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Original article

Phytochemical, analgesic, antibacterial, and cytotoxic effects of *Alpinia nigra* (Gaertn.) Burtt leaf extract

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ABSTRACT

This research evaluated the phytochemical contents as well as the analgesic, cytotoxic, and antimicrobial effects of the methanolic extract of *Alpinia nigra* leaf. Phytochemical analysis was carried out using established methods. The analgesic effects of the extract were measured with the formalin test and tail immersion test. The antibacterial activity of the extract was evaluated using the disc diffusion technique. Cytotoxicity was assessed with the brine shrimp lethality bioassay. Data were analyzed with one-way analysis of variance using statistical software (SPSS, Version 19.0). The qualitative phytochemical screening of *A. nigra* leaf extract showed the presence of medicinally active secondary metabolites such as alkaloids, glycosides, cardiac glycosides, flavonoids, steroids, tannins, anthraquinone glycosides, and saponins. The extract at a dose of 200 mg/kg revealed a prevailed central nociception increasing the reaction time in response to thermal stimulation. The extract also showed a response to chemical nociceptors, causing pain inhibition in the late phase. The leaf extract (2 mg/disc) showed mild antibacterial activity compared to tetracycline (50 μg/disc). In the brine shrimp lethality bioassay, the LC_{50} (lethal concentration 50) value of the extract was found to be 57.12 μg/mL, implying a promising cytotoxic effect. The results evidenced the moderate analgesic and antibacterial effects with pronounced cytotoxic capability.

1. Introduction

Medicinal plants are the richest natural sources of medicinal products used in traditional and orthodox medicine.[1] The search for medicinal values of different plants has attracted increasing interest in the past couple of decades, presumably because of their potential as sources of potent pharmacological activities, convenience to users, economic viability, as well as low toxicity.[2] Plant-derived drugs proved to be relatively safer and dependable even in long-term use, where synthetic drugs are always feared in chronic cases.[3] Plants have also formed the basis of sophisticated traditional medicine systems in different therapeutic areas for thousands of years in many countries.[4] Therefore, continuous efforts in the search for newer sources of traditional medicines as well as screening of existing ones for newer therapeutic indications are inevitably urgent.

Alpinia nigra (Gaertn.) B.L. Burtt, which belongs to the Zingiberaceae family, is known as *Jongly Ada* or *Tara* in Bengali. This aromatic and rhizomatous herb is also referred to as Galangal, False galangal, Greater galangal, Black-Fruited, or Kala.[5,6] It is found in most parts of Bangladesh including Chittagong. It is widely distributed in Yunnan and Hainan provinces of China, and other Southeast Asian countries.[5] This plant is used as vegetable and hot culinary spice. Different parts of this herb are used as folk remedies to treat dyspepsia, gastric disease, and insect bites.[7] A previous phytochemical investigation revealed the presence of two flavone glycosides, astragalin and kaempferol-3-*O*-glucuronide,[8] which possess several biological activities, including antibacterial,[9,10] antioxidant,[11–13] antiprotozoal,[14] hepatoprotective,[15,16] and glycation inhibitory effects.[17] The crude shoot extract is reported to cause destruction of surface tegument leading to paralysis and death of intestinal parasites.[18] Recent studies have reported the antibacterial effect of seed extracts, the anti-inflammatory effects of rhizome

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extracts, and the anthelmintic effect of the shoot ethanol extract of this plant.[19–21] Although the different parts of this plant have been studied for several biological effects, the leaf extract is yet to be studied extensively. This research, therefore, was aimed to investigate the *in vivo* analgesic and *in vitro* antibacterial and cytotoxic effects of *A. nigra* leaf extract. Apart from these, other species of *Alpinia* were found to have analgesic, antimicrobial, and cytotoxic effects, which assisted us to formulate our hypothesis on *A. nigra*, presumably because of the interspecies similarities of the biological activities of different plant species.

2. Materials and methods

2.1. Drugs and chemicals

All chemicals and reagents used in this study were of analytical grade. Methanol (99.5%), which was used for extraction, was procured from Sigma-Aldrich (Humburg, Germany). Nalbuphine HCl was obtained from Square Pharmaceuticals Ltd. (Kaliakoir, Gazipur, Bangladesh). Formalin was purchased from CDH, New Delhi-110002, India. Diclofenac sodium (powder form) was a gift from GlaxoSmithKline Ltd. (Chittagong-4217, Bangladesh). Normal saline solution was purchased from Beximco Infusion Ltd (Dhaka).

2.2. Collection of plants

A. nigra leaves were collected from the Bangladesh Centre for Scientific and Industrial Research (Chittagong, Bangladesh) plantation area in April 2010 and authenticated by Dr Sheikh Bokhtear Uddin, a taxonomist (Department of Botany, University of Chittagong, Chittagong, Bangladesh). A taxonomical voucher specimen (No. BS-05/2010) is preserved at the Bangladesh Centre for Scientific and Industrial Research as an accession.

2.3. Preparation of extract

Fresh leaves of *A. nigra* were cleaned, sun-dried for about 5 days, and ground into coarse powder with a mechanical grinder (3 in 1 blender; Miyako, China). A total of 292 g powder was macerated into 800 mL of 98% methanol (Sigma Chemicals Co., St. Louis, MO, USA) for 5 days at room temperature (25 ± 1°C) with occasional stirring. The filtered extract was concentrated under reduced pressure below 50°C through a cyclone vacuum evaporator (RE200; Bibby Starling, Staffordshire, England). The concentrated extract was collected in a Petri dish and allowed to air-dry for the complete evaporation of methanol in the absence of sunlight. The whole process was repeated three times, and finally 15 g of blackish green, concentrated extract was obtained (yield 5.14%, w/w), which was kept in a refrigerator at 4°C.

$$\text{Yield } (\%) = \frac{\text{Weight of particular extract}}{\text{Total amount of coarse powder}} \times 100$$

2.4. Phytochemical screening of the extract

The freshly prepared crude extract was qualitatively tested for the presence of secondary metabolites especially saponins, flavonoids, steroids, anthroquinone, alkaloids, terpenoids, phlobatannins, tannins, and cardiac glycosides through established methods.[22]

2.5. Analgesic study

The analgesic effects of the extract were evaluated using two different animal pain models: the tail immersion test and formalin test.

2.6. Experimental animals

The experiments were carried out on 6-week-old Swiss albino mice of both sexes weighing 25–30 g. The animals were housed in standard polypropylene cages, at five mice per cage, under a 12-hour light/12-hour dark schedule at an ambient temperature of 25 ± 1°C. The mice were maintained on standard laboratory animal feed and water *ad libitum*. The animals were acclimatized for a week prior to commencing the experiment. They were deprived of food but not water 4 hours prior to the experiment. All animal experiments were maintained and carried out in compliance with Helsinki Animal Ethics guidelines.

2.7. Formalin test

The most predictive of the models for acute pain is the formalin test. The method used in our study was similar to that described previously by Shibata et al.[23] Briefly, 20 μL of 5% formalin was injected subcutaneously into the right hind paw of mice to produce a biphasic pain response. The time (in seconds) spent in licking and biting responses of the injected paw was taken as an indicator of pain response. Responses were measured from 0 to 5 minutes (early phase, neurogenic) and 25–40 minutes after formalin injection (late phase, inflammatory).[24] The mice selected for this study were divided into three groups of five animals each. In the test group, the plant extract [200 mg/kg, intraperitonally (i.p.)] was administered 60 minutes prior to the formalin injection. Diclofenac sodium (10 mg/kg, i.p.) was administered 30 minutes prior to the formalin injection in the positive control group. The control group received the same volume of saline by oral administration.

2.8. Tail immersion test in mice

Analgesia was assessed according to the method described by Chandrashekar et al,[25] with slight modifications. The mice selected for this study were divided into three groups of five animals each and pretreated with 200 mg/kg (i.p.) of the extract for Group 1, whereas Group 2 received 10 mL/kg normal saline. As the reference standard, nalbuphine HCl (10 mg/kg) was administered to Group 3. After 30 minutes of drug administration, each mouse was held in position in a suitable restrainer with its tail extending out. About a 3 cm area of the tail was marked and immersed in the water bath thermostatistically maintained at 51°C. Within a few seconds, the mouse reacted by withdrawing the tail. The time it took for the mouse to remove its tail out of the water was recorded. The latency was evaluated at 0, 30, 60, and 90 minutes with 0 minute being the initial reading. The criterion for analgesia was postdrug latency, which was greater than two times the predrug average latency as reported by Janssen et al.[26] The mean increase in latency after drug administration was used to indicate the analgesia produced by test and standard drugs. To avoid injury to the tail, the maximum cutoff time for immersion was fixed at 15 seconds. The animals were allowed to adapt to the cages for 30 minutes prior to testing.

2.9. Assay of antibacterial activity

2.9.1. Bacterial strains used in the study

The bacterial strains selected for this research were *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, *Salmonella paratyphi*, *Escherichia coli*, *Vibrio cholerae*, and *Shigella sonnie*. All of these organisms were obtained from the Department of Microbiology, University of Chittagong.

2.9.2. Disc diffusion technique

The *in vitro* sensitivity of the bacteria to the extract was investigated using the disc diffusion method as described by Iennette.[27] Under aseptic conditions, a sterile nutrient broth was prepared and inoculated with the test organisms. It was incubated for 24 hours at 37°C and used as inoculum. Nutrient agar was prepared, autoclaved, and cooled to 45°C. For plate preparation, 0.5 mL of bacterial suspension and 15–20 mL of nutrient agar was poured, respectively, into the sterile Petri dish under aseptic conditions and rotated clockwise and counterclockwise for a few times to be seeded uniformly. Then the agar medium was allowed to solidify. A measured amount of plant extract was dissolved in dimethyl sulfoxide (DMSO) to obtain a solution of known concentration (200 μg/μL). Dried and sterilized filter paper discs (4 mm in diameter) were treated with test materials (2 mg/disc) using a micropipette, then carefully dried in air to evaporate the residual solvent. Reference antibiotic discs (Tetracycline; 50 μg/disc; Oxoid, Hampshire, England) were placed on the agar surface with sterile forceps gently pressed down to ensure complete contact of the disc with the agar surface. These plates were then kept for 2–4 hours at low (4–6°C) temperature and allowed to diffuse the test materials (antimicrobial) from the disc to the surrounding medium by this time. The plates were then incubated at 37°C for 24 hours to allow the maximum growth of the organisms. The zone of inhibition includes the diameter of the disc (in millimeters). Each experiment was repeated three times.

2.10. Brine shrimp lethality bioassay

Brine shrimp lethality bioassay is based on the ability to kill laboratory-cultured brine shrimp nauplii (*Artemia salina*) that can be used as a convenient monitor for the screening. The eggs of the brine shrimp were collected from an aquarium shop (Dhaka, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) at a temperature of about 37°C with constant oxygen supply for 48 hours to mature the shrimps called nauplii.

2.11. Preparation of test groups

The plant extract was dissolved in DMSO to give a concentration of 500 μg/mL. Fifteen test tubes were taken, each of which contained 5 mL seawater. Ten nauplii were transferred to each tube using Pasteur pipette. Then solutions of varying concentrations (from 1250 to 12.5 μg/mL) were obtained by adding a freshly prepared sample in different test tubes. Only 50 μL DMSO was added in 5 mL seawater containing 10 nauplii for control. Each concentration was tested in triplicate. The test tubes were maintained under illumination. After 24 hours, the percentage of mortality was counted using a magnifying glass.

2.12. Statistical analysis

All data are expressed as mean ± standard error of the mean. Data were analyzed with one-way analysis of variance using a statistical software (SPSS, Version 19.0; IBM Corporation, New York, NY, USA). A difference was considered significant at $p < 0.05$.

3. Results

3.1. Phytochemical contents

The qualitative phytochemical screening was carried out for *A. nigra* leaf extract. The results of various biochemical tests for the detection of chemical constituents are summarized in Table 1.

3.2. Analgesic effects

The results obtained in formalin test and tail immersion method are shown in Tables 2 and 3, respectively. The extract at the dose of 200 mg/kg body weigh displayed inhibition of the late phase of formalin-induced pain, which was significant ($p < 0.05$) compared to control. In the tail immersion method, the methanolic extract of *A. nigra* showed significant ($p < 0.05$) increase in reaction time up to 60 minutes after thermal stimulus was applied. The extract exhibited potent analgesic effect after 30 and 60 minutes of administration at a dose level of 200 mg/kg body weight.

3.3. Antibacterial effects

The antibacterial activity of the *A. nigra* leaf (2 mg/disc) extract tested against nine pathogenic bacteria was compared with that of standard antibiotic tetracycline. The results indicate that the leaf extract of *A. nigra* have different levels of *in vitro* antibacterial activity. Table 4 shows the zone of inhibition diameter expressed in millimeters.

3.4. Cytotoxic effects

Following the procedure of Meyer et al,[28] the lethality of the crude *A. nigra* was determined on *A. salina* after a 24-hour exposure of the samples. This technique was applied for the determination of general toxic property of the plant extract. From the mortality percentage of brine shrimp (Fig. 1), the probits were calculated for each concentration and plotted against the corresponding log concentration of the plant extract. From this plot, the LC_{50} (lethal concentration 50) value was found to be 57.12 μg/mL by regression analysis using the computer program “BioStat-2009” (Fig. 1).

4. Discussion

Plants are potential sources of new drugs for human benefit. Plants used in traditional medicine contain a vast array of substances that can be used to treat chronic and even infectious diseases.[29] The phytochemical analysis of the extracts of *A. nigra* showed the presence of alkaloids, glycosides, cardiac glycosides, flavonoids, steroids, tannins, anthraquinone glycosides, and saponins.

The tail immersion test is very sensitive to centrally acting drugs.[30] It measures the complex response to a noninflammatory, acute nociceptive input. To produce painful reactions in animals, thermal stimulus is used by dipping the tip of the tail in hot water. The tail immersion method in our study indicated that the central analgesic effect of the methanolic extract of the test drug was

Table 1
Screening test for phytochemical groups in the extract of *Alpinia nigra*.

Alkaloids	Glycosides	Cardiac glycosides	Anthraquinones	Terpenoids	Flavonoids	Steroids	Tannins	Phlobatannins	Saponins
+	+	+	+	−	+	+	+	−	+

+ = presence; − = absence.

Table 2
Effect *Alpinia nigra* leaf extract on formalin-induced pain in Swiss albino mice.

Treatment (mg/kg)	Mean pain scores			
	First phase	% Inhibition	Second phase	% Inhibition
Normal saline	1.44 ± 0.23	–	3.02 ± 0.14	–
Diclofenac Na (10 mL/kg)	1.09 ± 0.18**	24.30	1.01 ± 0.42**	66.55
ANEx (200 mg/kg)	1.79 ± 0.22*	−19.69	2.76 ± 0.26	08.60

Each value represents mean ± SEM.
*$p < 0.05$, **$p < 0.01$ compared with control (Student *t* test).
ANEx = *Alpinia nigra* extract ($n = 5$); SEM = standard error of the mean.

Table 3
Effects of *Alpinia nigra* leaf extract on the tail withdrawal reflex of mice induced by the tail immersion method.

Treatment (mg/kg)	Mean reaction time before and after treatment			
	0 min	30 min	60 min	90 min
Normal saline (10 mL/kg)	2.21 ± 0.05	2.5 ± 0.09	2.9 ± 0.18	2.39 ± 0.09
Nalbupin (10 mg/kg)	2.27 ± 0.07	6.05 ± 0.24**	9.23 ± 0.20**	11.93 ± 0.52*
ANEx (200 mg/kg)	2.46 ± 0.06	3.75 ± 0.47*	3.82 ± 0.66*	3.77 ± 0.23

Each value represents mean ± SEM.
*$p < 0.05$, **$p < 0.01$ compared with control (Student *t* test).
ANEx = *Alpinia nigra* extract ($n = 5$); SEM = standard error of the mean.

Table 4
In vitro antibacterial activity of *A. nigra* leaf extract and reference antibiotic (tetracycline).

Bacterial type	Test organism	Diameter of zone of inhibition (mm)	
		ANEx (2 mg/disc)	Tetracycline (50 μg/disc)
Gram (+)ve	*Bacillus cereus*	8.0	26
	Bacillus subtilis	8.6	25
	Staphylococcus aureus	9.0	13
Gram (−)ve	*Salmonella typhi*	6.0	16
	Salmonella paratyphi	7.0	15
	Shigella dysenteriae	7.5	15
	Escherichia coli	10.0	26
	Vibrio cholerae	9.0	15
	Shigella sonnie	9.2	15

ANEx = *Alpinia nigra* extract.

significant as revealed by the increased reaction time after giving thermal stimulus to the animal.[31] The latency in reaction time continued up to 90 minutes after the administration of 200 mg/kg extract, revealing a sustained and pronounced central analgesic effect when compared with the control group. However, its overall central analgesic effect was slightly weaker when compared with the standard drug nalbuphine HCl over different time intervals.

The formalin test is sensitive to nonsteroidal anti-inflammatory drugs[32] and other mild analgesics. The test uses a chemical nociceptive stimulus that elicits a spontaneous response indicative of pain. The test has two different phases, possibly reflecting the different types of pain.[24,32] The early phase may be due to direct effects on nociceptors and can be inhibited by centrally acting analgesics such as morphine. In contrast, the late phase may be attributable to an inflammatory response partly mediated by prostaglandins and can be inhibited by nonsteroidal anti-inflammatory drugs and steroids, as well as by centrally acting drugs. As inflammation occurs at the site of formalin injection, it is possible to elucidate the role of inflammation on the responses in the two phases. No inhibitory effect was shown in the first phase, but an insignificant inhibitory effect compared to the reference drug (diclofenac sodium) was observed in the late phase of our study, reflecting a lighter central antinociceptive effect of the extract. However, it is obvious from the formalin test and tail immersion test that *A. nigra* has prevailing central analgesic effects on the chemical nociceptors and thermal stimuli, although the effects are not very pronounced compared to reference drugs in both cases.

The antibacterial effect of the extract was analyzed in the disc diffusion technique. Reports from Sinha and Choudhury[33] showed that *A. nigra* has antimicrobial activity. In the present investigation, the extract exhibited better antibacterial activity for *E. coli*, *S. sonnie*, *V. cholerae*, and *S. aureus* compared to *S. typhi*, *S. paratyphi*, and *Shigella dysenteriae*. A narrow zone of inhibition was found against *B. subtilis* and *B. cereus*, whereas *S. typhi*, *S. paratyphi*, and *S. dysenteriae* showed insignificant inhibitory effect.

The brine shrimp lethality bioassay against *A. salina* is a simple method to test cytotoxicity. It is an indicator of antitumor and pesticidal activity,[34] as well as antiviral, antiplasmodial, antifilarial, and antimalarial activities.[35] The LC_{50} value of *A. nigra* leaf extract was found to be 57.12 μg/mL, which indicates the significant pharmacological actions of the crude leaf extract.[36] The inhibitory effect—i.e., ovicidal and larvicidal properties—of the extract might be attributable to the toxic compounds in the active fraction that either affected the embryonic development or slayed the eggs. Hence, the cytotoxic effects of the plant extracts can be selected for further cell line assay.[36]

Conclusion

From the results obtained, it can be concluded that the leaf extract of *A. nigra* possesses profound cytotoxic and analgesic activities. The extract also showed moderate antibacterial activity. Therefore, the present study offers a scientific basis for the folkloric use of this plant in the management of various illnesses. Still, as this

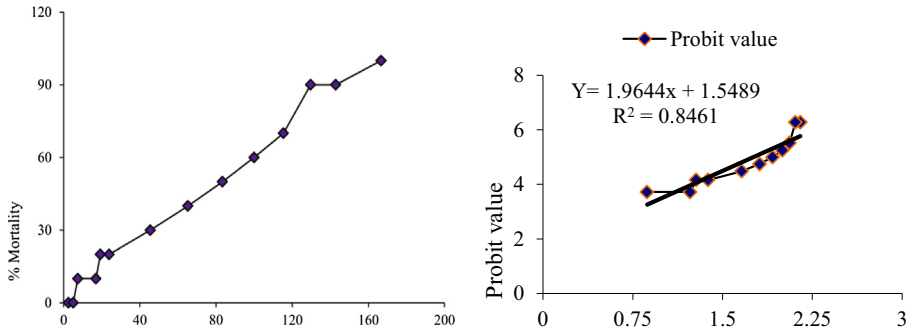

Fig. 1. Mortality and regression line for determining the LC_{50} value of ANEx. ANEx = *Alpinia nigra* extract; LC_{50} = lethal concentration 50.

is only preliminary study, further studies are necessary to identify the phytochemicals responsible for the different pharmacological effects.

Conflicts of interest

The authors declare that there is no conflict of interest.

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