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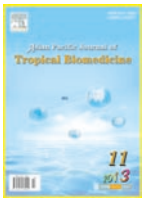

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Phytochemical investigation and *in vitro* antioxidant activity of an indigenous medicinal plant *Alpinia nigra* B.L. Burtt

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PEER REVIEW

Peer reviewer

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Comments

This is a good study in which the authors have evaluated the *in-vitro* antioxidant activity of the methanol extract of *A. nigra* leaves by DPPH, ABTS, H_2O_2 and nitric oxide radical scavenging assay and the content of total phenolics and flavonoids.
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ABSTRACT

Objective: To investigate antioxidant potential of methanol extract of *Alpinia nigra* leaves.
Methods: The study was done by using various *in vitro* methods such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), nitric oxide and hydrogen peroxide radical scavenging assays. Phytochemical constituents, total phenolic content and total flavonoid content of the extract at different concentrations (10-500 μg/mL) were determined.
Results: *Alpinia nigra* leaves showed high free radical scavenging activity as evidenced by the low IC_{50} values in DPPH (64.51 μg/mL), in ABTS (28.32 μg/mL), in nitric oxide (80.02 μg/mL) and in H_2O_2 (77.45 μg/mL) scavenging assays. Furthermore the TPC and TFC of the extract were found to be 69.25 mg gallic acid equivalent per gram of extract and 78.84 mg quercetin equivalent per gram of extract respectively.
Conclusions: The results of present comprehensive analysis demonstrated that *Alpinia nigra* leaves possess high phenolic, flavonoid contents and potential antioxidant activity, and could be used as a viable source of natural antioxidants and might be exploited for functional foods and neutraceutical applications.

KEYWORDS
Alpinia nigra, Phytochemical screening, TPC, TFC, Antioxidant activity

1. Introduction

The traditional medicine all over the world is nowaday revealed by an extensive activity of researches on different plant species and their therapeutic principles. Plants contain phytochemicals with various bioactivities including antioxidant, anti inflammatory and anticancer activities. Currently, about 25% of the active component was identified from plants that are used as prescribed medicines[1]. Reactive oxygen species (ROS) exert oxidative damaging effects by reacting with nearly every molecules found in living cells including protein, lipid, amino acids and DNA, if excess ROS are not eliminated by antioxidant system. They play important roles in aging and in the pathogenesis of age related disorders such as cancer, hypertension, atherogenesis, Alzheimers disease and Parkinsons disease. The most practical way to fight degenerative diseases is to increase antioxidant activity in our body and that could be achieved by consumption of vegetables, fruits or edible plants[2]. There is an increasing interest in natural antioxidants *e.g.* polyphenols, present in medicinal and dietary plants, which might help prevent oxidative damage. Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of diseases[3]. Different parts such as seeds, leaves and bark of stem and root known to contain substantial amounts of phytoconstituents such as phenolics, flavonoids, tannins having the ability to inhibit the free radicals that are excessively produced, hence can act as antioxidants[4]. The continued search among plant secondary metabolites for natural antioxidants has gained importance in recent years because of the increasing awareness of herbal remedies as potential sources of

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phenolic oxidants[5].

Several synthetic antioxidant agents including butylated hydroxyanisole and butylated hydroxytoluene (BHT) are commercially available, however, are reported to be toxic to animals including human beings which have stimulated the interest of many investigators to search natural antioxidant[6]. *Alpinia nigra* B.L. Burtt (*A. nigra*, family Zingiberaceae) is a perennial aromatic medicinal plant found in China, Bhutan, India, Srilanka and Thiland. It is commonly called as "Noh Kala" in Thailand. It is not only an edible vegetable, but also used in folk remedies to treat dyspepsia, gastric disease and insect bites. The shoot of this plant has traditional usage among the native tribes of Tripura, Northeast India who consume the raw juice of green shoot for its presumed anthelmintic properties[7]. The rhizomes of this plant are also used as vegetables in Thailand. Rhizomes of *A. nigra* are also closely related to galangal, curcuma and zinger. The crushed leaves and rhizomes of *A. nigra* produce an aromatic fragrance, indicating the presence of essential oils. Essential oils obtained from leaves and flowers contained 1,8-cineole as major component and β-pinene, camphor, carotol, α-pinene, camphene, α-fenchyl acetate, α-terpineol as minor components[8]. *A. nigra* is one of the most important herbal remedies and it is used in Thai traditional medicine for stomachicum, gastric diseases, antibacterial and antifungal activities[9]. Nevertheless, it is still unknown whether *A. nigra* has antioxidant activity on ABTS, H_2O_2 and nitric oxide radical scavenging and the content of total phenolics and flavonoids of *A. nigra* has not been described yet. Although leaves of ginger species have been used for food flavouring and in traditional medicine, very little research has been done in their antioxidant properties[10]. Keeping all these into account, the present study was undertaken to evaluate antioxidant activities as well as it deals with preliminary phytochemical screening, total phenolics and total flavonoid content (TFC).

2. Materials and methods

2.1. Plant material

The rhizomes of *A. nigra* were collected from Ganeshmani nursery, West Bengal and the specimen was authenticated by Dr. P.C. Panda, Senior Scientist, Taxonomy and Conservation Division, Regional Plant Resource Centre, Bhubaneswar. The collected rhizomes were planted in the greenhouse of Centre of Biotechnology. The leaves were taken from these plants, chopped into pieces and dried under shade for 10-15 d.

2.2. Preparation of extract

The shade dried leaves were grounded to coarse powder. The resulting materials were extracted with methanol for 24 h by using soxhlet apparatus. The methanol extract was filtered and then concentrated by using rotary evaporator to yield a semisolid mass (12.94%, w/w). The residue obtained was stored in refrigerator for further study.

2.3. Preliminary phytochemical screening

The leaf extract of *A. nigra* was subjected to different chemical tests for the detection of phytoconstituents such as carbohydrates, glycosides, alkaloids, amino acids, phenolics, flavonoids, triterpenoids, steroids, *etc*.

2.4. Determination of total phenolic content (TPC)

Determination of TPC of methanol extract of *A. nigra* was done by Folin-Ciocalteu method with little modifications, using gallic acid as a standard phenolic compound[11]. The extracts were diluted with distilled water to a known concentration in order to obtain the readings within the standard curve range of 0.0 to 600.0 μg of gallic acid/mL. A volume of 250 μL of diluted extract or gallic acid solution was mixed with 1 mL of distilled water in a test tube followed by the addition of 250 μL of Folin-Ciocalteu reagent. The samples were mixed well and then allowed to stand for 5 min at room temperature in order to allow complete reaction with the Folin-Ciocalteu reagent. Then 2.5 mL of 7% sodium carbonate aqueous solution was added and the final volume was made up to 6 mL with distilled water. After incubating the samples for 90 min the absorbance of the resulting blue colour solution was measured at 760 nm using spectrophotometer. The result was expressed as mg of gallic acid equivalents (GAE)/g of extract by using an equation that was obtained from standard gallic acid graph. All the experiment was conducted in three replicates.

2.5. Determination of TFC

TFC of leaf extract of *A. nigra* was estimated by the method with little modification[12]. This method is based on the formation of a complex flavonoid-aluminium. A volume of 1 mL of 2% $AlCl_3$ ethanol solution was added to 1 mL of extract solution and left in the dark at room temperature for 1 h. The absorbance was measured at 420 nm using UV-VIS spectrophotometer. TFC was calculated by extrapolating the absorbance of reaction mixture on calibration curve of quercetin. All the determinations were performed in triplicate and TFC was expressed as mg quercetin equivalent/g of the extract.

2.6. DPPH radical scavenging assay

The DPPH assay was carried out as described by Hsu *et al.* with some modifications[13]. A volume of 1.5 mL of 0.1 mmol/L DPPH solution was mixed with 1.5 mL of various concentrations (10 to 500 μg/mL) of leaf extract. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm by a spectrophotometer. The solution without any extract and with DPPH and methanol was used as control. The experiment was replicated in three independent assays. Ascorbic acid was used as positive controls. Inhibition of DPPH free radical in percentage was calculated by the formula:

DPPH radical scavenging activity (%)=$(A_{control}-A_{test})/A_{control}\times 100$

Where, $A_{control}$ is the absorbance of the control and A_{test} is the absorbance of samples.

The antioxidant activity of each sample was expressed in terms of IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%), calculated from the graph after plotting inhibition percentage against extract concentration.

2.7. ABTS radical scavenging assay

To determine ABTS radical scavenging assay, the method of Re *et al.* was adopted[14]. The stock solutions included 7 mmol/L ABTS solution and 2.4 mmol/L potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The resulting solution was then diluted by mixing 1 mL of freshly prepared ABTS solution to obtain an absorbance of (0.706±0.001) units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 mL) were allowed to react with 2.5 mL of the ABTS solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition was calculated as ABTS radical scavenging activity (%)=$(A_{control}-A_{test})/A_{control}\times100$

Where $A_{control}$ is the absorbance of ABTS radical+methanol; A_{test} is the absorbance of ABTS radical+sample extract/standard.

2.8. Nitric oxide scavenging activity

Nitric oxide was generated from sodium nitroprusside and was measured by the Griess reagent. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide[15]. Sodium nitroprusside (10 mmol/L) in phosphate buffer saline (PBS) was mixed with different concentrations of the extract and incubated at 25 °C for 150 min. The samples were added to Griess reagent (1% sulphanilamide, 2% $H_3PO_4$and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm and referred to the absorbance of standard solutions of ascorbic acid treated in the same way with Griess reagent as a positive control. All the tests were performed in triplicate and the graph was plotted with the mean values. The percentage of inhibition was measured by the following formula:

Radical scavenging activity (%)= $(A_{control}-A_{test})/A_{control}\times100$

Where $A_{control}$ is the absorbance of the control (without extract) and A_{test} is the absorbance in the presence of the extract/standard.

2.9. Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the plant extract was estimated using the method of Ruch *et al.* with little modification[16]. 4 mmol/L solution of H_2O_2 was prepared in PBS (pH 7.4). Plant extract (4 mL), prepared in distilled water at various concentration was mixed with 0.6 mL of 4 mmol/L H_2O_2 solution prepared in PBS and incubated for 10 min. The absorbance of the solution was taken at 230 nm against a blank solution containing the plant extract in PBS without H_2O_2. Ascorbic acid was used as positive control. The amount of hydrogen peroxide radical inhibited by the extract was calculated using the following equation:

H_2O_2 radical scavenging activity=$(A_{control}-A_{test})/A_{control}\times100$

Where $A_{control}$ is the absorbance of H_2O_2 radical+methanol; A_{test} is the absorbance of H_2O_2 radical+sample extract or standard.

3. Results

3.1. Phytochemical screening

Preliminary phytochemical screening of the *A. nigra* leaf extract revealed the presence of different phytoconstituents which are represented in Table 1.

Table 1
Preliminary phytochemical screening of *A. nigra* leaf extract.

Phytoconstituents	Test performed	Results
Alkaloids	Dragendroff's test, Mayer's test	−ve
	Wagner's test, Hager's test	+ve
Flavonoids	Alkaline reagent test, Shinoda test	+ve
Steroids	Libermann Burchard test, Salkowski test	+ve
Triterpenoids	Libermann Burchard test, Salkowski test	−ve
Carbohydrates	Molisch's test, Fehling's test	−ve
	Barfoed's test, Benedict's test	+ve
Aminoacids	Millon's test, Ninhydrin test	+ve
Tanins	$FeCl_2$ test	+ve
Saponins	Foam test	−ve
Glycosides	Killer-Kiliani test	+ve
	Brontrager's test	−ve

+: Indicates the presence of chemical constituents
−: Indicates the absence of chemical constituents

3.2. Total phenolic and TFCs

In our present study, *A. nigra* leaf extract possessed high phenolic contents (69.25 mg GAE/g of extract) and flavonoid contents (78.84 mg quercetin equivalent/ g of extract). TPC was calculated using the standard curve of gallic acid (standard curve equation: Y=0.004x+0.063, R^2=0.998) and TFC was calculated using the standard curve of quercetin (standard curve equation: Y= 0.013x+0.487, R^2=0.886).

3.3. DPPH radical scavenging assay

It is well known that the antioxidant activity of plant extracts containing polyphenol components is due to the capacity to be donors of hydrogen atoms or electrons and to capture the free radicals. In the present study, *A. nigra* methanolic leaf extract showed a significant effect in inhibiting DPPH, reaching up to 86.35% at concentration

of 500 μg/mL. Figure 1 showed the dose response curve of DPPH radical scavenging activity of *A. nigra* compared with standard ascorbic acid. The IC_{50} value of *A. nigra* leaf extract was 64.51 μg/mL while the IC_{50} value of standard antioxidant ascorbic acid was 6.58 μg/mL. DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts.

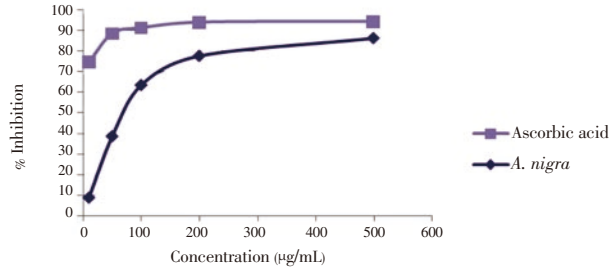

Figure 1. DPPH radical scavenging activities of the methanolic extract of *A. nigra* leaves and ascorbic acid.
Result represents means of triplicates of different concentrations analyzed.

3.4. ABTS radical scavenging activity

The methanolic leaf extracts of *A. nigra* were fast and effective scavengers of the ABTS radical (Figure 2) and this activity was comparable to that of BHT. It exhibited potent scavenging effects against ABTS with an IC_{50} value of 28.32 μg/mL almost equivalent to that of standard BHT (IC_{50} value19.47 μg/mL). The percentage of inhibition was 98.11% and 99.36% for the leaf extract and BHT respectively at 500 μg/mL concentration.

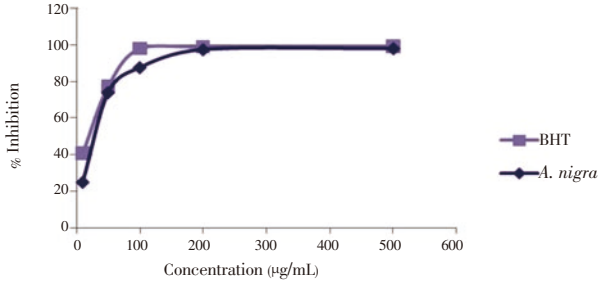

Figure 2. ABTS radical scavenging activity of methanolic leaf extract of *A. nigra* and the standard BHT.
Result represents means of triplicates of different concentrations analyzed.

3.5. H_2O_2 radical scavenging activity

As shown in Figure 3 , *A. nigra* leaf extract also demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner with an IC_{50} of 77.45 μg/mL. Scavenging activity of H_2O_2 by the extract may be attributed to their phenolics, which can donate electrons to H_2O_2 thereby neutralizing it into water[17].

3.6. Nitric oxide scavenging assay

In the present study, the crude methanol leaf extract of the *A. nigra* was investigated for its inhibitory effect on nitric oxide production. The present study revealed that the extract has a moderate activity in scavenging nitric oxide radical. Figure 4 illustrates a significant decrease in the nitric oxide radical due to scavenging ability of extract and the standard ascorbic acid. The leaf extract showed maximum activity of 80.45% at 500 μg/mL, where as ascorbic acid at the same concentration exhibited 87.46% inhibition.

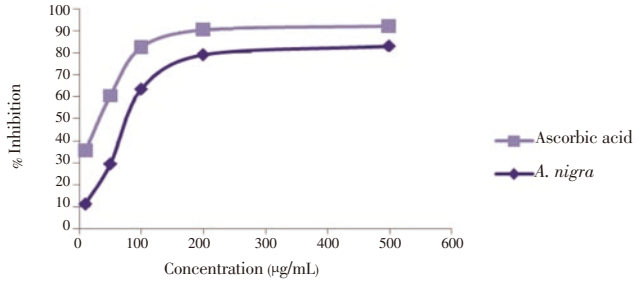

Figure 3. H_2O_2 scavenging activity of *A. nigra* leaf extract and ascorbic acid.
Result represents means of triplicates of different concentrations analyzed.

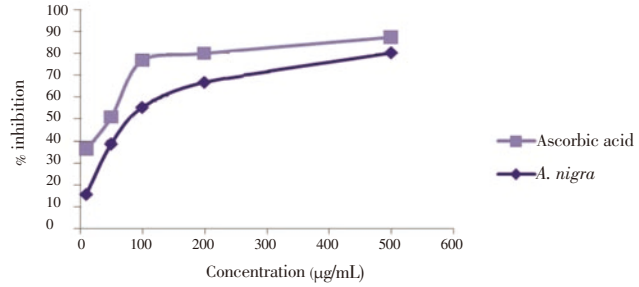

Figure 4. Nitric oxide scavenging activity of leaf extract of *A. nigra* and ascorbic acid.
Result represents means of triplicates of different concentrations analyzed.

4. Discussion

Plants are important source of potential compounds for the development of new therapeutic agents. Plant phenolics are widely distributed in the tissues of plants as well as play a vital role in the highly effective free radical scavengers and antioxidant activity. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers[17]. Typical phenolics that possess antioxidant activity are known to be mainly phenolic acid and flavonoids. These compounds possess diverse biological activities, such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities. These activities might be related to their antioxidant activity[18].The antioxidant activity of flavonoids is due to their ability to reduce free radical formation and to scavenge free radicals. Phenolic compounds are important plant antioxidants which exhibited considerable scavenging activity against radicals. Thus antioxidant capacity of a sample can be attributed mainly to its phenolic compounds[19]. Thus the antioxidant potential of the leaf extract of *A. nigra* may be possibly attributed to the presence of high phenolic compounds in it.

Plant has long been a very important source of drug and many plants have been screened if they contain compounds

with therapeutic activity[20]. Therefore, it is vital to evaluate the antioxidant activity of *A. nigra*. DPPH is a stable, nitrogen centered free radical which produces deep purple colour in methanol solution. The principle of this assay is based on the reduction of purple coloured methanolic DPPH solution in the presence of hydrogen donating antioxidants by the formation of yellow coloured diphenyl-picryl hydrazine. As the absorbance decreases the more efficient, the antioxidant activity of the extract in terms of hydrogen atom donating capacity. The more antioxidant present in the extract, the more DPPH reduction will occur. Our results suggested that different concentration have different activities and maximum activity was observed at 500 μg/mL concentration. The observed antioxidant of extracts may be due to the neutralization of free radicals (DPPH), either transfer of hydrogen atom or by transfer of an electron[21]. The scavenging effect can be attributed to the presence of active phytoconstituents in them.

The ABTS assay is based on the inhibition of the absorbance of radical cation, $ABTS^{+}$, which has a characteristic wavelength at 734 nm, by antioxidants. The principle behind the technique involves the reaction between ABTS and potassium per sulphate to produce the ABTS radical cation ($ABTS^{+}$) which is a bluegreen chromogen. In the presence of antioxidant reductant, the coloured radical is converted back to colourless ABTS[22]. The order of ABTS radical scavenging activity of the extract was almost similar to that observed for DPPH.

The H_2O_2 scavenging activity was detected and compared with ascorbic acid. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It rapidly transverses cell membrane and once inside the cell interior, H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects[23]. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate.

It is well known that nitric oxide play an important role in various inflammatory processes such as carcinomas, juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis[24]. Nitric oxide is a potent pleiotropic inhibitor of physiological process such as smooth muscle relaxation, neural signalling, 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, vasodilation and antimicrobial and antitumor activities[25]. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions[26].

In the present investigation, leaf extract of *A. nigra* exhibited outstanding scavenging effects on DPPH, ABTS, nitric oxide and H_2O_2 radicals. In addition, TPC and TFC of the extract were evaluated. It was observed that the leaf extract contained high level of phenolic and flavonoid content that might have accounted for the strong activity observed against the free radicals. Results revealed that leaves of *A. nigra* have many phytochemical constituents which may be responsible for many pharmacological activities. Since this investigation is a preliminary study, a detailed study of the antioxidant mechanisms of specific phenolic components is an absolute necessity. For further work on the profile and nature of chemical constituents of *A. nigra* leaves will provide more information on the active principles responsible for their pharmacological properties. This may also lead to the development of a new generation of drugs that possess both chemotherapeutic and chemopreventive properties which can result in ways of combating the serious problems of diseases. Never the less, based on the above presented results, leaf extract of *A. nigra* could be investigated as a possible new source of natural antioxidants in the food, nutraceuticals and cosmetic industry.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

ROS exert oxidative damaging effects by reacting with nearly every molecules found in living cells including protein, lipid, amino acids and DNA, if excess ROS are not eliminated by antioxidant system. They play important roles in aging and in the pathogenesis of age related disorders such as cancer, hypertension, atherogenesis, Alzheimers disease and Parkinsons disease. The most practical way to fight degenerative diseases is to increase antioxidant activity in our body and that could be achieved by consumption of vegetables, fruits or edible plants. There is an increasing interest in natural antioxidants *e.g.* polyphenols, present in medicinal and dietary plants, which might help to prevent oxidative damage.

Research frontiers

The presence of different phytoconstituents in the methanol extract of *A. nigra* leaves is being determined by performing the preliminary phytochemical analysis. The study is also being performed in order to determine the *in-vitro* antioxidant activity of *A. nigra* leaves by DPPH, ABTS, H_2O_2 and nitric oxide radical scavenging assay and the content of total phenolics and flavonoids.

Related reports

A. nigra is one of the important herbal remedies used in Thai traditional medicine for stomachicum, gastric diseases, antibacterial and antifungal activities. (Habash M *et al.*,

2000). Rhizomes of *A. nigra* are closely related to galangal, curcuma and zinger and the crushed leaves and rhizomes produce an aromatic fragrance, indicating the presence of essential oils. Even though leaves of ginger species have been used for food flavouring and in traditional medicine, still very little research has been done in their antioxidant properties as per the reports of Chan EWC, *et al.* (2008).

Innovations and breakthroughs

The leaf extract of *A. nigra* exhibited outstanding scavenging effects on DPPH, ABTS, nitric oxide and H_2O_2 radicals as evidenced from the results of the study. It is also observed that the leaf extract contained high level of phenolics that might have accounted for the strong activity observed against the free radicals.

Applications

Since the leaf extract of *A. nigra* exhibited significant free radical scavenging effect in different *in-vitro* experimental methods, therefore it could be further investigated as a possible new source of natural antioxidants in the food, nutraceuticals and cosmetic industry.

Peer review

This is a good study in which the authors have evaluated the *in-vitro* antioxidant activity of the methanol extract of *A. nigra* leaves by DPPH, ABTS, H_2O_2 and nitric oxide radical scavenging assay and the content of total phenolics and flavonoids. The extract exhibited outstanding scavenging effect as evidenced from the study. The results are interesting and it is suggested that the leaf extract contained high level of phenolics that might have accounted for the strong activity observed against the free radicals.

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