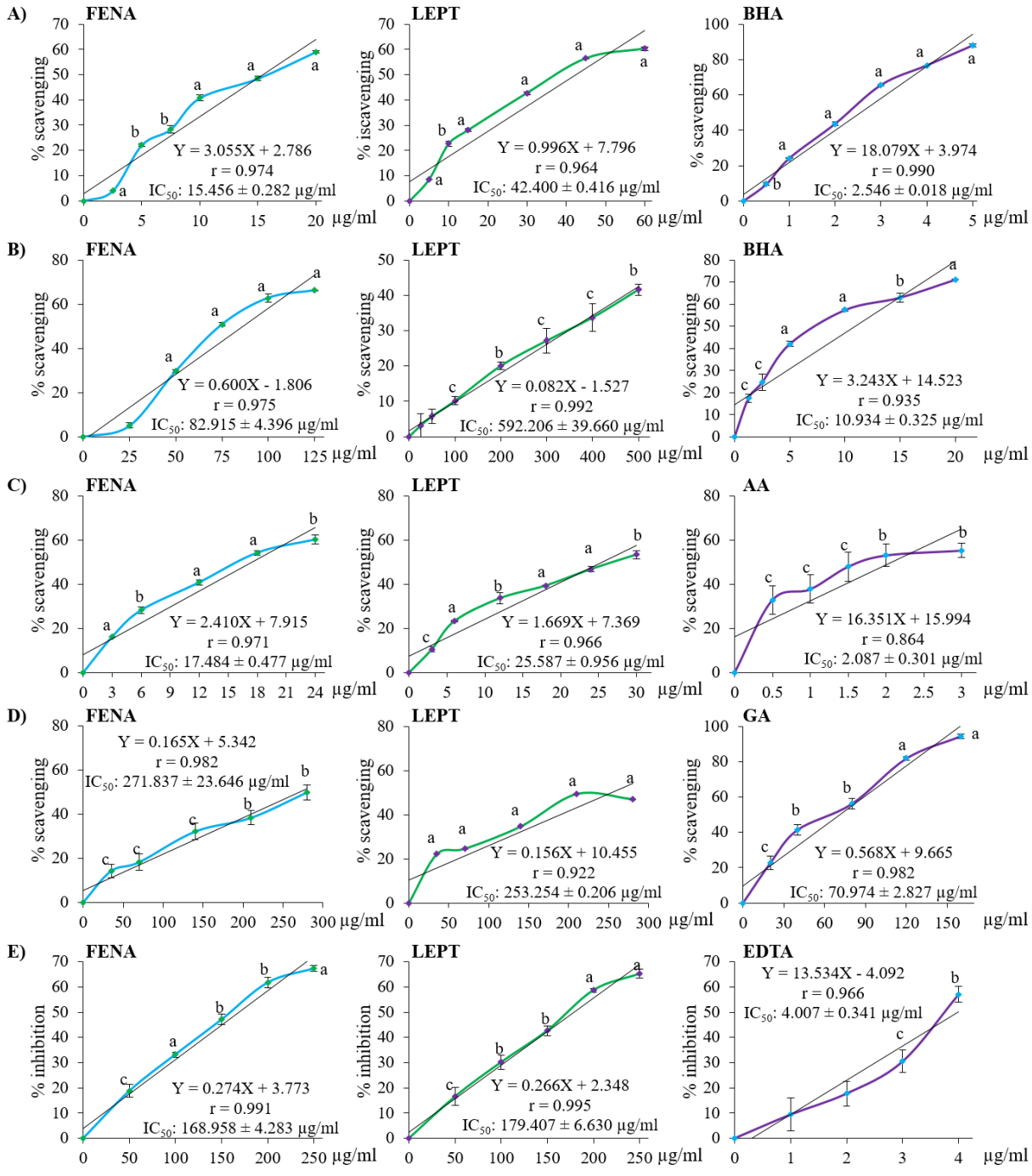


**Figure 16: Antioxidative and free radical scavenging activity of FENA and LEPT :** A) ABTS, B) DPPH, C) Superoxide, D) Hydroxyl radical scavenging and E) $Fe^{2+}$ chelation inhibitory activities were evaluated by *in vitro* cell free chemical based reactions as described in 'Material and methods'. Values are mean ± sem; n=3; $^{a}p\leq0.001$ compared to control; $^{b}p\leq0.01$ compared to control; $^{c}p\leq0.05$ compared to control.

$IC_{50}$ value 42.40 ± 0.42, 592.21 ± 39.66, 25.59 ± 0.96, 253.25 ± 0.21 and 179.41 ± 6.63 µg/ml respectively. For similar assay, the $IC_{50}$ value for the standard compounds were 2.55 ± 0.02 (BHA), 10.93 ± 0.33 (BHA), 2.09 ± 0.30 (AA), 70.97 ± 2.83 (GA) and 4.01 ± 0.34 (EDTA) µg/ml respectively [Figure 16]. The FENA, LEPT and ascorbic acid reduces the $Fe^{3+}$ to $Fe^{2+}$ with $OD_{0.50}$ at 143.29 ± 0.79, 1094.75 ± 24.75 and 13.44 ± 1.43 µg/ml respectively [Figure 17]. Qualitative phytochemical reveals the presence of total phenolic content 71.2 ± 0.6 and 19.8 ± 2.0 µg gallic acid equivalent (GAE) per mg FENA and LEPT respectively. The total flavonoid content in the FENA and LEPT was 520.9 ± 16.2 and 220.2 ± 11.3 µg Epicatechin equivalent (ECE) per mg extract respectively.

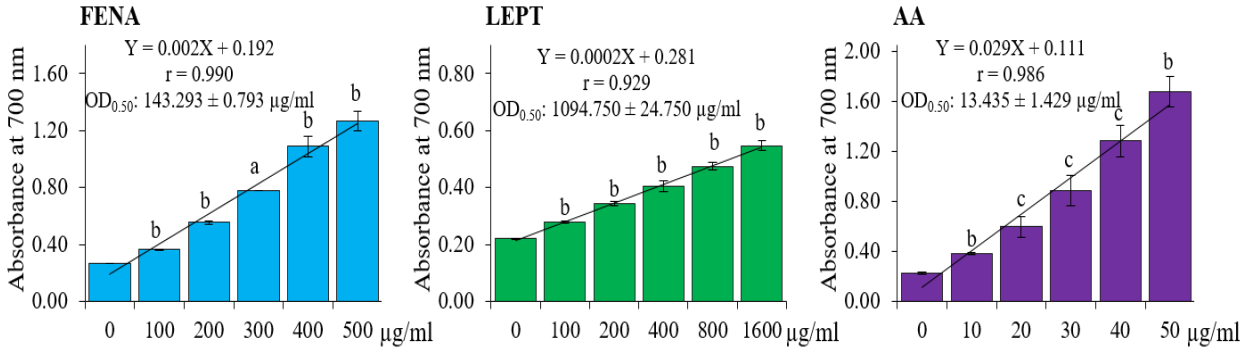


**Figure 17: $Fe^{3+}$ reducing ability of FENA and LEPT:** The reductive ability of FENA, LEPT and ascorbic acid was evaluated by *in vitro* cell free chemical based reactions as described in 'Material and methods'. Values are mean ± sem; n=3; $^{a}p\leq0.001$ compared to control; $^{b}p\leq0.01$ compared to control; $^{c}p\leq0.05$ compared to control.

### 5.3.2 UV-VIS and FTIR spectroscopic analysis

The UV-VIS spectra of FENA and LEPT was scanned from 200 to 800 nm due to sharpness of the peaks and proper baseline. As shown in Figure 18 and Table 8, FENA and LEPT showed 05 and 07 Nos of peak respectively. The results of the FTIR peak values and functional groups of the components are presented in Figure 18 and Table 9. The analysis confirmed the presence of phenolic compound and flavonoids from the plant materials.

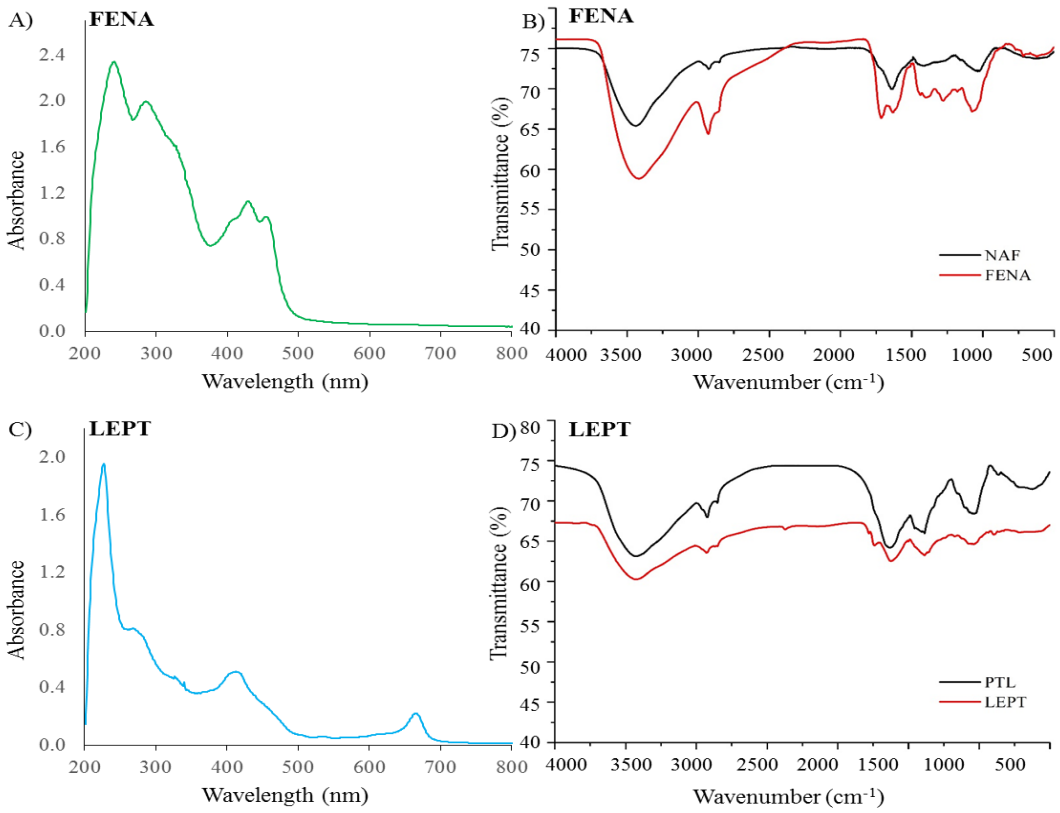


**Figure 18: Ultraviolet-visible (A,C) and FTIR spectra (B,D) of FENA and LEPT**
Abbreviation: NAF: *Nyctanthes arbor-tristis* flower and PTL: *Phlogacanthus thyrsiflorus* leaf.

**Table 8:** UV-VIS peak values of FENA and LEPT

| FENA | | LEPT | |
|---|---|---|---|
| Wavelength (nm) | Absorption | Wavelength (nm) | Absorption |
| 240 | 2.348 | 226 | 1.960 |
| 184 | 1.985 | 267 | 0.812 |
| 327 | 1.591 | 325 | 0.478 |
| 428 | 1.083 | 339 | 0.439 |
| 453 | 0.951 | 367 | 0.370 |
| | | 412 | 0.510 |
| | | 664 | |

**Table 9:** FTIR peak values and functional groups of FENA and LEPT

| | Frequency ($cm^{-1}$) | Bond | Functional group [275-277] |
|---|---|---|---|
| FENA | 3640–3610 | O–H stretch | Free hydroxyl alcohols, phenols |
| | 2830–2695 | H–C=O: C–H stretch | Aldehydes |
| | 1600–1585 | C–C stretch (in–ring) | Aromatics |
| | 1550–1475 | N–O asymmetric stretch | Nitro compounds |
| | 1500–1400 | C–C stretch (in–ring) | Aromatics |
| LEPT | 3500–3200 | O–H stretch, H–bonded alcohols | Phenols |
| | 2830–2695 | H–C=O: C–H stretch | Aldehydes |
| | 1750–1735 | C=O stretch | Esters, saturated aliphatic |
| | 1360–1290 | N–O symmetric stretch | Nitro compounds |
| | 1320–1000 | C–O stretch | Alcohols, carboxylic acids, esters, ethers |

### 5.4 Discussion:

The use of natural products in the form of crude preparation and active principle as a therapeutic regime has been widely established. Several medicinal plants have been studied for their potentials to modulate cellular antioxidants and free radicals, and numbers of active principles have also isolated from plants with anti-oxidative efficacy.[278,279] But, there is still lack of magic principle with maximum efficacy and least toxicity. The present study explores the antioxidative and free radical scavenging activity of flower extract of *Nyctanthes arbor-tristis* and leaf extract of *Phlogacanthus thyrsiflorus* by employing chemical based *in vitro* reaction systems.

Quantitative phytochemical analysis revel that FENA and LEPT are rich in flavonoids and phenolic content. Since, these two classes of compounds are known to possess potent antioxidative and free radical scavenging activity, we evaluated their activity by chemical based *in vitro* reaction. The total antioxidant activity of the FENA/LEPT was evaluated using ABTS radical. In this assay, $ABTS^{*+}$ radical is generated by mixing ABTS with potassium persulfate overnight. The antioxidant reduces this blue color $ABTS^{*+}$ radical into ABTS resulting in decrease in blue color. [280]

As shown in Figure 16, FENA/LEPT demonstrates potent antioxidant activity. The DPPH method is a widely used method of determination of antioxidant and free radical scavenging property. The blue colored DPPH is a synthetic, stable radical which accepts an electron or hydrogen from antioxidant and become stable DPPH-H. The discoloration of the DPPH can be spectrophotometrically measured at 517nm. [281] The DPPH radical scavenging activity of FENA/LEPT has been shown in Figure 17. It is evident that both the FENA and LEPT scavenged the DPPH radical in dose dependent manner. The Superoxide anion radical is generated as a by-product of mitochondrial respiration and fatty acid oxidation and known to be very harmful to the cellular component as it can result into hydrogen peroxide, hydroxyl radical ($OH^*$), singlet oxygen ($O^-$), peroxynitrite ($ONOO^*$) etc. [280] Here, in this study superoxide radical scavenging activity is evaluated by alkaline DMSO method. [280,281] In this method, superoxide radical is generated by addition of sodium hydroxide to air saturated DMSO and the generated superoxide which remain stable in solution, reduces nitroblue tetrazolium (NBT) into formazan dye at room temperature which can be measured at 560 nm. As shown in Figure 16, FENA and LEPT exhibit dose dependent superoxide radical scavenging activity. The hydroxyl radical ($OH^*$) is one of the major active oxygen species that cause lipid peroxidation and exerts various biological damages. In the present investigation, $OH^*$ radical was generated by incubating $Fe^{3+}$-EDTA with ascorbic acid and $H_2O_2$ and reacted with 2-deoxyribose to yield a malondialdehyde (MDA) like product which upon heating form pink color chromogen. Antioxidant substances remove the hydroxyl radicals from the sugar and prevent the reaction. [282,283]. Results of the present study indicates that the FENA/LEPT are potent hydroxyl radical scavenger.

Transitional metal such as iron stimulates lipid peroxidation through Fenton reaction and accelerates lipid peroxidation by converting lipid hydroperoxides into peroxyl and alkoxyl radical which perpetuate chain reaction. Therefore, metal chelating activity is significant in maintaining healthy cellular status. [284,285] The finding of the present data, clearly demonstrated that FENA/LEPT has iron chelating activity. The reducing potential of substances from $Fe^{3+}$ to $Fe^{2+}$ is a significant indicator of antioxidant

activity, as it exerts antioxidant activity by preventing free radical chain initiation by donating hydrogen atom, decomposing peroxides and scavenging free radical. [286] Here, we found that, FENA/LEPT exhibited dose dependent reducing potentials.

It is evident from this study that the the hydro-alcoholic flower extract of *Nyctanthes arbor-tristis* and leaf extract of *Phlogacanthus thyrsiflorus* are rich in phytochemicals like polyphenol and flavonoids and exhibit potent antioxidative and free radical scavenging activity. The encouraging results shown by extract further indicates that they can be used as a supplement to eliminate the harmful effects of free radicals in order to manage healthy life.