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 50^0C. 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

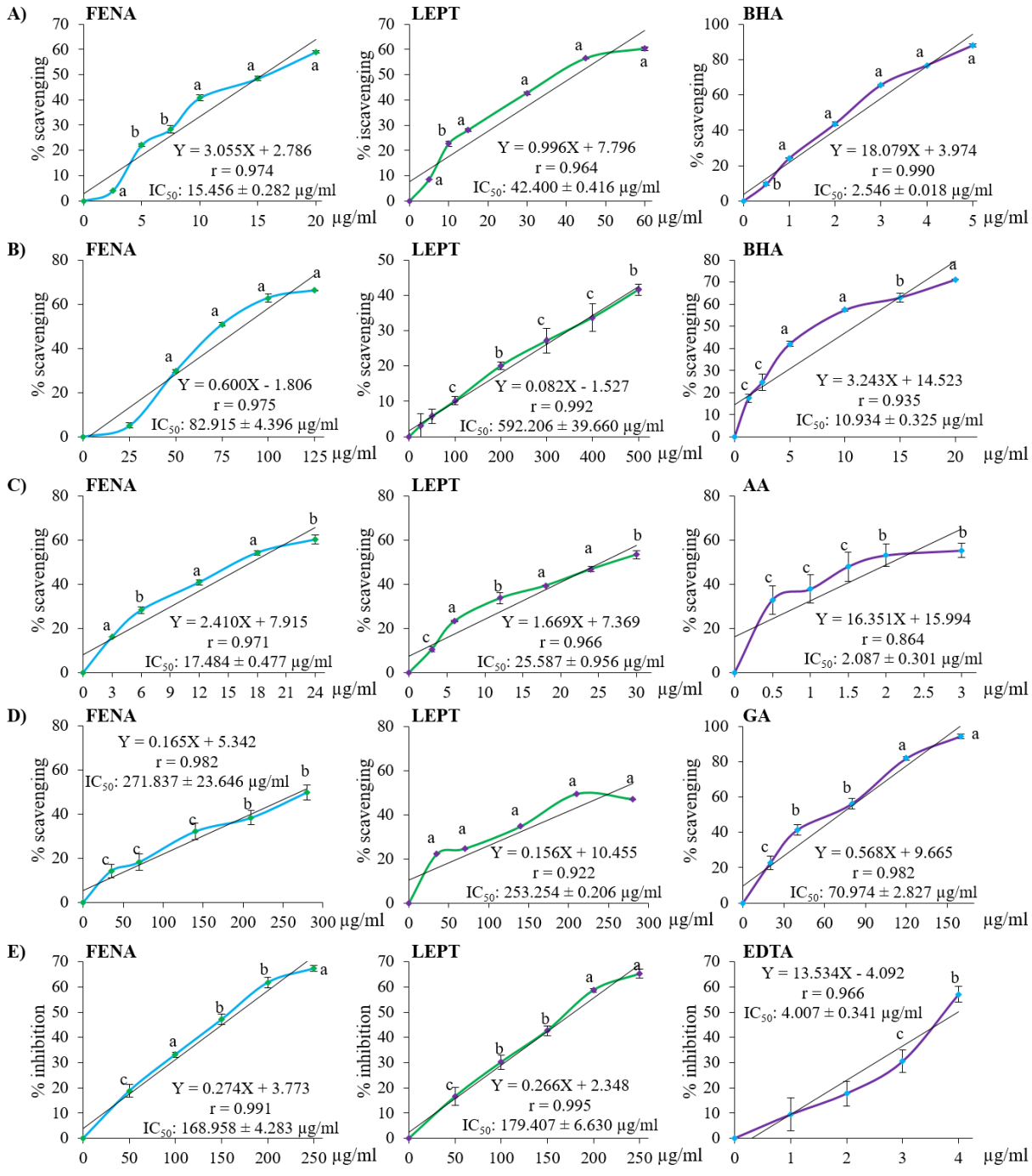

Figure 16: Antioxidative and free radical scavenging activity of FENA and LEPT : A) ABTS, B) DPPH, C) Superoxide, D) Hydroxyl radical scavenging and E) Fe^{2+} chelation inhibitory activities were evaluated by *in vitro* cell free chemical based reactions as described in 'Material and methods'. Values 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.

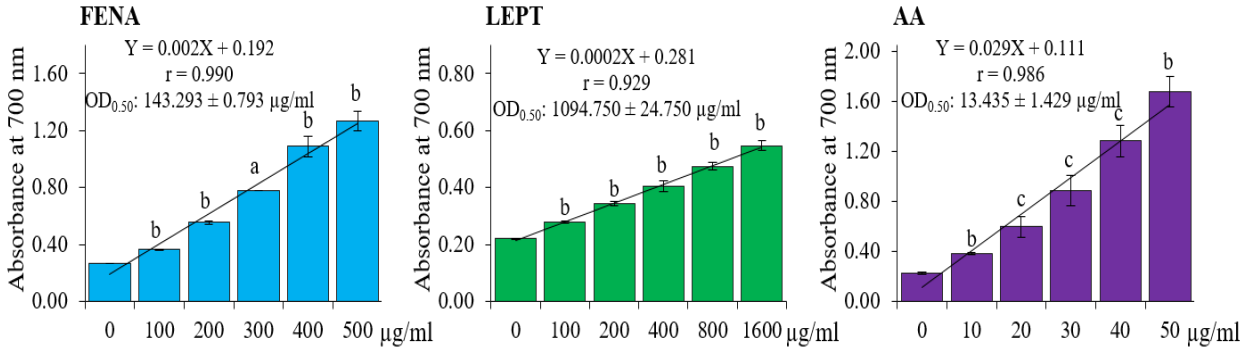

Figure 17: Fe^{3+} reducing ability of FENA and LEPT: The reductive ability of FENA, LEPT and ascorbic acid was evaluated by *in vitro* cell free chemical based reactions as described in 'Material and methods'. Values are mean ± sem; n=3; $^{a}p\leq0.001$ compared to control; $^{b}p\leq0.01$ compared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

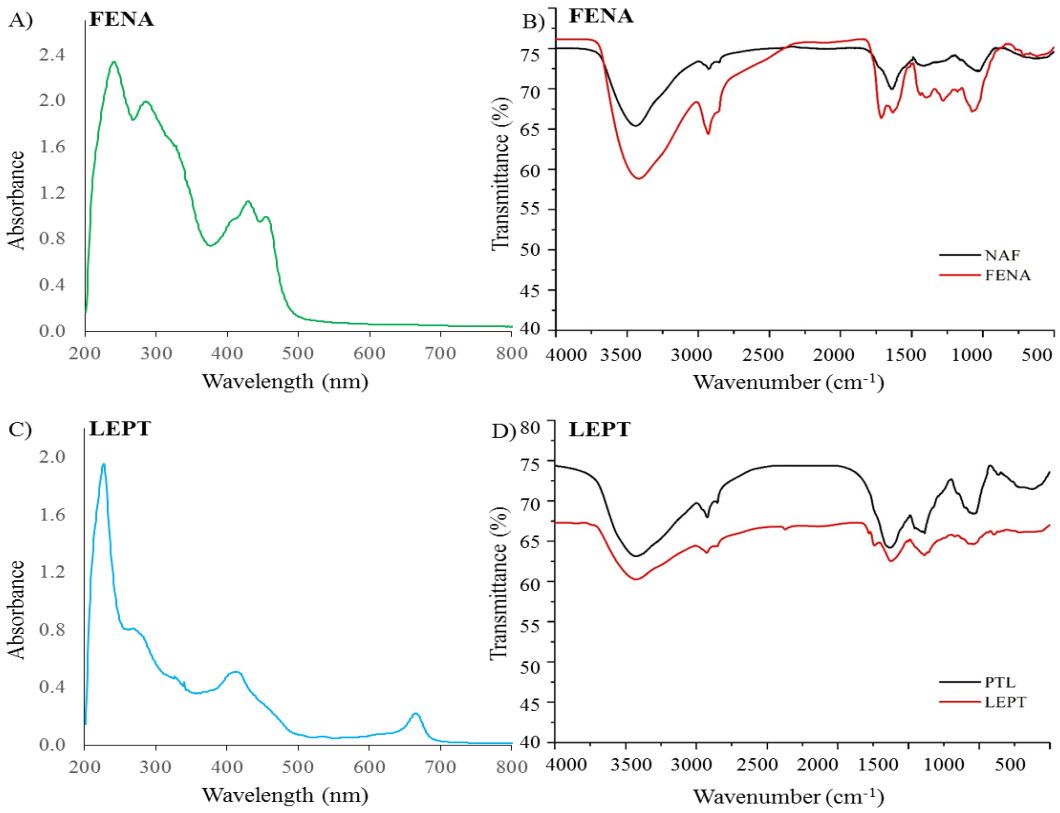

Figure 18: Ultraviolet-visible (A,C) and FTIR spectra (B,D) of FENA and LEPT
Abbreviation: NAF: *Nyctanthes arbor-tristis* flower and PTL: *Phlogacanthus thyrsiflorus* leaf.

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.