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Original Research

Evaluation of analgesic and antioxidant potential of ethanolic extract of *Nymphaea alba* rhizome

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Abstract
Nymphaea alba is rich in phytoconstituents that posses pharmacological actions. But any scientific literature about its antioxidant and analgesic actions using its rhizome part is not documented. So the objective of the present research is focused to evaluate the analgesic activity and antioxidant potential by different models of scavenging properties of *N.alba* extract. *Nymphaea alba* rhizome ethanol extract was investigated for their analgesic activity by acetic acid- and formalin-induced analgesia and antioxidant activities employing 3 *in vitro* assay systems, *i.e.* 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO) and superoxide anion radicals scavenging. The reducing properties and total phenolic content was also observed. The extract at a dose of 600 mg/kg was found more potent in acetic acid induced pain and showed more licking activity in both the phases of formalin acid induced pain than the standard drug. IC_{50} values for DPPH, NO and superoxide radical-scavenging activity were 63.9, 49.21 and 79.56 µg/ml, respectively. The IC_{50} of extract is more comparable to the standard drug for scavenging superoxide anion than the other methods. The antioxidant activities of *N.alba* ethanolic extract was moderate in comparison to the standard antioxidant ascorbic acid, achieved by the scavenging ability observed against DPPH, NO radicals and superoxide anions. The antioxidant effects observed in this study may be due to the presence of tannins and phenolic compounds. However, unless a thorough compositional analysis of the extract along with activity correlation is not carried out there can be no statements with high degree of certainty.

INTRODUCTION

Antioxidants have the ability of protecting organisms from damage caused by free radical-induced oxidative stress. Oxidation reactions are crucial for life but they can also be damaging. Due to oxidation the reactive oxygen species produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and free radicals such as the hydroxyl radical (•OH) and the superoxide anion ($O_2•^-$) [1]. The hydroxyl radical is particularly unstable and will react rapidly and nonspecifically with most biological molecules. These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, oxidizing DNA or proteins [2]. Currently, the possible toxicity of synthetic antioxidants has been criticized. It is strongly believed that regular consumption of plant-derived phytochemicals may drift the balance toward an adequate antioxidant status. Thus, interest in antioxidant, especially of plant origin, has greatly increased in recent years [3].

Analgesic therapy is dominated by two major classes; namely opioids and non-steroidal anti-inflammatory drugs (NSAIDs). These drugs produce serious side effects such as dyspeptic symptoms, gastrointestinal erosions and peptic ulcers to more serious complications, such as over bleeding or perforation [4]. So, new compounds with improved pain management capacity and fewer side effects are being sought with urgency.

Nymphaea alba L, also known as the European White Waterlily, White Lotus, or Nenuphar, is an aquatic flowering plant of the family Nymphaeaceae. It grows

in water from 30-150 cm deep and likes large ponds and lakes. The leaves may be up to thirty centimeters in diameter and they take up a spread of 150 cm per plant. The flowers are white and they have many small stamens inside. It contains the active alkaloids nupharine and nymphaeine, and is a sedative and an aphrodisiac/anaphrodisiac depending on sources. The root of the plant was used by monks and nuns for hundreds of years as an anaphrodisiac, being crushed and mixed with wine. It is rich in tannic acid, gallic acid, alkaloids, sterols, flavonoids, glycosides, hydrolyzable tannins and high-molecular-weight polyphenolic compounds [5]. All the parts of the plant have medicinal uses in traditional system of medicine. It is used as an aphrodisiac, anodyne, antiscrophulatic, astringent, cardiotonic, demulcent, sedative and antiinflammatory. Further, it also produces calming and sedative effects upon the nervous system, and is useful in the treatment of insomnia, anxiety and similar disorders [6-8]. Its anticarcinogenic action and inhibition of renal oxidative stress and hyperproliferative response were reported [9-11]. It also posses good anxiolytic activity [12].

Another literature review suggests that the aqueous and ethanolic extracts of leaves of *N.alba* possess antioxidant activity [13]. But there are no any scientific detailed reports of rhizomes as antioxidant or analgesic activity. So, our present investigation was to evaluate the antioxidant and analgesic activity of ethanolic extract of rhizome of *N alba*.

MATERIALS AND METHODS

Chemicals

Acetic acid, formalin, potassium ferricyanide, ferric chloride, phosphate buffer, trichloroacetic acid, gallic acid (Merck, Mumbai, India), Folin-Ciocalteu reagent (Sisco Research Laboratory, Mumbai, India), 1,1-diphenyl-2-picrylhydrazyl (DPPH), methanol, sulphanilamide, phosphoric acid, naphthylethylenediaminedihydrochloride, sodium nitroprusside, xanthine, ethylenediaminetetraacetic acid, nitroblue tetrazolium (NBT), xanthine oxidase, sodium bicarbonate, potassium dihydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), potassium hydroxide (KOH), sodium hydroxide (NaOH) (HiMedia Laboratories Pvt Ltd, Mumbai, India), paracetamol and ascorbic acid (Loba Chemical, Mumbai, India) were purchased from their appropriate sources given in parentheses.

Plant material

The rhizomes of young matured plants were collected in bulk from Salipur, Cuttack district, Orissa, India, in the month of September and authenticated by the taxonomists of Botanical Survey of India, Shibpur, Howrah. A voucher specimen has been retained in our laboratory for future reference (Chem.11/2010).

Preparation of extracts

The rhizome were shade dried and pulverized using an electric grinder and was extracted by taking 200 grams of the powder and soaked in 2000 ml of 90% ethanol for 3 days. The mixture was filtered using muslin cloth followed by Whatman filter paper (No.1). The resultant filtrate was evaporated to dryness on steam bath to give a yield of 8% (w/w) of the extract. The crude extract was dissolved in tragacanth (5 ml/kg of b.w) prior to the experiment and used.

Animals

Male BALB/c mice (20-30 g) obtained from Chakraborty Animal Center, Kolkata, West Bengal, India, were used. The animals were kept in a well-ventilated room in the Laboratory Animal Center of Institute of Pharmacy & Technology, Orissa. They received standard food and water *ad libitum*. The study was undertaken with due to approval of the study protocol by the Institution's Animal Ethics Committee (1053/ac/07/CPCSEA) and the experiments were performed according to the current guidelines for the care of the laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals.

Phytochemical screening

Conventional standard protocols [14] for detecting the presence of different chemical constituents in the plant extract were employed. Secondary metabolites tested include alkaloids, tannins, saponins, glycosides, flavonoids, digitalis, phenols, resins and volatile oils.

Analgesic activity

Acetic acid-induced writhing test: according to Sawadogo *et al* [15], nociception was induced by an intraperitoneal injection of 0.6% acetic acid solution in a value of 10 ml/kg b.w. Five groups of six mice each were used. The mice received ethanol extract (200, 400 and 600 mg/kg), paracetamol (100 mg/kg) and distilled water 1 h orally before acetic acid injection. The number of writhes occurring between 5 and 20 min after acetic acid injection was recorded. The analgesic effect was expressed as the percentage reduction of writhes in treated mice compared to those in the control group.

Formalin-induced nociception: the analgesic effect of the extract was also evaluated using formalin-induced paw licking method according to Wibool *et al* [16]. Five groups of six mice each were used. The ethanol extract (200, 400 and 600 mg/kg), paracetamol (100 mg/kg) and water were orally administered. One hour after drug administration, 20 µl of formalin (2.5%

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in normal saline) was injected into the plantar surface of the left hind paw of mice. The time spent in licking the injected paw was recorded and expressed as the total licking time in early phase (0 to 5 min) and late phase (15 to 30 min) after formalin injection.

Antioxidant activity

Reducing power: the reducing power of *N.alba* was determined according to method followed by Srinivas *et al* [17]. Different concentrations of extract (10-100 µg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloro-acetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_2$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the reference.

Determination of total phenol content: the total phenol content of extracts was determined using the Folin-Ciocalteu method [18]. The extracts were oxidized with Folin-Ciocalteu reagent and were neutralized with sodium carbonate. The absorbance of the resulting blue color measured at 760 nm after 60 min using gallic acid as standard total phenol content was expressed as mg gallic equivalent/g of extract.

DPPH radical scavenging assay: the free radical scavenging activity of the extracts, based on the scavenging activity of the stable DPPH free radical, was determined by the method described by Braca *et al* [19]. Different concentrations ranging from 10-100 µg/ml of plant extract (0.1 ml) was added to 3 ml of a 0.004% methanol solution of DPPH. Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration of extracts of *N.alba.* Absorbance at 517 nm was determined after 30 min and the percentage inhibition activity was calculated from the optical density of the treated and control samples. The inhibition curves were prepared and IC values were obtained by probit analysis.

Nitric oxide scavenging assay: sodium nitroprusside (5 µM) in standard phosphate buffer solution was incubated with different concentration of the test extracts dissolved in standard phosphate buffer (0.025 M, pH 7.4) and the tubes were incubated at 25°C for 5 h. After 5 h, 0.5 ml of incubation solution was removed and diluted with 0.5 ml Griess reagent (prepared by mixing equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% naphthylethylenediaminedihydrochloride in water). The absorbance of chromophore formed was read at 546 nm and the percentage inhibition activity was calculated from the optical density of the treated and control samples [20].

Superoxide anion scavenging activity: superoxide radicals generated by the xanthine-xanthine oxidase system was determined spectrophotometrically by monitoring the product of NBT [21]. Various concentrations of the extract (10, 25, 50, 75, 100 µg/ml) were added to the reaction mixture containing: 3 mM xanthine, 3 mM ethylenediaminetetraacetic acid, 0.75 mM NBT, 50 mU/ml xanthine oxidase, and 50 mM carbonate buffer (pH 10.2), making up a final volume of 2.4 ml. After incubation of the mixture at 25°C for 10 min, the absorbance was read at 560 nm and compared with the control samples. The percentage of superoxide anions scavenging was calculated from the optical density of the treated and control samples.

Statistical analysis

The data are represented as mean ± SEM, where n (number of samples) was taken as 6. Student's *t*-test is used for statistical analysis and $P < 0.05$ is considered significant.

RESULTS

The Phytochemical study revealed the presence of alkaloids, tannins, glycosides and flavonoids in the ethanol extract of the rhizome of *N.alba.*

Analgesic activity

Table 1 shows the effect of ethanol extract of rhizome of *N.alba* on acetic acid-induced writhing in mice. Different doses of extract of *N.alba* showed significant reduction ($P < 0.001$) of writhing induced by acetic acid after oral administration in a dose dependent manner. After oral administration of three different doses (200, 400 and 600 mg/kg body weight) of the extract and standard drug paracetamol (100 mg/kg body weight), the percent inhibitions were compared with control values and found to be 57.55%, 64.52%, 76.55% and 51.18%, respectively. The efficacy of the plant extract at the dose of 400 mg/kg body was comparable to the standard drug, and 600 mg/kg was found more potent than the standard drug.

Table 2 shows the effect of ethanol extract of rhizome of *N.alba* on formalin-induced pain in mice. The different doses of extract of *N.alba* (200, 400 and 600 mg/kg b.w) significantly suppressed the licking activity in either phase of the formalin-induced pain in mice in a dose dependent manner. The reference analgesic drug paracetamol also significantly inhibited the licking activity against both phases of formalin-induced nociception. But the extract of *N.alba* at the dose of 600 mg/kg body weight showed the more licking activity in both the phases of formalin-induced pain which is more than the standard drug.

Table 1. Effect of ethanol extract of *N.alba* on writhing induced by acetic acid.

Samples	Dose (mg/kg of b.w)	Number of writhing	% Inhibition
Control	-	34.16 ± 1.35	-
Paracetamol	100	16.66 ± 0.88	51.18
Ethanol extract	200	14.5 ± 1.26	57.55
Ethanol extract	400	11.83 ± 0.94	64.52
Ethanol extract	600	9.56± 0.88	76.55

Values are expressed as mean ± SEM (n = 6) and are significantly different (P < 0.001) when compared to control by using Student's *t*-test.

Table 2. Effect of *N alba* on licking the hind paw induced by formalin injection.

Samples	Dose (mg/kg b.w)	Formalin induced licking of hind paw			
		1st phase (0 to 5 min)	% inhibition	2nd phase (15 to 30 min)	% inhibition
Control	-	90.5 ± 4.5	-	133.67 ± 5.7	-
Paracetamol	100	55.5 ± 6.4	10.69	58.17 ± 4.6	15.09
Ethanol extract	200	80.83 ± 3.7	17.87	113.5 ± 3.7	43.39
Ethanol extract	400	74.33 ± 5.3	36.64	75.67 ± 4.4	55.73
Ethanol extract	600	57.5 ± 4.2	38.67	59.17 ± 5.4	56.48

Values are expressed as mean ± SEM (n = 6) and are significantly different (P < 0.001) when compared to control by using Student's *t*-test.

Antioxidant activity

Reducing power assay: the reducing power of the extract of *N.alba* and the standard increased in a non-linear manner (Fig.1). The reducing power of the extract of *N.alba* rhizome was found to be remarkable, which increased gradually with a rise in the concentration (P < 0.001). As illustrated, Fe^{3+} was transformed to Fe^{2+} in the presence of the extract and the reference compound ascorbic acid to measure the reductive capability. At 10 µg/ml, the absorbance of the plant extract and ascorbic acid was 0.016 and 0.026 respectively, while at 75 µg/ml, the absorbance of both the extract and ascorbic acid were almost the same and it was calculated by plotting ascorbic acid equivalence versus concentration of the extract and ascorbic acid.

Determination of total phenolic content: phenolic compounds may contribute directly to the antioxidative action. The total phenolic content was estimated to be 22.69 ± 0.084 mg/ml gallic acid equivalent per 100 mg plant extract.

DPPH radical scavenging assay: the DPPH antioxidant assay is based on the ability of the antioxidants present in the sample to decolorize DPPH free radical by virtue of their scavenging activities. The DPPH radical contains an odd electron that is responsible for the absorbance at 540 nm and also for the visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. Comparison of the antioxidant activity of the extract and ascorbic acid is shown in Fig.2. The ethanol extract of *N.alba* exhibited a significant dose-dependent inhibition of DPPH activity (P < 0.001), with a 50% inhibition (IC_{50}) at a concentration of 63.9 µg/ml as compared with the standard ascorbic acid (24.18 µg/ml).

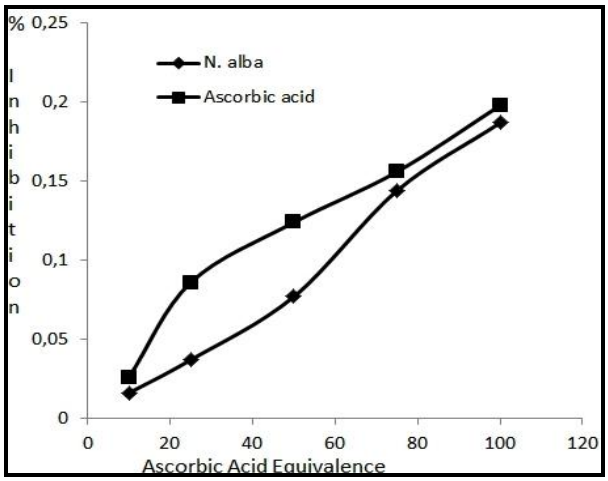

Figure 1. Reducing power activities of the ethanolic extract of *N.alba* in comparison with a standard (ascorbic acid). Values are expressed as mean ± SEM (n = 6) and are significant at the level of P < 0.001 when compared to control by using Student's *t*-test.

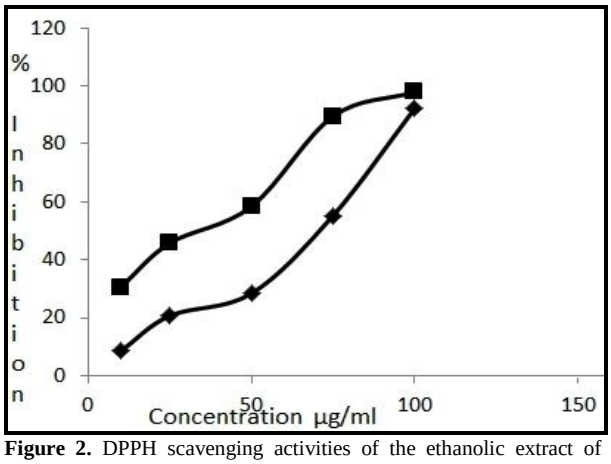

Figure 2. DPPH scavenging activities of the ethanolic extract of *N.alba* in comparison with a standard (ascorbic acid). Values are expressed as mean ± SEM (n = 6) and are significant at the level of P < 0.001 when compared to control by using Student's *t*-test.

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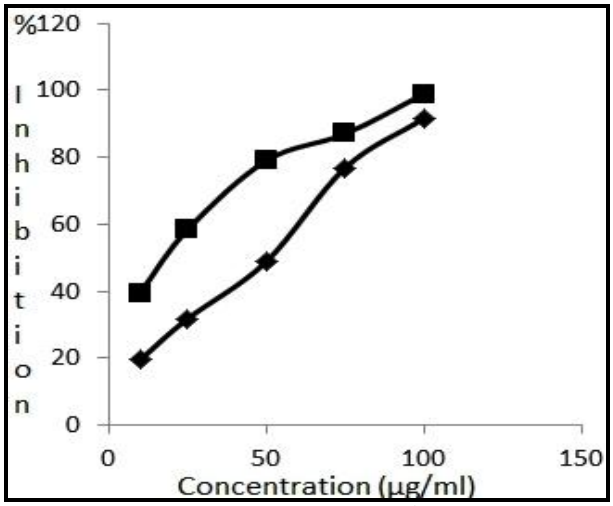

Figure 3. Nitric oxide scavenging activities of the ethanolic extract of *N. alba* in comparison with a standard (ascorbic acid). Values are expressed as mean ± SEM (n = 6) and are significant at the level of P < 0.001 when compared to control by using Student's *t*-test.

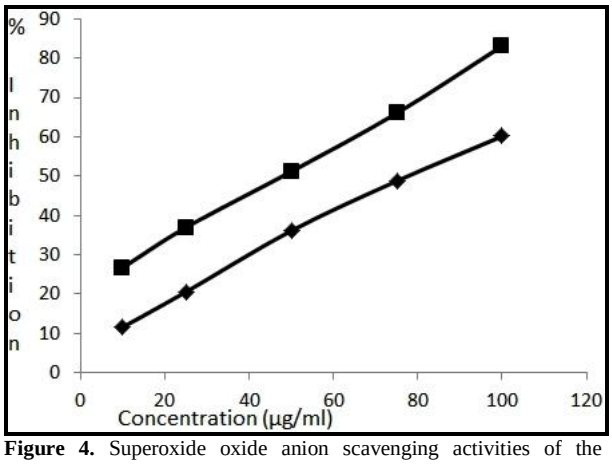

Figure 4. Superoxide oxide anion scavenging activities of the ethanolic extract of *N. alba* in comparison with a standard (ascorbic acid). Values are expressed as mean ± SEM (n = 6) and are significant at the level of P < 0.001 when compared to control by using Student's *t*-test.

Nitric oxide scavenging assay: the scavenging of nitric oxide (NO) by the extracts was increased in dose dependent manner (P < 0.001). Fig.3 illustrates a significant decrease in the NO radical due to the scavenging ability of extracts and ascorbic acid. The ethanol extracts showed maximum activity of 91.7% at 100 µg /ml, whereas ascorbic acid at the same concentration exhibited 98.99% inhibition. The IC_{50} values were found to be 20.78 µg/ml and 49.21 µg/ml for ascorbic acid and ethanol extracts, respectively.

Superoxide anion scavenging activity: the superoxide anion radical scavenging activity of ethanol extract of *N.alba* is given in Fig.4, which shows the percentage inhibition of superoxide radical generation by the extract and comparison with ascorbic acid (P < 0.001). The percentage inhibition of superoxide generation by 100 µg/ml concentration of the extract was found as 60.15% compared to ascorbic acid at 50 µg/ml concentration having 51.20% inhibition of superoxide radical. The IC_{50} values were found to be 51.45 µg/ml and 79.56 µg/ml for ascorbic acid and ethanol extracts, respectively.

DISCUSSION

Naturally antioxidants present in many plants, foods and beverages offer health benefits in preventing various diseases by fighting cellular damage caused by free radicals in the body [22]. Due to the presence of flavonoids and polyphenolic compounds in the plant extracts it may have antioxidant activity. Furthermore, recent studies suggest that the inflammatory tissue damage is due to the liberation of reactive oxygen species (ROS) form phagocytes invading the inflammation sites [23]. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom and may have great relevance in the prevention and treatment of diseases associated with oxidants or free radicals.

The DPPH assay is based on the ability of the antioxidants present in the sample to decolorize DPPH free radical by virtue of their scavenging activities. Ascorbic acid was chosen as the reference antioxidant for this test. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. Additionally, it has been determined that the antioxidant effect of plant products is mainly due to radical scavenging activity of phenolic compounds such as flavonoids, polyphenols, tannins, and phenolic terpenes [24]. As the plant *N.alba* is having high content of phenolic compounds and flavonoids it shows antioxidant activity which is due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [25].

Nitric oxide 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 antitumor activities [26]. Preliminary phytochemical studies of ethanol and aqueous extract of *N.alba* rhizome shows the presence of alkaloids,

saponin, terpenoids, flavonoids and polyphenols.

Suppression of released NO may be partially attributed to direct NO scavenging, as the extracts of *N.alba* decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro.*

N.alba extract showed an inhibitory effect on the superoxide anion ($O_2^{\bullet-}$) generation. The high percentages of antioxidant activity is observed at all concentrations evaluated, evidencing that polyphenolic components present in the fraction are mainly responsible for the inhibitory effect on the $O_2^{\bullet-}$ production observed in the extract [27, 28]. The inhibitory effect shown by the extract on the $O_2^{\bullet-}$ generation in an acellular system using the xanthine-xanthine oxidase assay, could be explained by 2 different mechanisms: the direct scavenging of this ROS or inhibition of the production of the responsible enzyme xanthine oxidase [21].

In conclusion, in the present study, the *N.alba* rhizome ethanol extract showed a moderate antioxidant activity by inhibiting DPPH, $O_2^{\bullet-}$, NO and reducing power activities when compared with the standard antioxidant ascorbic acid. In addition, the *N.alba* was found to contain a noticeable amount of total phenols and tannins, which play a major role in controlling oxidation. The results of this study show that the *N.alba* rhizome can be used as an easily accessible source of natural antioxidant. However, the phyto-constituents responsible for the antioxidant activity of *N.alba* are not much clear. Therefore, a further study is needed to determine the mechanism behind the antioxidant activity of this plant.

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