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Research Article

Phytochemical Screening and *in Vitro* Antioxidant Activity of Methanolic Extract of Flowers of *Allamanda neriifolia Hook*

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ABSTRACT
Antioxidants play the essential role in reducing the free radicals. Methanolic extract of *Allamanda neriifolia hook* flower was studied for its *in vitro* antioxidant activity using different models of screening *viz.* DPPH and ABTS radical scavenging activity, reducing power ability and Nitric oxide scavenging activity. The extract showed a good dose dependent free radical scavenging property in all the models. Phytochemical analysis revealed the presence of major phyto compounds like alkaloids, flavonoids, phenol, tannins, glycosides and terpenoids. Its antioxidant activity was estimated by IC50 value and the values are 192.0µg/ml (DPPH radical scavenging), 127.0 µg/ml (ABTS radical scavenging), and 147.0 µg/ml (nitric oxide scavenging). The antioxidant property may be related to the polyphenols and flavonoids present in the extract. It indicates that the methanolic extract of the flower has the potency of scavenging free radicals in vitro and may provide leads in the ongoing search for natural antioxidants to be used in treating diseases related to free radical reactions.

Keywords: Antioxidants, Free radicals, *Allamanda neriifolia hook*, free radical scavenging activity, total phenolic content, flavonoids.

INTRODUCTION

Free radicals are atoms, molecules or ions with unpaired electrons, which are highly active to chemical reactions with other molecules. In the biology system, the free radicals are often derived from oxygen, nitrogen and sulphur molecules. These free radicals are parts of groups of molecules called reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulphur species (RSS). ROS are produced during cellular metabolism and functional activities, and have important roles in cell signalling, apoptosis, gene expression and iont transportation[1]. However, excessive amounts of ROS can have deleterious effects on many molecules including protein, lipid, RNA and DNA since they are very small and highly reactive. ROS can attack bases in nucleic acids, amino acid side chains in proteins and double bonds in unsaturated fatty acids, in which ▪OH is the strongest oxidant. ROS attacking macromolecules is often termed oxidative stress. However, during times of environmental stress and cell dysfunction, ROS levels can increase dramatically, and cause significant cellular damage in the body. Thus, oxidative stress significantly contributes to the pathogenesis of inflammatory disease, cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts, autism and aging[2,3]. In order to prevent or reduce the ROS-induced oxidative damage, Cells are equipped with different kinds of mechanisms that includes enzymatic, metal chelating and free radical scavenging activities to neutralize these radicals after they have formed. In addition, When the mechanism of antioxidant protection becomes unbalanced in human body, antioxidant supplement may be used to help reduce oxidative damage. Antioxidants are substances that can neutralize free radicals by accepting or donating electron(s) to eliminate the unpaired condition of the radical. Medicinal plants are an important source of antioxidants[4]. Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of certain diseases such as cancer, heart diseases and stroke[5]. The secondary metabolites like phenolics and flavonoids from plants have been reported to be potent free radical scavengers. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark[6]. There are many synthetic antioxidants in use. It is reported, they have several side effects[7] such as risk of liver damage and carcinogenesis in laboratory animals. There is therefore a need for more effective, less toxic and cost effective antioxidants. Comparative advantages, hence the growing interest in natural antioxidants from plants. Research for natural antioxidants agents have become increasingly important due to health implications since the synthetic antioxidant such as BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole), and TBHQ (*tert*-butyl hydroquinone) could be toxic and could cause carcinogenic effects[8]. For this reason, there is an increasing interest in identifying natural antioxidant sources from medicinal plants to replace synthetic antioxidants and eliminate these health concerns. Several medicinal plants (Rasayana) have also been extensively used in the Indian traditional (Ayurveda) system of medicine for the treatment of number of diseases[9]. Some of these plants

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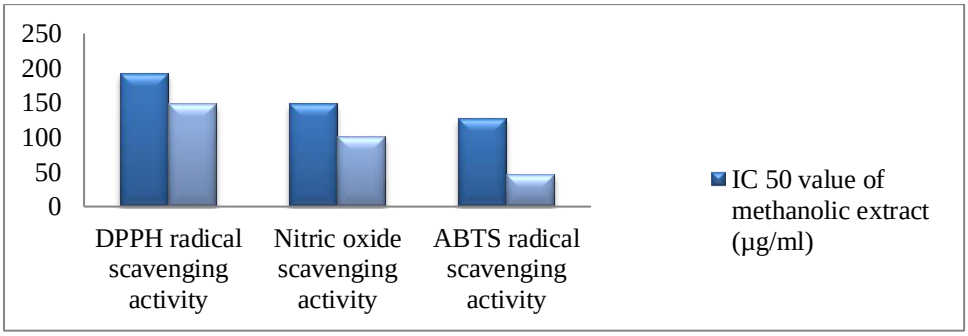

Figure 1: Comparison of IC 50 values of methanolic extract with standard

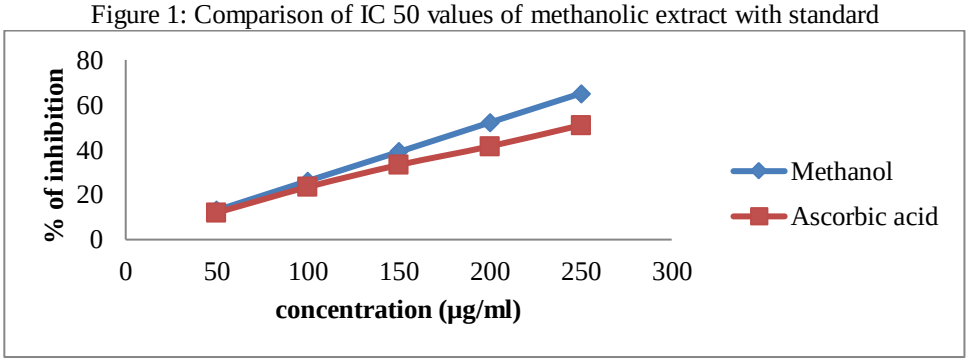

Figure 2: DPPH scavenging activity of methanolic extract of flowers of *Allamanda neriifolia hook*

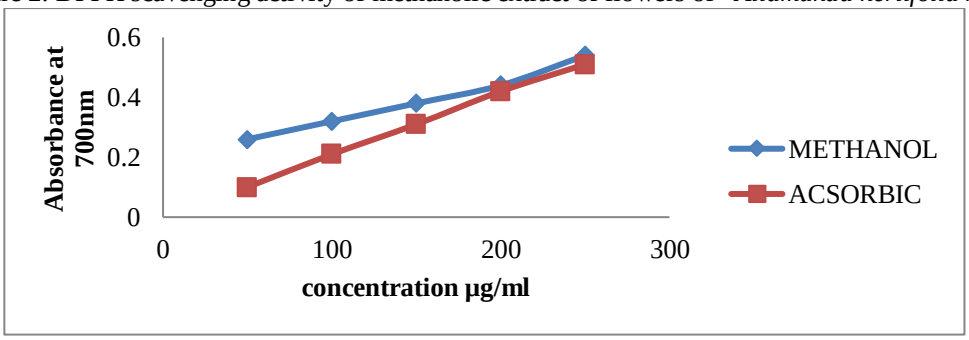

Figure 3: Reducing power abilityof methanolic extract of flowers of *Allamanda neriifolia hook*

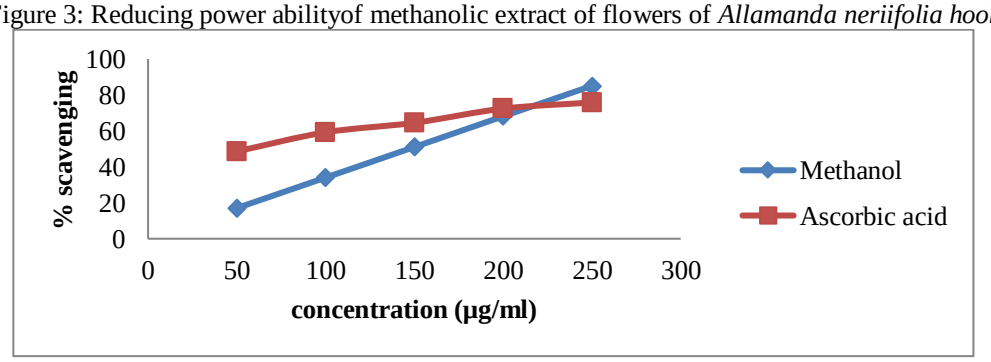

Figure 4: Nitric oxide scavenging activity of methanolic extract of flowers of *Allamanda neriifolia hook*

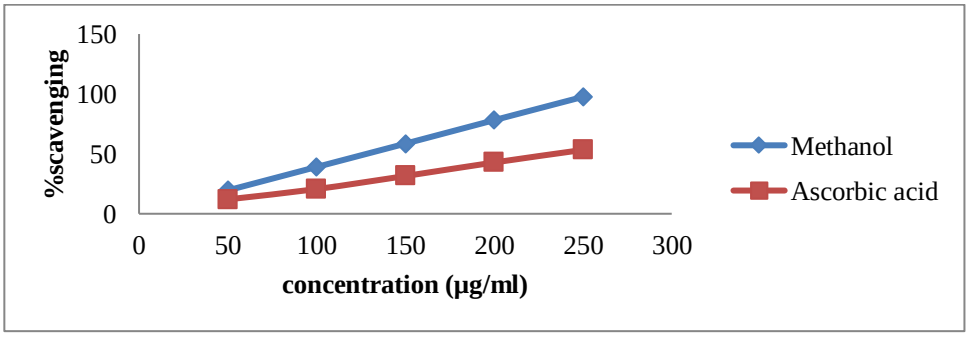

Figure 5: A BTS radical scavenging activity of methanolic extract of flowers of

have shown potent antioxidant activity[10]. *Allamanda* is a genus of flowering plants in the dogbane family, Apocynaceae. They are native to the Americas, where they are distributed from Mexico to Argentina. Some species are familiar as ornamental plants cultivated for their large, colorful flowers. Most species produce yellow flowers *Allamanda* also known as angel's trumpt, golden trumpet, yellow bell, is an evergreen tree, shrubs or wines they contain white latex. *Allamanda* species have been used in systems of traditional medicine for various purposes.

MATERIALS AND METHODS

Collection of plant

The selected medicinal plant *Allamanda neriifolia hook* was collected from Thiruvarur (Dt), Tamil Nadu, India. The Plant was authentified by Dr.S.JohnBritto, The Director, The RAPINAT Herbarium and Centre for Molecular Systematic, in St.Joseph'sCollege,Tiruchirappalli. The flower was washed well and dust was removed from the flower and were dried at room temperature. These dried materials were macerated to powder form with a mixer grinder and stored in air tight container for further use.

Preparation of extracts

The coarsely powder was packed into soxhlet column and extracted with 70% methanol for 48 hours (64.5-65.5℃). The extract was concentrated under reduced pressure (bath tem 50℃) then the dried extract was stored in air tight container for further use.

Phytochemical screening

Phytochemical screening of the extract was carried out to identify the secondary metabolites such as alkaloids (Mayer's and Draggendorff's test), flavonoids (Shinodatest), terpenoids (Salkowski test), tannins (gelatin test), saponins (Frothing test), cardiac glycosides (Keller-Killiani test) and phenols (ferric chloride test) according to standard phytochemical methods as described by[11].

Determination of of total flavonoids

The total flavonoid content in the extracts were determined by the method of[12] different concentration of extracts in methanol (3 ml) was mixed with 0.3ml of 10 % aluminum chloride followed by 2 ml of 1 M sodium hydroxide solution. Add 4 ml of water and kept for incubation at room temperature for 30 min. The absorbance was measured at 415 nm. The total flavonoid content is expressed as Quercetin equivalent (mg/100 g) of the dried weight. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically.

Determination of Total Phenolic Content

The total phenolic content was determined by the method[13] different concentration of extracts were made up to 3.5 ml, then 0.5 ml of Folin-Ciocalteu reagent followed by 2 ml of 7.5 % sodium carbonate solution. The above solution is incubated at room temperature for 10 min and absorbance was measured at 650 nm. Total phenolic content are expressed as gallic acid equivalent (mg/g) of the dried weight.All determinations were carried out in triplicate.

In Vitro Antioxidant Activity

Determination of total antioxidant capacity (phosphomolybednum antioxidant assay)

The total antioxidant capacity of the extracts was evaluated by the phosphomolybednum assay method of [14] which is based on the reduction of Mo (VI) to Mo (V) by the compounds and subsequent formation of a green phosphate - Mo (V) complex in acidic condition. 0.3ml of the extracts were combined with 3ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and the reaction mixture in the tubes were capped and incubated in a boiling water bath at 95℃ for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 3 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under the same conditions. The antioxidant capacity was expressed as the number of gram equivalents of ascorbic acid which was also processed and incubated under the same conditions.

DPPH Radical Scavenging Assay

The free radical scavenging activity of the different extracts were measured *in vitro using* 1,1-diphenyl-2-picrylhydrazyl (DPPH) by[15] About 200 µM solution of DPPH in 75% Ethanol was prepared and 0.5ml of this solution was added to 3 ml of different extracts dissolved in ethanol at different concentrations (50- 250 µg/ml). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance of the solution was measured at 517 nm against a blank using spectrophotometer. The % scavenging activity at different concentrations was determined and the IC50 value of the fractions was compared with that of ascorbic acid, which was used as the standard. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical scavenging ability. DPPH radical scavenging activity was calculated according to the following equation:

Scavenging effect (%) = [(A0-A1)/A0 x 100]

The results were expressed as mean values ± standard deviation. The extract concentration providing 50% inhibition (IC50) was calculated from the graph of scavenging effect percentage against the extract concentration. Ascorbic acid was used as standard.

Reducing Power Assay

Like the antioxidant activity, the reducing power increased with increasing amount of the extract.when potassium ferricyanide react with ferric chloride in the present of antioxidant, potassium ferrocyanide and ferrous chloride are found as a product. Presence of reducers causes the conversion of the Fe3+/ferricyanide complex used in this method to the ferrous formThe reducing power was determined by the Fe3+ and Fe2+ transformation in the presence of fractions described by[16].

The Fe2+can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. One ml of the different extracts (50-250 µg/ml), 2.5ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide was incubated at 500C for 30 min and 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. About 2.5 ml of supernatant was diluted with 2.5 ml of water and shaken with 0.5 ml of freshly prepared 0.1% ferric chloride

Table 1: Qualitative analysis of aqueous and methanolic extract of flowers of Allamanda neriifolia hook

S. No	Phytoconstituents	Allamanda neriifolia hook	
		Aqueous	Methanol
1	Alkaloids	+	+
2	Flavonoids	-	+
3	Phenol	+	+
4	Glycosides	+	+
5	Tannins	-	+
6	Terpenoids	+	+

(+) and (-) signs indicates the presence and absence of the compound

Table 2: Determination of total flavonoids and total phenol

Methanolic extract of *allamanda neriifolia*	Total flavonoids (mg QE/g)	Total phenol (mg GAE/g)
	10.096±0.2	8.49 ± 0.1

Table 3: Comparison of IC50 values of methanolic extract offlowers of *Allamanda neriifolia hook*

S. No	Particulars	IC50 values (Flower extract)	IC 50 values of standard
1	DPPH	192	147
2	Nitric oxide	147	100
3	ABTS	127	46.9

solution. The absorbance was measured at 700 nm. Ascorbic acid was used as standard.

Nitric Oxide Scavenging Activity

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griese reagent SNP in aqueous solutiion at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitric ions that can be estimated by the use of Griess reagent colour absorbance at 546 nm by the method of[17] Sodium Nitroprusside(5 µm) in standard Phosphate buffer solution was incubated with different concentrations(50-250 µg/ml) of the methanol and aqueous plant extract made upto 5 ml with Phosphate buffer(0.025 m, pH 7.4) and tubes were incubated at 25 °C for 3 hours. After 3 hours 0.5 ml of the incubated solution was removed and diluted with 0.5 ml of Griess reagent (1% Sulphanilic acid, 5% Phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophoic formed during diazotization of nitrite ions with Sulphanilic acid and it subsequent coupling with napthylethylenediamine was read at 546 nm.

ABTS$^+$ Radical Scavenging Activity[18]

A ferryl myoglobin radical is formed from metmyoglobin and hydrogen peroxide. The ferryl myoglobinradical can oxidize ABTS (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid) to generate a radical cation, ABTS$^+$ that is green in color and can be measured by absorbance at 405nm. Antioxidants suppress this reactionby electron donation radical scavenging and inhibit the formation of the colored ABTS radical. The concentrationof antioxidant in the test sample is inversely proportional to the ABTS radical formation and 730nm absorbance.

Metmyoglobin + H_2O_2 ⟶ ferryl myoglobin + H2O

ABTS + ˙ferryl myoglobin⟶ ABTS$^{\cdot+}$ + metmyoglobin

[Antioxidants inhibit the oxidation of ABTS by electron transfer radical scavenging]

Varying concentration (100 -500µg) of plant extract and different concentration (50 -250 µg/ml) of standard Ascorbic acid solution were taken, 0.3 mlof ABTS Solution was addded and the volume was made upto 2.5 ml with phosphate buffer. The solution was read immediately at 734 nm.

I (%)= $(A_0 - A_1)/A_0 x100$

Where A_0 is absorbance of the control, A_1 is absorbance of the extract/standard. A percent inhibition versus concentration curve was plotted and the concentration of sample required for % 50 inhibition was determined and expressed as IC_{50} value indicates high antioxidant capacity.

RESULTS AND DISCUSSION

Phytochemical screening

The present study was conducted on preliminary phytochemical analysis in both aqueous and methanolic extracts. The secondary metabolites such as alkaloids, flavonoids, phenol, tannins, glycosides and terpenoids were found to be universally in methanolic extract of the plant.

Medicinal plants contain secondary metabolites, which are organic compounds that are not directly involved in the normal growth, development, or reproduction of organism. However, they often play an important role in plant defenses[19]. and are also capable of destroying or inhibiting the growth of microorganism[20].

Determination of total flavonoids

Total flavonoid content was calculated using the standard curve of quercetin (y=0.016x-0.044; R^2=0.999) and was expressed as quercetin equivalents (QE) per gram of the plant extract. The total flavonoids content of the methanolic extract of *A.neriifolia* was found to be 10.096± 0.2mg/g in terms of quercetin equivalent (Table 2).Result indicate that methanolic extract of flower showed highest flavonoid content.

Flavonoids are one of the most diverse and widespread group of natural compound and it has been shown to possess a broad spectrum of biological activities including radical scavenging properties, antiallergenic, antiviral, antiinflammatory, and vasodilating actions[21]. The antioxidant properties of flavonoid depend on their structureparticularly hydroxyl position in the molecule and their ability as electron donor to free radical[22].

Determination of total phenolic content

Total phenolic content of the methanolic extracts *of A. neriifoliahook* was determined by using the Folin-Ciocalteau reagentand were expressed as GAE per gram of plant extract. The total phenol content of the test fractions was calculated using the standard cure of gallic acid

Table 4: In vitro antioxidant activity

concentration (µg/ml)	DPPH scavenging activity	Reducing power assay	Nitric oxide scavenging activity	ABTS radical scavenging activity
	% of inhibition	Absorbance at 700nm	% of inhibition	% of inhibition
50	13.03 ± 0.03	0.28±0.03	17.06± 0.04	19.52±0.02
100	26.10 ± 0.05	0.36±0.03	34.01 ± 0.01	39.04 ±0.02
150	39.03 ± 0.03	0.39±0.01	51.02 ±0.01	58.51 ± 0.01
200	52.10 ± 0.06	0.50 ±0.05	68.1 ±0.05	78.10 ±0.06
250	65.04 ± 0.04	0.56±0.05	85.05 ±0.04	97.54 ±0.03

Assays were performed in triplicates. Values are expressed as mean±SD

(y=0.001x+0.005; R^2=0.998). The total Phenolic content of the methanolic extract were found to be 8.49± 0.1mg/g respectively in terms of gallic acid equivalent (Table 2). Phenolic compounds are known as powerful chain breaking antioxidant[23] and they are very important plant constituents because of their particularly hydroxyl position in the molecule and their ability as electron donor to free radical[22] which can stabilize and delocalize the unpaired electron and from their potential to chelate metal ions. The results strongly suggest that phenols are important components of the tested flower extract.

Comparison of IC50 values

DPPH scavenging activity

In the present study the percentage of scavenging effect on the DPPH radical was increased with the increase in the concentration of methanolic extract of flower from 50 to 250µg/ml. The percentage of inhibition was existing from 13.03 at 50µg/ml to 65.04 at 250µg/ml for flower extract. (Table 4& fig 2). The methanolic extract of *A.neriifolia* showed DPPH scavenging activity and compared with ascorbic acid as standard. The result of IC50 values are 192µg/ml and 147µg/ml (table 3). DPPH is one of the free radicals widely used for testing preliminary radical scavenging activity of the plant extract[24]. Scavenging of DPPH radical is related to the inhibition of lipid per oxidation. DPPH is usually used as a substance to evaluate the antioxidant activity[25]. Antioxidants either transfer an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character[26]. DPPH test, which is based on the ability of DPPH, a stable free radical, to decolorize in thepresence of antioxidants, is a direct and reliable method for determining radical scavenging action[27]. The DPPH assay has been largely used as a quick, reliableand reproducible parameter tosearch the *in vitro* general antioxidant activity of pure compounds as well as plant extracts[28].

Reducing power ability

In this assay the reducing power activity of the methanolic extract of flowers of *Allamanda neriifolia hook* was increased with increase in the volume of concentration from 50 to 250 µg/ml (Table 4). The methanolic extract of *A.neriifolia hook* showed higher absorbance in 0.54 at 250µg/ml (fig 3). Reducing capacity of the extract component may serve as significant indicator of its potential antioxidant activity[29]. Different studies have indicated that the electron donation capacity of bioactive components is associated with antioxidant activity[30.] Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes that they can act as primary and secondary antioxidants.

Nitric oxide scavenging activity

In the present study, nitric oxide scavenging activity of methanolic extract of *A.neriifolia hook* has the percentage of inhibition was existing from 17.06 at 50µg/ml to 85.05 at 250µg/ml(Table 4& fig 4). The methanolic extract of *A.neriifolia* showed a potent nitric oxide scavenging activity and compared with ascorbic acid as standard. The results of IC50 values are 147µg/ml and 100µg/ml (table 3&fig 1). From the above results, the flower has a potent nitric oxide scavenging activity. The nitric oxide scavenging assay showed the half maximum inhibitory concentration of methanolic extracts quiet equivalent to standard. The result indicatethat the extracts contain compounds able to inhibit nitric oxide and offers scientific evidence for the use of the plant in inflammatory condition. NO is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and anti-tumour activities[31]. NO is a very unstable species and reacting oxygen molecule produce stable nitrate and nitrite which can be estimated by using Griess reagent. In the presence of a scavenging test compound, the amount of nitrous acid will decrease which can be measured at 546 nm.

ABTS radical scavenging activity

This study reports that the methanolic extract offlowersof *A. neriifolia* has radical scavenging activity. The percentage of inhibition was existing from 19.52 at 50µg/ml to 97.54 at 250µg/ml (Table 4& fig 5). From the results, the methanolic extract *A.neriifolia h*as showed ABTS radical scavenging activity and compared with ascorbic acid as standard and the IC50 values are 127µg/ml and 46.9µg/ml(table 3&fig 5).Based on the above results indicated, the methanolic extract of flowers of *A.neriifolia* was found to most effective in exhibiting *in vitro* antioxidant activity in various methods.

Allamandaneriifolia hook

The decolorization of the ABTS, through measuring the reduction of the radical cation as the percentageinhibition of absorbance at 734nm. ABTS was generated by

incubating ABTSchromophore through the reaction[32].The presence of specific chemical compound in the extract of *A.neriifolia* may inhibit the potassium persulfate activity and hence reduced the production of ABTS.

CONCLUSION

In this study, all the antioxidant methods (DPPH, Reducing power assay,Nitric oxide and ABTS radical scavenging activity) showed that the methanol extracts of *A.neriifolia* contain more antioxidant activities. More- over, this study demonstrated that,methanolic extracts of flowers of *A.neriifolia is* an important source of phenol flavonoid compounds, which are a good source of antioxidant activity. The phenol component has a high inhibi- tory effect that prevents lipid peroxidation andflavonoids has an good scavenging activities.Thus, we concluded that flowers of *A.neriifolia* - act via its free radical scavenging to prevent lipidperoxi- dation. Finally, it can be concluded that the methanolic extracts of *Allamanda neriifolia hook* have a potent antioxidant activity and free radical scavenging activity. Further work on isolation and identification of active compounds and its efficacy needs to be done.

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