Volume : 5 | Issue : 5 | May 2016 • ISSN No 2277 - 8179 | IF : 3.508 | IC Value : 69.48

Research Paper

Evaluation of in vitro Antioxidant and Free Radical Scavenging Properties of Different Parts of Medicinal Plant Nothapodytes foetida (Family: Icacinaceaae)

Biological Science

KEYWORDS : Nothapodytes foetida, Antioxidants, DPPH, ABTS, Reducing Power

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ABSTRACT

This study was designed to examine the in vitro antioxidant properties of different parts (leaves, stem, bark, fruits and seeds) of Nothapodytes foetida, which is a major source of anticancer drug, camptothecin. Antioxidant activites of the aqueous and methanolic extracts have been evaluated by various in vitro models. In vitro antioxidant activity was evaluated by percentage inhibition in different assays including 2, 2'-diphenyl-1-picrylhydrazyl (DPPH), (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS•+), hydroxyl radical scavenging assay, superoxide radical scavenging assays and reducing power capacity. The results obtained from the present study indicated that the N. foetida leaf, fruit and seed extract is a potent source of natural antioxidants and both the aqueous and methanolic extracts possesses excellent anti-oxidant and free radical scavenging activity. It was also observed that the leaves, fruits and seeds of N. foetida possessed potent free radical scavenging ability as compared to stem and bark. In all the assays performed, aqueous extract was found to possess higher antioxidant activity than methanolic extracts.

Introduction:

Free radicals are reactive oxygen species (ROS) generated as byproducts of various physiological and biochemical processes during a range of metabolic activities or by environmental conditions imposing oxidative stress burden in the living organisms. The most common ROS include superoxide anion (O2), hydrogen peroxide (H2O2), peroxyl radicals (ROO) and reactive hydroxyl (OH) radicals. These free radicals can directly react and cause injury to various cellular constituents including proteins, lipids and DNA of healthy cells which leads to cellular damage. Also, deregulation of these ROS is the major cause of various chronic and degenerative diseases such as cardiovascular disease, neurodegenerative disease, diabetes mellitus, ageing and cancer (Scalbert et al 2005). The most effective way to get rid of free radicals causing the oxidative stress is with the help of antioxidants. Antioxidants help to protect the body against ROS toxicity either by preventing the formation or by scavenging reactive molecules (Hedge and Joshi 2009). Most synthetic antioxidants such as Butylated Hydroxytoluene (BHT) and Butylated Hydroxyl Anisole (BHA) are widely used but have more damaging than beneficial effects. Therefore, considerable interests in development of antioxidants of natural origin have received much attention in recent years, especially within biological, medical and nutritional areas because they are considered as safer and cause fewer adverse health effects than synthetic antioxidants (Anusuya N and Manian S 2013).

Different medicinal plants are known to be important source of biologically active phytochemicals which have already been reported to act as natural antioxidant by scavenging free radicals, and many have therapeutic potential against free radical associated disorders (Lee et al 2000). Recently, the pharmacological properties of medicinal plants have begun to receive more attention in the scientific community and have become important research focus. The medicinal plant products derived from plant parts such as leaves, fruits, seeds, roots, stem and stem bark have been part of phytomedicine that produce a definite physiological action on the human body. The most important of these natural bioactive constituents of plants are secondary metabolites like alkaloids, flavonoids, tannins and phenolic compounds. The bioactivity of these secondary metabolites may be related to their ability to scavenge free radicals or prevent the adverse effects of ROS on normal physiological function in humans (Huang et al 2005).

The medicinal plant *Mapia foetida* is commonly known as Amruta (Family: Icacinaceae) now renamed as *Nothapodytes foetida* is a moderate sized tree. *N. foetida* occupies important position in the plant based anticancer drugs. The active constituent extracted from this endangered plant species is camptothecin, an alkaloid which is a major essential component used in chemotherapy. Foetidine-1 and 2 alkaloids have anti-cancer properties which are also present in this plant. These alkaloids are soluble in water and present in all parts of the plant. They are precursors of campothecin and 9- methoxycampothecin known to have pharmacodynamic properties (Hsiao et al 2008). It was thought to be necessary to understand the antioxidative status of this medicinal plant used in the herbal medicine, Therefore the present study was proposed to explore the antioxidant potential and free radical scavenging properties of different plant parts of medicinal plant *N. foetida*. The in vitro free radical scavenging properties of aqueous and methanolic extracts of various parts (Leaves, stem, bark, fruit, seed) of *N. foetida* plant was evaluated by DPPH, ABTS, FRAP, superoxide radical scavenging and hydroxyl radical scavenging assays.

Materials and methods

Chemicals & Reagents

2, 2'-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS), 1,1-diphenyl-2-picryl hydrazine (DPPH) were obtained from Sigma Aldrich. Butylated hydroxyl toluene (BHT), Deoxy-ribose, Nitroblue tetrazolium (NBT), Phenazine methosulphate (PMS),

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Ascorbic acid, Potassium persulfate, Phosphate buffer, Potassium Ferricyanide, Thiobarbituric Acid (TBA) Trichloroacetic acid (TCA), Ferric chloride (FeCl3), Ethylene diamine tetra acetic acid (EDTA) and the solvents were obtained from Sisco Research Laboratories. All aqueous solutions were prepared using double-distilled water.

Collection and processing of plant materials.
Plant parts were collected from Amboli Ghat region of Maharashtra from India and authenticated by Department of Botany, University of Pune, India. The plant parts were thoroughly washed with double-distilled water for removal of adhered dust particles, shade dried and then ground into fine powder using mortar and pestle. The powdered samples were stored in an air-tight container in dark at room temperature for further experiments.

Preparation of aqueous extracts.
Based on an analysis of published literature the following plant parts were considered for the assessment of antioxidant properties. To prepare aqueous extract ten grams (gms) powder of plant parts like leaves, stem bark, fruit pulp and seeds was mixed with 100 milliliter (mL) of double distilled water in a 250 mL capacity Erlenmeyer flask. The mixture was boiled at 80^0C for 30 minutes. After cooling, the aqueous extract was filtered through a series of Whatman filters and finally passed through a 0.22µm filter and stored at 4^0C for further experiments.

Preparation of methanolic extracts by soxlet extraction.
The methanolic extract of different dried plant parts were prepared by soxhletion. In this extraction process, 10 gms of dried powder was extracted with 250 ml of 90 % methanol at 70^0C temperature for 8-9 extraction cycles. All the resulting extracts were filtered through filter paper and poured on crucible dishes kept in desicators to evaporate the liquid solvents from the extract to get dry extracts. After drying, crude extracts were weighed and stored in stock vials and kept at 4°C for further experiments.

Evaluation of Antioxidant properties of *Nothapodytes foetida* plant extracts:
Antioxidant properties were determined by different free radical scavenging assays.

DPPH radical scavenging assay
The DPPH scavenging activity of aqueous and methanolic extracts of different plant parts was measured according to the method described by Rajan Rushender et al., 2012 with some modifications. The reaction mixture consisted of a mixture of freshly prepared 1 mL DPPH (0.1 mM) solution in methanol with different concentrations (25, 50, 100,200 and 400 µg/mL of aqueous and methanolic extracts in final volume of 0.1 mL in distilled water or methanol respectively. The blank sample consisted of 2 mL of methanol, while the control contained 2 mL DPPH methanolic solution only. The absorbance of sample, blank and control was measured at 517 nm, after 30 min incubation of reaction in the dark at room temperature, using a UV-Visible 1800 spectrophotometer (Shimadzu). The percentage of DPPH scavenging activity was calculated using the expression below: Percentage inhibition (%) = (A0-A1) / A0) × 100, where: A0 is the Absorbance of control and A1 Absorbance of test. The results were compared with Ascorbic acid and Butylated hydroxy toluene (BHT) as standard

ABTS radical scavenging assay
ABTS radical scavenging assay was carried out using procedures described by Arnao *et al.* 2001. ABTS•+ radical cations are produced by reacting ABTS (7 mM) and potassium persulfate (2.4 mM) and incubating the mixture at room temperature in the dark for 16 hr. Different concentrations of aqueous as well as methanolic extracts in final volume of 0.1 mL were added and allowed to react with 1 mL of ABTS for 30 minutes and the absorbance was recorded at 734 nm after incubation. The blank sample consisted of 1 mL of methanol, while the control contained 1 mL ABTS solution prepared in Methanol. Ascorbic acid and BHT were used as standard. The percent inhibition was calculated from the following equation: Percentage inhibition (%) = (A0 –A1) / A0) × 100

Ferric-reducing antioxidant power assay
The ferric-reducing antioxidant power of the aqueous and methanolic extract fractions was determined using the modified method of Ganesan et al. (2008). Initially, 0.250mL of 0.2M phosphate buffer (pH 6.6) and 0.250 mL of 1% potassium ferricyanide were added to varying concentrations of extracts in 0.1 mL final volume either in distilled water or methanol. The mixture was then incubated at 50°C for 20 minutes. After cooling, 0.250 mL of 10% trichloroacetic acid was added to the mixture and then centrifuged at 5000 rpm for 10 minutes. Next, to the upper layer of the solution, 0.250mL of distilled water and 0.05 mL of freshly prepared 0.1% ferric chloride was mixed. After 10 min incubation at room temperature, the absorbance was measured at 700 nm using a UV-Visible spectrophotometer. The reducing power of the extract was linearly proportional to the concentration of the sample. Increased absorbance of the reaction mixture indicated increased reducing power.

Superoxide radical scavenging assay
Measurement of superoxide anion scavenging activity was done based on the reduction of NBT according to a previously described method of Nishikimi et al., (1972). The reaction mixture consisted of 1ml of NBT solution (156µM) and sample solution at different concentrations of aqueous and methanolic extracts. The reaction was started by adding 0.1 mL of PMS solution (60µM) in phosphate buffer pH 7.4) to the reaction mixture followed by incubation at 25^0C for 5 minutes and the absorbance was measured at 560 nm against blank. The percent inhibition of superoxide radicals was calculated from the following equation:

Percentage inhibition (%) = (A0 –A1) / A0) × 100

Hydroxyl radical scavenging assay
The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell et al., (1987). The assay was performed by adding 0.1 mL of EDTA (100 µM) 0.01 mL of FeCl3 (100 µM), 0.1 mL of H202 (1mM), 0.36 mL of deoxyribose (2.8 mM),0.1 mL of aqueous as well as methanolic plant extracts, 0.33 mL of phosphate buffer (50 mM, pH 7.4) and 0.1mL of ascorbic acid in sequence. The mixture was then incubated at 37°C for 60 minutes. About 1.0 mL portion of the incubated mixture was mixed with 1.0 mL of 10 % TCA and 1.0 mL of 0.5% TBA and the mixture was incubated at 95°C for 15 minutes to develop the pink chromogen. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution (Phosphate buffer pH 7.4) The hydroxyl radical scavenging activity of the extract was reported as % inhibition of deoxyribose degradation and was calculated as equation: Percentage inhibition (%) = (A0 –A1) / A0) × 100

Statistical analysis
All experiments were done in four replicates and then values were expressed as mean ± standard error (SE) of four measurements. Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Student's t-test by SPSS 11 for windows..

Results
***In Vitro* DPPH radical scavenging activity:**
The results of the DPPH scavenging activity of different plant parts of *M. foetida* extracts are shown in figure 1A & B. As shown

in figure 1A that the maximum percentage scavenging of DPPH for aqueous extracts of leaves, stem, bark, fruit and seed was 90.32%, 41.78%, 23.57%, 78.44% and 87.93% whereas for methanolic extracts was 88.42%, 33.34%, 15.93%, 49.75% and 57.80% respectively (Figure 1B) at highest concentration of 400µg/mL. The results showed that the aqueous extract of leaves exhibited strong DPPH radical scavenging ability (IC50 value of 127.59 ± 0.26 µg/mL) than the methanolic extract (IC50 value of 165.87±1.273µg/ml). IC50 value of aqueous extract of fruit and seeds was observed as 270.08 ± 1.76 and 173.0 µg/mL which were significantly lower than methanolic extracts i.e. 500 µg/mL and 320 µg/mL respectiveluy. There was no DPPH radical scavenging seen in the stem and bark even at higher concentration of aqueous as well as methanolic extracts.

ABTS radical scavenging activity

As shown in figure 2A that the maximum percentage scavenging of ABTS for aqueous extracts of leaves, stem, bark, fruit and seed was 99.30%, 58.91%, 35.27%, 98.79% and 98.86% whereas for methanolic extracts was 81.66%, 57.00%, 31.57%, 83.42% and 82.54% respectively (Figure 2B) at highest concentration of 400µg/mL. There were no ABTS radical scavenging seen in the bark (35.50 %) even at higher concentration of aqueous as well as methanolic extracts (31.57 %)

Assessment of reducing power of extracts.

The reducing capacity serves as a significant indicator of its potential antioxidant activity. As illustrated in figure 3A & B at 0.4 mg/ml the absorbance of the aqueous and methanolic extract of leaves were 1.76 and 1.04 respectively, while the absorbance of the standard BHT was 1.25. The absorbance at 700nm of aqueous extract of stem, bark fruit and seeds were 0.532, 0.531, 1.35 and 1.39 respectively where as methanolic extracts were 0.525, 0.326, 0.755 and 0.795 respectively. Thus, the reducing power of the selected aqueous as well as methanolic extracts of *N. foetida* increased steadily with increasing concentration.

Superoxide radical scavenging activity

The ability of the plant extract to quench superoxide radicals from reaction mixture is reflected in the decrease of the absorbance at 560 nm. The maximum inhibition was found to be 92.00%, 35.0%, 23.0%, 85.80% and 82.75% for aqueous extracts of leaves, stem, bark fruit and seeds respectively and 90.07%, 24.0%, 16.0%, 80.0% and 75.0% for methanolic extract at 0.4 mg/mL (Figure 4 A &B). The aqueous extract exhibited an IC50 value of 184.54±0.84 µg /mL for leaves, 177.65 ± 1.10 for fruits and 255.92 ± 0.77 for seeds whereas for the methanolic extracts of leaves 220.70 ± 1.18, for fruits 221.16 ± 0.27 and for seeds 277.25 ± 2.22 which was found to be an effective scavenger of superoxide radical

Hydroxyl radical scavenging activity

The estimated IC50 values of aqueous extracts of leaves, fruit and seeds were 143.31 ± 2.88, 267.04 ± 2.06 and 158.18 ± 1.76 respectively and for methanolic extracts of leaves, fruits and seeds were 167.22 ± 0.10, 433.75 ±2.38 and 390.33 ± 0.77 respectively. At a concentration of 0.4 mg/mL, the scavenging activity of methanolic and the aqueous extracts of leaves, stem, bark, fruits and seeds was found to be 93.05%, 31.93%, 31.93%, 78.44%and 88.77% respectively and the methanolic extracts was 86.59%, 24.60%, 24.66%, 55.61%, 74.59% respectively (Figure 5 A & B). Of these, the aqueous extracts were found to be more effective in quenching the hydroxyl radicals produced in the reaction mixture than methanolic extracts

Discussion

Among the phytochemicals, phenolic compounds are known as powerful chain breaking, antioxidants because of their scavenging ability due to their hydroxyl groups which contribute directly to antioxidative action (Sawant et al 2009). The *in vitro* antioxidant assays demonstrates that plant extracts are important sources of natural antioxidants, which might be useful as preventive agents against oxidative stress. No single assay accurately reflects the mechanism of action of all radical sources in a complex system; therefore multiple methods were employed in order to evaluate the total antioxidant activity. For *in vitro* antioxidant screening, DPPH, ABTS, and reducing power assay are most commonly used (Arulmozhi et al 2010). Superoxide radical is implicated as one of the strongest ROS among the free radicals and get converted to other harmful reactive oxygen species such as singlet oxygen, hydrogen peroxide and hydroxyl radical, damaging biomolecules which results in chronic diseases (Al-Mamun et al 2007). Therefore, to determine the antioxidant activity of this medicinal plant, superoxide radical scavenging assay was preferred which showed significant increase in aqueous extracts of leaves, fruits and seeds than methanolic extracts. The hydroxyl radical is one of the reactive free radicals formed in biological systems which can also induce oxidative damage to DNA, lipids and proteins and causes enormous damage to biomolecules of the living cells. In this study, hydroxyl radical scavenging ability of the extracts was determined by its ability to compete with deoxyribose for hydroxyl radical. The antioxidant activity has also been reported to be associated with development of reducing power. Since the reducing capacity serve as a significant indicator of its potential antioxidant activity, the reducing ability of phytoextract of different parts were also measured in this study which showed comparatively higher reducing power in aqueous extracts than in increased concentrations of methanolic extracts.

Extensive research has been carried out on the free radical scavenging properties of phytochemicals of different parts of various medicinal plants including *Withania somnifera* (Sumathi et ap 2007) *Plumbago indica* (Eldhose et al 2013), *Aloe vera* (Patel et al 2012), *Aegle marmaloes* (Modi et al 2012), *Auricularia auricula* (Acharya et al 2003*)*. However, the components responsible for the antioxidative activity of *N foetida* extracts are currently unclear. Therefore, it was suggested that further work be performed on the identification of the antioxidant properties of *N. foetida*. Earlier, only a single report available on antioxidant properties of *Nothaphodytes nimmoniana* stating that when antioxidant capacity of stem and leaves compared stem showed higher antioxidant activity (Uma et al.,2013), but in contrary to that report our results showed marginal increase in antioxidant capacity in leaves than stem and bark. The phytochemical analysis of *M. foetida* already revealed the presence of alkaloids, have been associated with medicinal uses and one of their common biological properties is their cytotoxicity in cancer cells (Wu et al 2008**).** The presence of these phenolic compounds in this plant extracts could be contributed to its antioxidant properties. Thus, the *in vitro* antioxidant activity of *M foetida* indicates that the plant extracts may contain compounds that are capable of donating hydrogen to a free radical in order to remove electrons responsible for the radicals reactivity.

Conclusion:

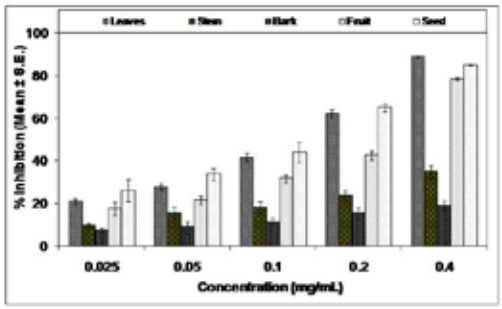
The results obtained in the present study revealed that *Nothapodytes foetida* exhibits powerful antioxidant as well as free radical scavenging activity. It was also observed that the leaves, fruits and seeds of *Nothapodytes foetida* possessed potent free radical scavenging ability as compare to stem and bark. In all the assays performed aqueous extract was found to possess higher antioxidant activity than methanolic extracts.

Acknowledgements:

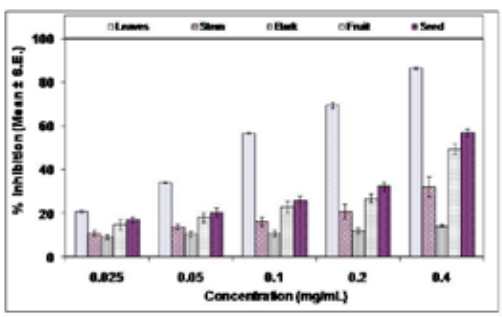
The authors acknowledge Krishna Institute of Medical Sciences for financial support to the experimental work and all the facilities. We greatly acknowledge Dr. R K. Ayachit, Director, Health Sciences of Krishna Institute of Medical Sciences for guidance & support to carry out this research.

Volume : 5 | Issue : 5 | May 2016 • ISSN No 2277 - 8179 | IF : 3.508 | IC Value : 69.48

Conflict of Interest: None declared

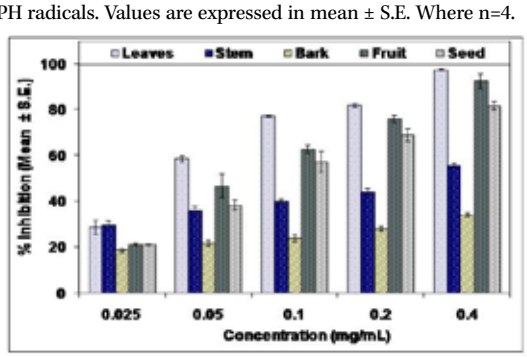


(A) DPPH radical scavenging Assay (Aqueous extract)

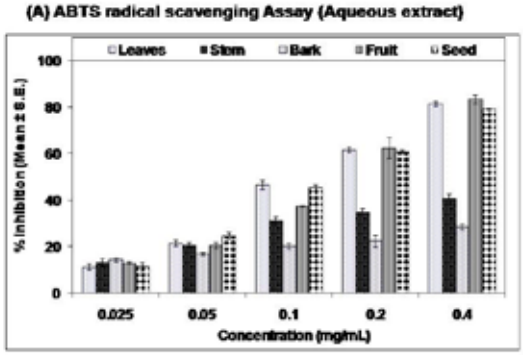


(B) DPPH radical scavenging Assay (MeOH extract)

Figure 1 (A) Effect of different concentrations of aqueous extract and (B) methanolic extracts of *N. foetida* on DPPH radical scavenging activity. The data represent the percentage inhibition on DPPH radicals. Values are expressed in mean ± S.E. Where n=4.

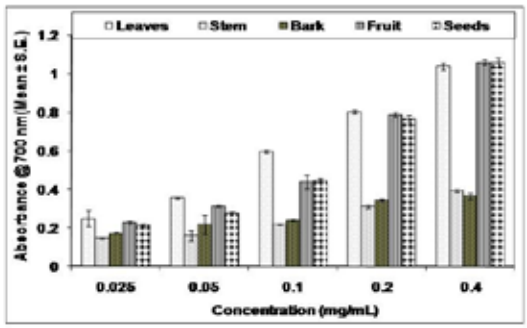


(A) ABTS radical scavenging Assay (Aqueous extract)

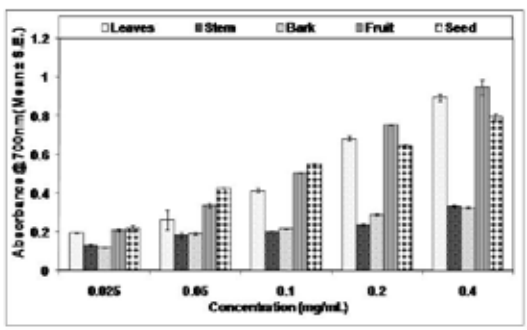


(B) ABTS radical scavenging Assay (MeOH extract)

Figure 2 (A) Effect of different concentrations of aqueous extract and (B) methanolic extracts of *N. foetida* on ABTS radical scavenging activity. The data represent the percentage inhibition on ABTS radicals. Values are expressed in mean ± S.E. Where n=4.

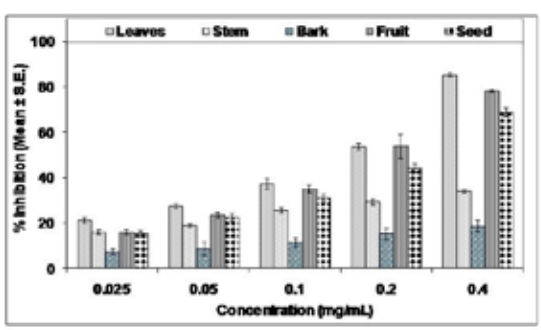


(A) Reducing power of aqueous extract of *N. foetida*

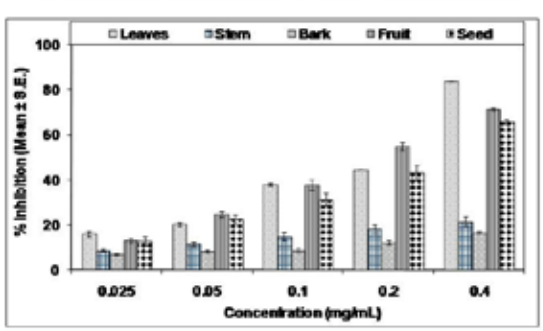


(B) Reducing power of methanolic extract of *N. foetida*

Figure 3 Reducing power of different concentrations of (A) aqueous extract and (B) methanolic extracts of *N. foetida*. The data represent the reducing power of *M. foetida*. Values are expressed in mean ± S.E. Where n=4.

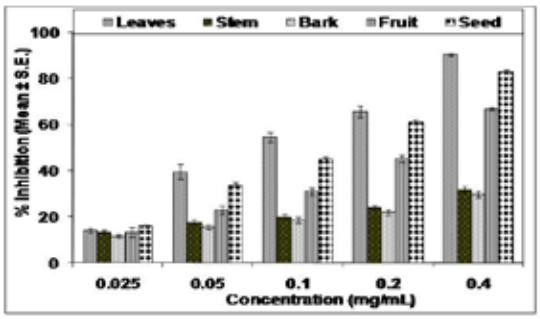


(A) Superoxide radical scavenging Assay (Aqueous extract)

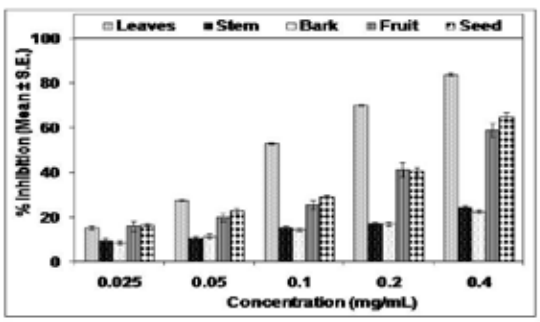


(B) Superoxide radical scavenging Assay (MeOH extract)

Figure 4 (A) Effect of different concentrations of aqueous extract and (B) methanolic extracts of *N. foetida* on superoxide radical scavenging activity. The data represent the percentage inhibition on superoxide radicals. Values are expressed in mean ± S.E. Where n=4.



(A) Hydroxyl radical scavenging Assay (Aqueous extract)



(B) Hydroxyl radical scavenging Assay (MeOH extract)

Figure 5 (A) Effect of different concentrations of aqueous extract and (B) methanolic extracts of N. foetida on hydroxyl radical scavenging activity. The data represent the percentage inhibition on hydroxyl radicals. Values are expressed in mean ± S.E. Where n=4.

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