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Assessment of *in vitro* Antioxidant Potential of Whole Plant of *Naregamia alata* W. & A.

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Abstract: As a new source of natural antioxidants, there is now an expansion of interest in phytochemicals to be used in foods and pharmaceutical preparations to substitute synthetic antioxidants, which are being restricted due to their potential health risks and toxicity. The present investigation deals with the *in vitro* antioxidant activity of different solvent extracts of whole plant of *Naregamia alata* using various antioxidants model systems viz., DPPH, Hydroxyl, superoxide and $ABTS^{+}$ radical cation scavenging activities. The ethanol extract of *N. alata* whole plant showed strong superoxide radical scavenging activity. The reducing power of the extracts were determined and compared with standard ascorbic acid. The reducing power increased with increase in concentration. Among the solvent tested, methanol extract of *N. alata* possessed strong reducing activity. The antioxidants that are present in the whole plant of *N. alata* studied in this investigation could be used to inhibit or prevent the harmful consequences of oxidative stress. The antioxidant effect could be related to the free radical scavengers like flavonoids and phenolic compounds.
Keywords: *Naregamia alata*, DPPH, Hydroxyl, Superoxide, ABTS.

INTRODUCTION

Reactive Oxygen Species (ROS), hydrogen peroxide (H_2O_2) and hypochlorous (HoCl) acid free radicals, hydroxyl radical (OH) and superoxide anion (O_2-) are produced as cellular metabolism's normal products. Rapid production of free radicals can cause oxidative damage to biomolecules. It may result in disorders such as cancer, diabetes, inflammatory disease, asthma, cardiovascular diseases, neurodegenerative diseases and premature ageing [1].

Many varieties of medicinal plants contain large amounts of antioxidants: polyphenols, vitamin C, vitamin E, selenium, β-carotene, lycopene, lutein and other carotenoids. These antioxidants are vital as they are involved in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [2]. Flavonoids and terpenoids are the plant secondary metabolites which play a vital role in defense against free radicals [3].

Various studies signify the data that a vast diversity of natural antioxidants like phenolics, flavonoids and tannins exist in the medicinal plants and it has more potent antioxidant activity than common dietary plants. These antioxidant compounds can be isolated and it is used for the prevention and treatment of free radical disorders [4]. As a result, concentration has enlarged recently to find naturally occurring antioxidants for use in food or medicine to replace synthetic antioxidants that are being restricted because of their carcinogenicity.

Naregamia alata commonly known as 'Nilanarakam' belongs to the family Meliaceae. It is a small branching undershrub and used traditionally for its curative property in treating anaemia, enlarged spleen, ulcers, rheumatism, malaria and acute dysentery. Root contains an alkaloid 'Naregamin', and other chemical constituents are Hannesane, β sitosterol, palmitic and stearic acid [5, 6]. Taking into the consideration of the medicinal importance of *Naregamia*, the present study has been undertaken to assess the total phenolic content, flavonoids and *in vitro* antioxidant properties of different solvent extracts of whole plant of *N. alata* using different models.

MATERIALS AND METHODS

Collection of Plant Material

Whole plant of *Naregamia alata* W. & A. was collected from Petchiparai, Kanyakumari District, Tamil Nadu. The collected samples were cut into small fragments and shade dried until the fracture is uniform and smooth. The dried flowers was granulated or powdered by using a blender and sieved to get uniform

particles by using sieve No. 60. The final uniform powder was used for the extraction of active constituents of the plant material.

Preparation of plant extract

In a Soxhlet apparatus for 24 hrs, the coarse powder (100 g) of the whole plant of *N. alata* was extracted sequentially with petroleum ether, benzene, ethyl acetate, methanol and ethanol, each 250 ml. Using Whatman No.41 filters paper, all the extracts were filtered and concentrated in a rotary evaporator. The concentrated extracts were used for *in vitro* antioxidant activity. The methanol extract was used for the estimation of total phenolics and flavonoids.

Estimation of total phenolic content

Total phenolic contents were estimated using Folin-Ciocalteau reagent based assay as previously described [7] with little modification. To 1 mL of each extract (100 µg/mL) in methanol, 5 mL of Folin-Ciocalteau reagent (diluted ten-fold) and 4 mL (75 g/L) of Na_2CO_3 were added. The mixture was allowed to stand at 20ºC for 30 min and the absorbance of the developed colour was recorded at 765 nm using UV-VIS spectrophotometer. For calibration curve 1mL aliquots of 20, 40, 60, 80, 100 µg/mL methanolic gallic acid solutions were employed as standard. The absorbance of solution and gallic acid calibration curve are compared. The total phenolic content was stated as gallic acid equivalents (GAE g/100g dry weight of extract).

Estimation of flavonoids

The flavonoids content was determined according to Eom *et al* [8]. An aliquot of 0.5ml of sample (1 mg/mL) was mixed with 0.1 mL of 10% aluminium chloride and 0.1 mL of potassium acetate (1 M). In this mixture, 4.3 ml of 80% methanol was added to make 5 mL volume. This mixture was vortexes and the absorbance was measured spectrophotometrically at 415 nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

DPPH radical scavenging activity

A stable free radical and is extensively used to assess the radical scavenging activity of antioxidant component is the DPPH. This method is supported by the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the creation of the non radical form DPPH-H [9].

According to the previously reported method [18] the free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) Briefly, an 0.1 mM solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3 mL of the solution of all extracts at different concentration (50,100,200,400 & 800 µg/mL). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the subsequent formula.

$$\% \text{ inhibition} = \{(A_0 - A_1)/A_0)*100\}$$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed thrice and the results were averaged.

Hydroxyl radical scavenging activity

According to the modified method of Halliwell [10] the scavenging capacity for hydroxyl radical was calculated. Stock solutions of EDTA (1 mM), $FeCl_3$ (10 mM), Ascorbic Acid (1 mM), H_2O_2 (10 mM) and Deoxyribose (10 mM) were prepared in distilled deionized water. The assay was executed by adding 0.1 mL EDTA , 0.01 mL of $FeCl_3$**,** 0.1 mL H_2O_2, 0.36 mL of deoxyribose, 1.0 mL of the extract of different concentration (50,100,200,400 & 800 µg/mL) dissolved in distilled water, 0.33 mL of phosphate buffer (50 mM , pH 7.9), 0.1 mL of ascorbic acid in succession. The mixture was then kept warm at 37^0C for 1 hour. 1.0 mL portion of the incubated mixture was mixed with 1.0mL of 10%TCA and 1.0mL of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm. The percentage inhibition was computed by comparing the results of the test with those of the control using the above formula.

Superoxide radical scavenging activity

As described by Srinivasan *et al* [11] the superoxide anion scavenging activity was measured. The superoxide anion radicals were produced in 3.0 ml of Tris – HCL buffer (16 mM, pH 8.0), containing 0.5 mL of NBT (0.3 mM), 0.5 mL NADH (0.936 mM) solution, 1.0 mL extract of different concentration (50,100,200,400 & 800 µg/mL), and 0.5 mL Tris – HCl buffer (16mM, pH 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12 mM) to the mixture, kept warm at 25ºC for 5 min. The absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was computed by comparing the results of the test with those of the control using the above formula.

Antioxidant activity by radical cation (ABTS +)

ABTS assay was based on the slightly modified method of Huang *et al* [12]. ABTS radical cation (ABTS+) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate. The

mixture was then allowed to stand in the dark at room temperature for 12-16 h before use. The ABTS + Solution were diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm. Exactly after 6 minutes, the absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) after addition of sample or trolox standard to 3.9 mL of diluted ABTS+ solution, Results were expressed as trolox equivalent antioxidant capacity (TEAC). The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Reducing power

The reducing power of the extract was determined by the method of Kumar and Hemalatha [13]. 1.0 mL of solution containing 50,100,200,400 & 800 µg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 mL, 1.0%): The mixture was incubated at 50^{o}C for 20 minutes. Then 5mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5^{o}C) in a refrigerator centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

STATISTICAL ANALYSIS

Antioxidant activities like DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical activity, ABTS radical cation scavenging activity and reducing powers were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS Inc., Chicago IL, USA) Estimates of mean, standard error for aforesaid parameters were calculated.

RESULTS AND DISCUSSIONS

Plant phenols, one of the main groups of compounds act as prime antioxidants or free radical terminators [14]. Phenolic compounds which act as the chief components own a substantial role in nutritional values, organoleptic properties, commercial properties and stabilization of lipid peroxidation because of the scavenging skill of their hydroxyl group [15]. Flavonoids and other phenolic compounds contain effective water soluble antioxidants and free radical scavengers. This is because it prevents oxidative cell damage and exhibit strong anticancer activity [16, 17, 18]. Flavonoids possess important effects on human health which are employed to treat hypertension and diabetes [19]. Further, they have antioxidant, antimicrobial, anticarcinogenic and antiinflammatory activities and influence against diarrhea [20, 21, 22]. Intaking flavonoids prevents coronary heart disease and owns a protective role in our diet [23, 24]. Flavonoids's antioxidant properties depend on their structure, specifically on the hydroxyl position in the molecule, and also as their strength as electron donor to a free radical [25]. Hence the total amount of phenolics and flavonoids in the plant extract chosen for the study has to be essentially determined. Compounds like total phenolics and flavonoids exist in *N. alata* whole plant extract may contribute credence to its local usage for the management of oxidative stress persuaded ailments.

The total phenolic and flavonoid content of the methanol extract of whole plant of *N. alata* were found to be 0.98 g 100 g^{-1} and 1.08 g 100 g^{-1} respectively. *In vitro* antioxidant activity of the petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *N. alata* whole plant were investigated in the present study by DPPH, hydroxyl, superoxide and ABTS radical cation scavenging activities. These methods have proven the effectiveness of the extracts in comparison to that of the reference standard antioxidants, ascorbic acid and trolox.

With a characteristic absorption at a wavelength of 517nm DPPH is a stable free radical compound. Antioxidants upon interaction with DPPH either transfer an electron or hydrogen atom to DPPH. This neutralizes its free radical characters and the colour of the reaction mixture changes from purple to yellow resulting in an absorbance decrease. The degree of discolouration indicates the scavenging potential of the antioxidants [26]. In the present study among the five solvent extracts, methanol extract of *N. alata* whole plant showed highest DPPH radical scavenging activity. The present study explains that the methanol extracts have the proton donating ability. This could also serve as free radical inhibitors or scavenging, acting possibly as primary antioxidants.

DPPH radical scavenging action of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of whole plant of *N. alata* were represented in figure 1.

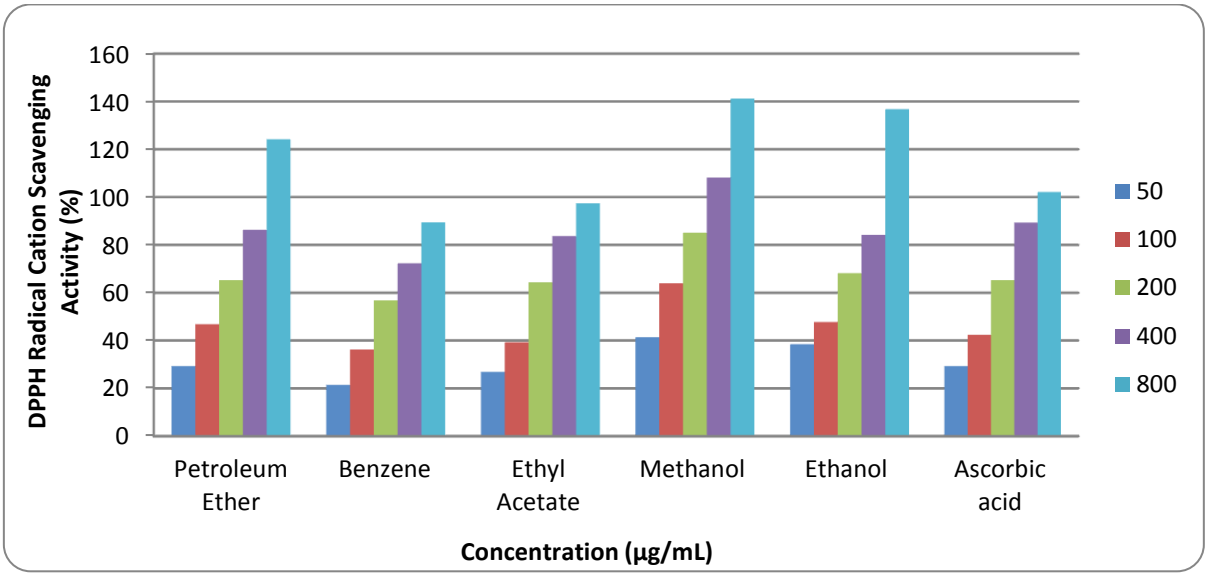

Fig-1: **DPPH radical scavenging activity of different extracts of whole plant of *N. alata***

The different solvent extracts of *N. alata* whole plant demonstrated a concentration dependent scavenging activity by quenching DPPH radicals. Among the solvents tested, methanol extract exhibited highest DPPH radical scavenging activity. At 800 µg/mL concentration methanol extract of *N. alata* showed 141.18% scavenging activity on DPPH. The concentration of methanol extract of *N. alata* whole plant needed for 50% inhibition (IC_{50}) was found to be 39.06 µg/mL, whereas 31.45 µg/mL was needed for ascorbic acid (Table 1).

Hydroxyl radical is an extremely reactive and highly damaging free radical species. This also has the capacity to join nucleotides and cause strand breakage in DNA leading to possible carcinogenesis, cytotoxicity and mutagenesis [27]. Hydroxyl radical scavenging capacity of *N. alata* whole plant extract is directly related to its antioxidant activity. This method involves *in vitro* generation of hydroxyl radicals using Fe^{3+}/ascorbate/EDTA/H_2O_2 system using Fenton reaction. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid [28]. When *N. alata* extract was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction. Among the solvents treated methanol possessed more hydroxyl radical scavenging activity when compared with standard ascorbic acid.

Hydroxyl radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *N. alata* whole plant was shown in figure 2.

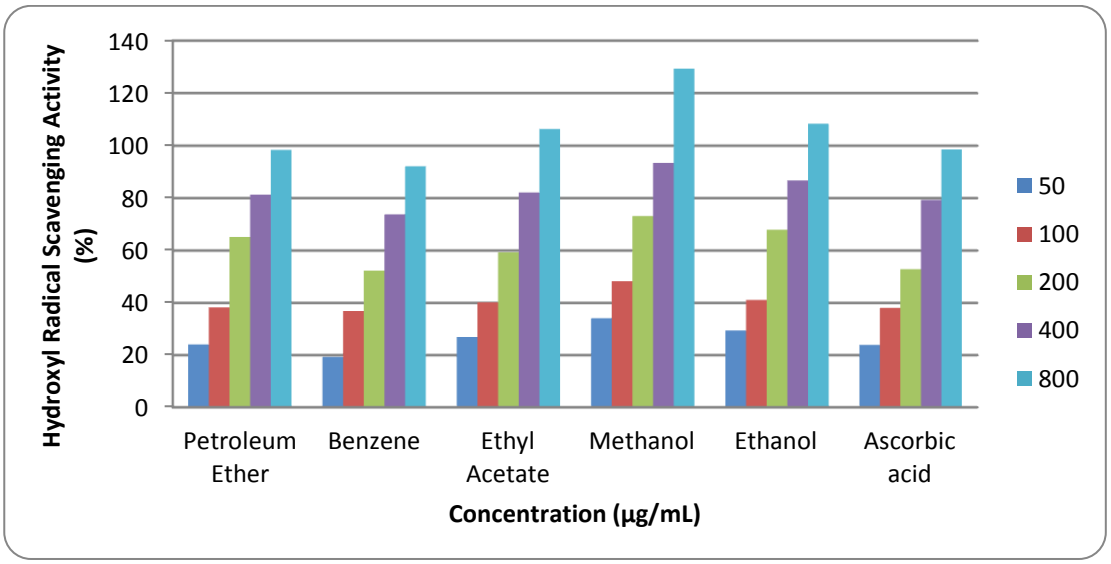

Fig-2: **Hydroxyl radical scavenging activity of different extracts of whole plant of N. *alata***

Methanol extract showed very potent hydroxyl radical scavenging activity. At 800 µg/mL concentration, *N. alata* whole plant exhibited 129.36% scavenging activity on hydroxyl radical. The IC_{50} value of methanol extract of *N. alata* whole plant on hydroxyl radical were found to be 38.94 µg/mL and 29.49 µg/mL for ascorbic acid, respectively (Table 1).

Superoxide anion is an initial free radical and a weak oxidant. This ultimately produces stronger oxidative species such as singlet oxygen species and hydroxyl radicals. At similar concentration the *N. alata* whole plant extracts showed a potent superoxide radical scavenging activity in a concentration dependent manner when compared with standard ascorbic acid. Ethanol extract showed potent superoxide radical scavenging activity with IC_{50} value 42.86 µg/mL compared to ascorbic acid 29.56 µg /ml. Therefore, studying the scavenging activity of plant extract on superoxide radical is one of the most important ways of clarifying the mechanism of antioxidant activity.

The different solvent extracts of *N. alata* whole plant were subjected to superoxide radical scavenging assay and the results were shown in figure 3.

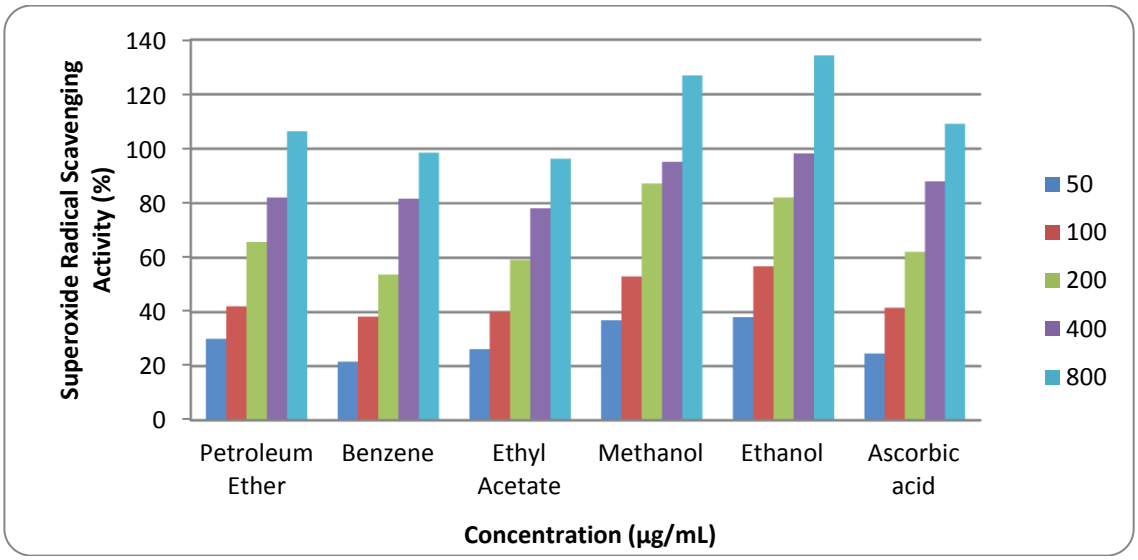

Fig-3: **Superoxide radical scavenging activity of different extracts of whole plant of *N. alata***

It indicates that ethanol extract of *N. alata* whole plant (800 mg/mL) exhibited the maximum superoxide radical scavenging activity of 134.22% which is higher than the standard ascorbic acid whose scavenging effect is 29.56%. Results showed the percentage of inhibition in a dose dependent manner. The quantity of *N. alata* whole plant ethanol extract required to produce 50% inhibition of superoxide radical was 42.86 µg/mL whereas 29.56 µg/mL was needed for ascorbic acid (Table 1).

The ABTS method depends on the inhibition of the absorbance of radical cation ABTS, which has a feature wave length at 734nm. Decolorization of ABTS reflects the capacity of the antioxidant species to donate electrons or hydrogen atoms to inactivate these radical actions. In the presence of antioxidant reductant, the colored radical is converted back to colorless ABTS [29]. ABTS radical cation scavenging activity is relatively recent, often used for screening complex antioxidant mixtures such as plant extracts, and involves a more drastic radical, chemically produced [30]. The prevent study, methanol extract of whole plant of *N. alata* were fast and effective scavenging of ABTS radical and this activity was higher than that of standard trolox.

The different solvent extracts of *N. alata* whole plant were subjected to be ABTs radical cation scavenging activity and the results were shown in figure.4.

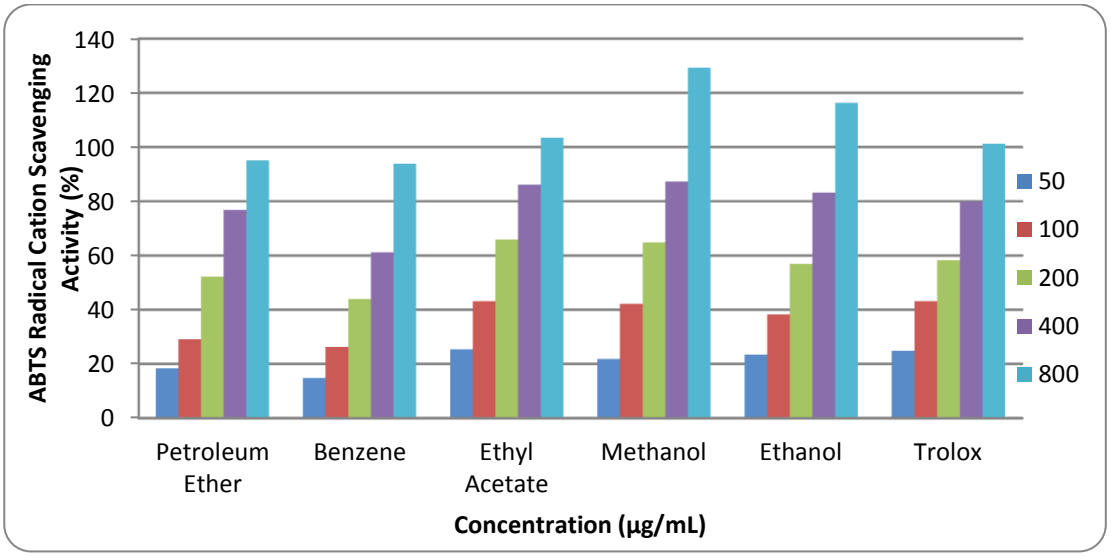

Fig-4: **ABTS Radical scavenging activity of different extracts of whole plant of *N. alata***

The methanol extract exhibited potent ABTS radical cation scavenging activity in concentration dependent manner. At 800 µg/mL concentration, *N. alata* whole plant showed 129.31% scavenging activity an ABTS which is higher than the standard trolox whose scavenging activity is 101.22%. The IC_{50} value of methanol extract of *N. alata* whole plant on ABTS radical were found to be 41.84 µg/mL and 33.50 µg/mL for trolox, respectively (Table 1).

From the formation of Perl's Prussian blue colour complex the reducing power of the *N. alata* extracts was assessed using ferric to ferrous reducing activity as determined spectrophotometrically [31]. The reducing power of different extracts of *N. alata* whole plant was compared with ascorbic acid. Among the extracts, methanol extract exhibited the most reducing power which was found even more than ascorbic acid.

Figure 5 showed the reducing ability of different solvent extracts of *N. alata* whole plant compared to ascorbic acid.

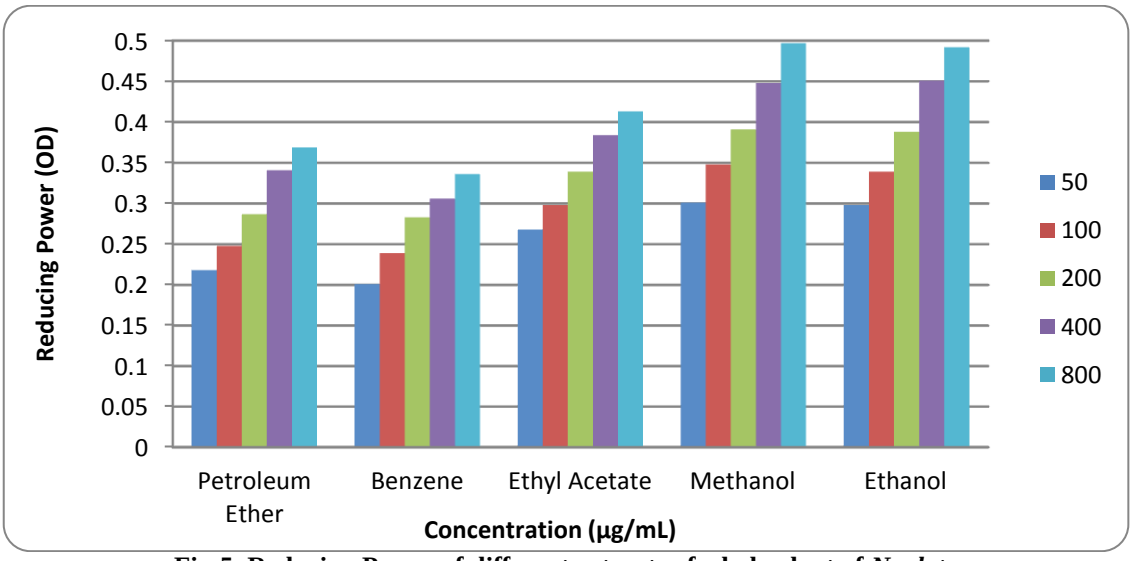

Fig-5: **Reducing Power of different extracts of whole plant of *N. alata***

Absorbance of the extracts was increased when the concentration increased. A higher absorbance indicates a higher reducing power. Among the solvent tested, methanol extract exhibited higher reducing activity.

Table-1: **IC_{50} values of different extracts of whole plant of *N. alata***

Solvent	IC_{50} (µg/mL)			
	DPPH Radical Scavenging Activity	Hydroxyl Radical Scavenging Activity	Superoxide Radical Scavenging Activity	ABTS Radical Cation Scavenging Activity
Petroleum Ether	34.89	27.23	34.85	27.38
Benzene	24.92	26.18	26.88	24.92
Ethyl Acetate	28.36	33.56	29.65	32.82
Methanol	39.06	38.94	37.83	41.84
Ethanol	38.24	32.83	42.86	36.84
Ascorbic Acid	31.45	29.49	29.56	-
Trolox	-	-	-	33.56

CONCLUSION

Based on the results of the prevent study, it can be suggested that the extracts of *N. alata* whole plant possess antioxidant effects. Almost all extracts exhibited potential antioxidant activity. Methanol extract showed highest antioxidant activity. Presence of total phenolics and total flavonoids in *N. alata* whole plant might be responsible for DPPH, hydroxyl, superoxide and ABTS radical scavenging capacities and reducing power. This indicated that *N. alata* contained potential antioxidant bioactive compounds, which if properly and extensively studied, could provide many chemically interesting and biologically active drug.

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