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Biochemistry

In Vitro Antioxidant Evaluation of Root Methanol Extract of *Naregamia Alata* W&A

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Abstract

The present research work was carried out to evaluate the antioxidant potential and phytochemical analysis of methanol root extract of *Naregamia alata*. Standard methods were adopted to screen antioxidant and phytochemical nature of the plant. Antioxidant and radical scavenging activity were determined by using different in vitro assays including DPPH (2, 2 –diphenyl -1-picryl hydrazyl radical) free radical scavenging assay, Hydroxyl radical scavenging assay, Nitric oxide scavenging assay, reducing power and superoxide free radical scavenging assay. Ascorbic acid was used as standard. The methanol root extract of the plant at concentrations of 12.5, 25, 50, 100 and 200 µg/mL were studied. The root extract of *Naregamia alata* showed significant antioxidant activity in a dose-dependent manner in all these assays. *In Vitro* antioxidant activity of the methanolic root extract showed good antioxidant power in DPPH assay (91.39%), Hydroxyl radical (76.90%) and Superoxide free radical scavenging assay (80.00%) with IC_{50} values 7.5µg/ml, 45µg/ml, and 20µg/ml respectively which is almost comparable to standard antioxidant ascorbic acid. The other two assays such as Nitric oxide scavenging (55.44%) with IC_{50} 82.5 µg/ml and Ferric reducing power (0.618) showed moderate scavenging activity. Results of phytochemical screening of the extract showed the presence of alkaloids, glycosides, terpenoids, phenols, tannin, flavanoids and coumarin.

Highlights

- Antioxidant activity of root extract incrases in a dose-dependent manner in all assays.
- The methanolic root extract of *Naragamia alata* possessed significant antioxidant activity.

Keywords: *Naregamia alata*, Antioxidant assays, Ascorbic acid

ROS are produced naturally as a by-product of cellular metabolism and have an important role in cell signaling and homeostasis. During times of environmental stress, ROS level can increase dramatically and this may result in significant damage to cell structures. Many diseases are caused by oxidative stress (Kumar *et al.* 2012). Accelerated cell oxidation contributes to age – related disorders such as cancer, hypertension, atherogenesis, Alzheimer's disease and Parkinson's disease. The antioxidants play a vital role in delaying, intercepting or preventing oxidative reactions catalyzed by free radical. Antioxidants eradicate chemicals in the body and protect against heart disease, arthritis, cancer and many other chronic diseases. Hence sufficient intake of antioxidants daily protects the cells from decomposition. Plant-derived antioxidant compounds can effectively eliminate the free radicals mediated toxicity, and many of these activities have been already reported for a wide range of plants (Sulekha *et al.* 2009) Therefore, search for natural antioxidant has significantly been increased in the recent scenario. The phytochemical constituents detected in the root extract of *Naregamia alata* could contribute to the traditional therapeutic use of the whole root. Alkaloids and glycosides have hypoglycaemic activities; anti-inflammatory activities (Augusti, 2008). Terpenoids exhibit various important pharmacological activities anti-inflammatory, anticancer, anti-malarial, inhibition of cholesterol synthesis, antiviral and anti-

bacterial activities (Kappers *et al.* 2005). Steroids showed the analgesic properties and central nervous system activities (Shaikh *et al.* 2010). Phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites and possess diverse biological properties including anti-apotosis, anti-aging, anti-carcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection, inhibition of angiogenesis and cell proliferation as well as the improvement of endothelial function (Han *et al.* 2007). Tannins have also received immense attention in many fields especially in the fields of nutrition, health and medicine due to their antioxidant, antimicrobial and anti-inflammatory activity (Buzzini *et al.* 2008). Flavonoids, a group of polyphenolics, are free radical scavengers, super antioxidants that have anti-inflammatory activity, prevent oxidative cell damage through their water soluble property and also possess strong anti-cancer activity (Gurib-Fakim, 2006). Coumarins are potential antioxidants, according to studies (Kostova *et al.* 2011), with the ability of scavenging free radicals and chelating metal ions.

The plant *Naregamia data* W&A. (Family: Meliaceae) Commonly known as "Nilanarakam" is a small branching undershrub. It is distributed throughout western and southern India. The literature survey showed scanty information available on this plant. It has antimicrobial property against gram-positive and gram negative bacteria. It is used in the treatment of itch, wounds, anaemia, enlarged spleen, ulcers, vitiated conditions of pitta, vata, halitosis, cough, dyspepsia and catarrh. The root is cholagogue, emetic, and expectorant, it is used in the treatment of rheumatism and acute dysentery. The leaves and stems, combined with bitters and aromatics, are given in decoction for the treatment of biliousness. It is used to prevent malaria, particularly useful in fever (Prajapathi *et al.* 2009).

Also, the previous studies on this herb have shown its pharmaceutical (Shinya *et al.* 2012), pharmacological (Jacob *et al.* 2012), antioxidant (Anagha, 2012), and antimicrobial (Mukesh *et al.* 2010) activities. The compounds such as sesquiterpenoids, caryophyllene oxide, daucene, beta-caryophyllene, and alpha-transbergamotene were reported essential oil of *Naregamia alata* (Geetha and Rameshkumar, 2010). Efforts to gain extensive knowledge regarding the relationship between phytochemical constituents and antioxidant activity of *Naregamia alata*,is an area of interest.

Methanolic extract of root was screened for their antioxidant activities by DPPH (2, 2 –diphenyl -1-picryl hydrazyl radical) free radical scavenging assay, Hydroxyl radical scavenging assay, Nitric oxide scavenging assay, reducing power and superoxide free radical scavenging assay. The methanol root extract of the plant at concentrations of 12.5, 25, 50, 100, and 200 µg/mL were studied. The objective of present study was to investigate the antioxidant and phytochemical analysis of methanolic root extract of *Naregamia alata.*

Materials and Methods

Plant materials

The plant root was collected from Elavoor, Ernakulam district, Kerala. The plant was identified based on its floral description given in the literature. The plant root was washed with running tap water and shade dried. The dried material was powdered using the mechanical method and stored in the air tight container.

Extraction of plant material

The root extract of *Naregamia alata* (50) gms was extracted exhaustively in a Soxhlet Apparatus with methanol, (250ml) for 12-16 hrs at 50°C. The extract obtained was later kept for evaporation to remove the excessive solvents. This crude extract was stored at low temperature in the refrigerator and used for evaluation of antioxidant activity.

Preliminary Phytochemical analysis

The root extract of *Naregamia alata was* screened for the presence of various bioactive compounds such as alkaloids, glycosides, terpenoids, phenols, tannin, flavanoids, and coumarin by using standard methods (Harborne, 1998).

Alkaloids (Dragendorff's method)

The extract was warmed with 10 ml of 2% sulphuric acid for 2 minutes and filtered. A known quantity of aliquot was treated with a few drops of Dragendorff's reagent (glacial acetic acid in a solution of bismuth nitrate and potassium iodide) orange-brown precipitate denoted the presence of alkaloids.

Glycosides (Keller – Killani test)

The extract was dissolved in distilled water and added with 2 ml of glacial acetic acid containing one drop of ferric chloride solution followed by 1 ml of concentrated sulphuric acid along the side of the test tube. The brown ring at the interface denoted the presence of glycosides.

Terpenoids (Libermann – Burchard method)

A little of the extract was dissolved in dry chloroform and added three drops of acetic anhydride followed by the addition of two to three drops of concentrated sulphuric acid. The appearance of green colour for steroids while pink colour indicated the presence of terpenoids.

Phenols (Lead Acetate test)

Alcoholic extract was diluted to 5 ml with distilled water and to this few drops of 1% aqueous solution of lead acetate was added. A yellow precipitate was formed, which indicates the presence of phenols.

Tannins (Ferric Chloride test)

To 1-2 ml of aqueous extract, few drops of 5% aqueous $Fecl_3$ solution were added. A bluish-black colour, which disappears in addition of a few ml of H_2SO_4, there is formation of the yellowish brown precipitate.

Flavonoids (Shinoda test)

The extract was dissolved in methanol and a few pinch of magnesium turnings followed by the addition of concentrated hydrochloric acid drop by drop. Presence of pink colour confirmed the presence of flavonoids.

Coumarins

The extract was dissolved in a few drops of alcoholic sodium hydroxide. The appearance of yellow colour on the addition of concentrated hydrochloric acid through the sides of the test tube indicated the presence of coumarins.

Antioxidant Assays

The antioxidant activity of plant material was assayed by employing the following methods.

DPPH assay

DPPH free radical scavenging assay was measured using the method of (Blois, 1958). Different volumes (12.5-200µg/ml) of plant extracts were made up to 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm.3ml of DPPH was taken as control. Ascorbic acid (10mg/ml DMSO) was used as a standard.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay was measured according to the method of (Halliwel *et al.* 1987). The reaction mixture contained in the final volume of 1 mL.2 deoxy 2 ribose (2.8mM) KH_2PO_4—KOH buffer (20 mM pH 7.4), FeCl3 (100µm), EDTA (100µm), H_2O_2 (1.0mM), ascorbic acid (100µm) and various concentrations (12.5-200 µg/ml) of the test sample. After incubation for 1hour at 37°C, 0.5 ml of the reaction mixture was added to 1ml of 2.8% TCA, then 1ml aqueous TBA was added and the mixture was incubated at 90°C for 15 minutes to develop the colour. After cooling the absorbance was measured at 532nm against an appropriate blank solution.

Nitric oxide scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction this assay was done by the method of (Marcocci *et al.* 1994). Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitro prusside (5mmolL^{-1})in phosphate buffered saline pH 7.4,was mixed with different concentration of the extract (12.5-200µg/ml)prepared in methanol and incubated at 25°C for 30minutes .A control without the test compound, but an equivalent amount of methanol was taken. After 30minutes, 1.5mL of the incubated solution was removed and diluted with 1.5mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diamine dihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphathyl ethylene diamine dihydrochloride was measured at 546nm and the percentage scavenging activity was measured concerning the standard.

Ferric reducing power assay

The reducing power of extract was determined by the method of Yen and Duh (1993). Different concentrations of extract (12.5-200µg/ml) were mixed with 2.5ml of phosphate buffer (200mM) (pH 6.6) and 2.5ml of 1% potassium ferric cyanide was added and boiled for 20 minutes at 50°C. After incubation, 2.5 ml of 10% TCA were added to the mixtures followed by centrifugation at 650xg it for 10 minutes The upper layer (5ml)was mixed with 5ml of distilled water and 1ml of 0.1% ferric chloride was added and the absorbance was read at 700nm.

Superoxide free radical scavenging assay

Superoxide free radical scavenging activity of extract determined by the method Nishikimi *et al.* 1972. Different concentrations of extracts (12.5-200µg/ml), 0.05ml of Riboflavin solution(0.12mM) , 0.2 ml of EDTA solution

[0.1M] and 0.1 ml NBT (Nitro-blue tetrazolium) solution [1.5mM] were mixed in test tube and reaction mixture was diluted up to 2.64ml with phosphate buffer [0.067M]. The absorbance of solution was measured at 560nm using DMSO as blank after illumination for 5 min and difference in OD was determined after 30 minutes incubation in fluorescent light. Absorbance was measured after illumination for 30 min. at 560 nm on UV visible spectrophotometer.

Results and Discussion

Phytochemical analysis conducted on the plant root extract revealed the presence of constituents such as alkaloids, glycosides, terpenoids, phenols, tannin, flavanoids, and coumarin. The results obtained confirm earlier reports of some of the phytochemical constituents found in the leaf extracts of *Naregamia alata* (Shinya *et al.* 2012). More research is required to determine the specific roles of these phytochemical constituents present in *Naregamia alata.*

Table 1. Phytochemical screening of methanol root extract of *Naregamia alata* W&A

Phytochemical Tests	Result
Alkaloid	+
Glycosides	+
Terpenoids	+
Phenols	+
Tannin	+
Flavanoids	+
Coumarin	+

Free radical scavenging and reducing power tests conducted in this study sought to establish the in vitro antioxidant properties of the plant root extract. The detection of phenolic compounds and other secondary metabolites in root extract strongly suggests the possible antioxidant activity of the extract. Phenolic antioxidants are potent free radical terminators and the high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups (Ben *et al.* 2013).

In vitro antioxidant assays

Several concentrations ranging from 12.5–200 µg/ml of the methanolic extract of root of *Naregamia alata* was tested for their antioxidant activity in different in vitro models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner up to the given concentration in all the models. The percentage scavenging and IC_{50} values were calculated for all models.

DPPH radical scavenging activity

The effect of antioxidants on DPPH radical was thought to be due to their hydrogen donating ability to the free radicals and reducing it to nonreactive species (Wang *et al.* 2008). The antioxidant compounds reduce the purple coloured DPPH radical to yellow. Here the reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidant. The DPPH radical scavenging activity of the methanol root extract at 200µg/ml was 91.39 % and that of the standard ascorbic acid was 81.49% (Table 1). The results showed that the methanol extract of root exhibited strong DPPH radical scavenging ability (IC_{50} value of 7.5 µg/ml) than the ascorbic acid (IC_{50} value of 22.5µg/ml) in (Figure 1). These observations support the earlier reports of Mohammed *et al.* (2012) who established the fact that ethanol extract of *Melia azedarach* showed highest antioxidant activity.

Table 2. DPPH Assay on the methanol root extract of *Naregamia alata*

Sample concentration (µg/ml)	% of inhibition	
	Root of *Naregamia alata*	Standard (Ascorbic acid)
12.5	70.32	46.52
25	72.10	51.15
50	79.82	73.77
100	82.78	78.40
200	91.39	81.49

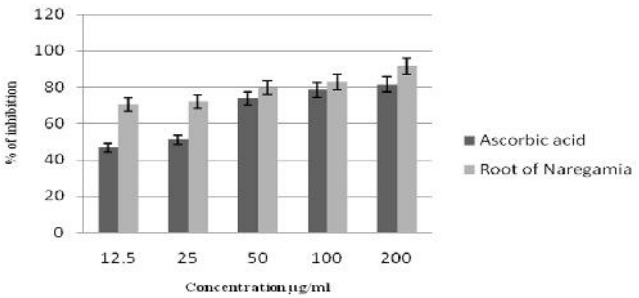


Fig. 1. DPPH assay on the methsnol root extract of *Naregamia alata* W&A

Hydroxyl radical scavenging activity

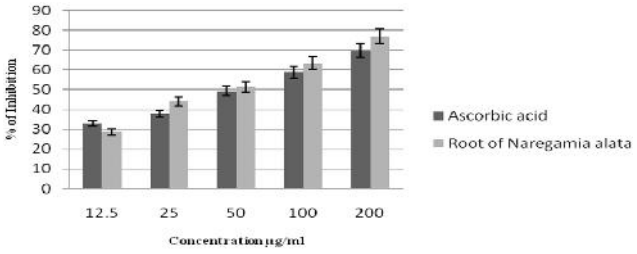
At a concentration of 200µg/ml, the scavenging activity of methanolic root extract and the standard ascorbic acid was found to be 76.90% and 69.77% respectively (Table-2). Of these, the methanolic root extract (IC_{50} value of 45µg/ml) was found to be more efficient compared with

the standard ascorbic acid (IC_{50} value of 55µg/ml) in quenching the hydroxyl radicals produced in the reaction mixture (Figure 2). The hydroxyl radical can induce oxidative damage to DNA, lipids, and proteins.

Table 3. Hydroxyl radical scavenging assay on the methanol root extract of Naregamia *alata*

Sample concentration (µg/ml)	% of inhibition	
	Root of *Naregamia alata*	Standard (Ascorbic acid)
12.5	28.50	33.00
25	44.22	37.90
50	51.47	49.34
100	63.39	58.82
200	76.90	69.77

µg/ml- Microgram/Millilitre



µg/ml- Microgram/ Millilitre

Fig. 2. Hydroxyl radical scavenging assay on the methanol root extract of *Naregamia alata* W&A

Nitric oxide scavenging Assay

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases (Panda *et al.* 2011). In the present study, the extract competes with oxygen to react with nitric oxide and thus inhibits generation of the anions. The percentage inhibition of nitric oxide generation by the extract in concentration 12.5, 25, 50, 100 and 200 µg/ml that significantly scavenged 38.14%, 41.34%, 46.79%, 52.56 and 55.44% of the nitric oxide radicals respectively (Table 3). The methanol extract of root showed the greatest NO scavenging effect of 55.44% at 200 µg/ml with IC_{50} values 82.5 µg/ml as compared to the standard ascorbic acid where 58.97% scavenging was observed at a similar concentration with IC_{50} value of 110 µg/ml. The result indicated that the extract might contain compounds able to inhibit Nitric oxide activity. The compounds such as flavonoids, which contain hydroxyl functional groups, are responsible for antioxidant effect in plants (Subathraa and Poonguzhali, 2012).

Table 4: Nitric oxide scavenging on the methanol root extract of *Naregamia alata*

Sample concentration (µg/ml)	% of inhibition	
	Root of *Naregamia alata*	Standard (Ascorbic acid)
12.5	38.14	34.29
25	41.34	38.78
50	46.79	39.42
100	52.56	49.67
200	55.44	58.97

µg/ml- Microgram/ Millilitre

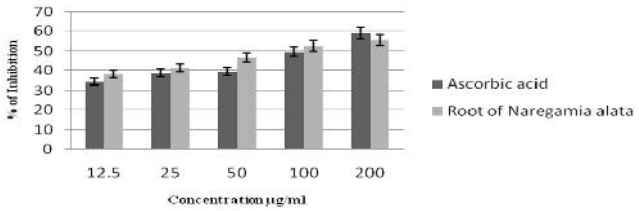


Fig. 3. Nitric oxide scavenging assay on the methanol rot extract of *Naregamia alata* W&A

Reducing power activity

Reducing the power of methanolic extract of root increased with increase in concentration. The methanolic root extract showed effective reductive ability when compared to that of standard ascorbic acid (Figure 4). At concentration 200µg/ml, methanol root extract and standard ascorbic acid showed absorbance with about 0.618 and 0.89 respectively (Table 4). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Reducing power is to the measure of the reductive ability of antioxidant and it is evaluated by the transformation of Fe^{3+} to Fe^{2+} in the presence of extracts (Kumar *et al.* 2012).

Table 5: Ferric reducing power assay on the methanol root extract of *Naregamia alata*

Sample concentration (µg/ml)	% of inhibition	
	Root of *Naregamia alata*	Standard (Ascorbic acid)
12.5	0.256	0.26
25	0.263	0.52
50	0.328	0.58
100	0.422	0.66
200	0.618	0.89

µg/ml- Microgram/ Millilitre

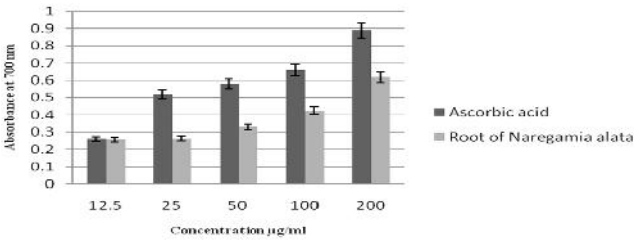


Fig. 4. Ferric reducing assay on methanol root extract of *Naregamia alata* W&A

Superoxide radical scavenging activity

The maximum inhibition was found to be 80.00% for methanolic root extract and 81.81 % for ascorbic acid at 200µg/ml (Table 5). The methanolic root extract exhibited an IC_{50} value of 20µg/ml and standard ascorbic acid was 20µg /ml (Figure 5). The methanolic root extract was found to be an efficient scavenger of superoxide radical. Superoxide anion radical is one of the strongest ROS among the free radicals and gets converted to other harmful reactive oxygen species such as hydrogen peroxide and hydroxyl radical, damaging biomolecules which results in chronic diseases (Al-Mamun *et al.* 2007)

Table 6: Superoxide free radical scavenging assay on the methanol root extract of *Naregamia alata*

Sample concentration (µg/ml)	% of inhibition	
	Root of *Naregamia alata*	Standard (Ascorbic acid)
12.5	29.09	41.41
25	58.18	54.54
50	72.72	63.63
100	76.36	72.72
200	80.00	81.81

µg/ml- Microgram/ Millilitre

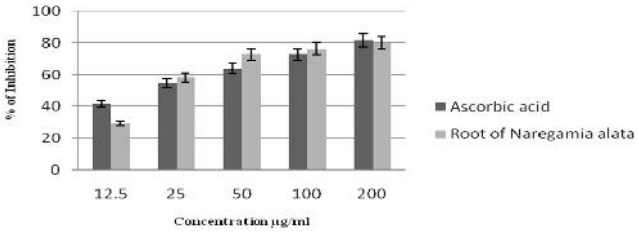


Fig. 5. Superoxide free radical scavenging assay on the methanol root extract of Naregamia alata W&A

Conclusion

This study suggests that the methanol root extract of *Naregamia alata* have many bioactive compounds which show antioxidant activity. The methanol root extract of *Naregamia alata* exhibited significant antioxidant activity compare to standard ascorbic acid. The root extract might be helpful in preventing and slowing the progress of various oxidative stress induced diseases. It might be useful for the development of newer and more potent natural antioxidants. The findings of the present study suggest that *Naregamia alata* could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of oxidative stress related degenerative diseases. These results might be helpful for providing the platform for researchers for the development of precious medicines which will be helpful for treatment of oxidative stress generated diseases.

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