

Evaluation of analgesic activity and toxicity of alkaloids in *Myristica fragrans* seeds in mice

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Aim: To examine the analgesic effect of alkaloids in *Myristica fragrans* seed in a mouse model of acetic acid-induced visceral pain.

Methods: Alkaloids were extracted from ground nutmeg seed kernels with 10% acetic acid in 95% ethyl alcohol. Visceral pain was induced in male and female BALB/c mice by intraperitoneal injection of 0.6% acetic acid. Analgesic effect of alkaloids (0.5 gram or 1 gram per kilogram [g/kg], by mouth) was assessed by evaluating writhing response. Acute toxicity was tested in response to 2, 3, 4, 5, or 6 g/kg of alkaloid extract; the median lethal dose (LD₅₀) was determined by probit analysis.

Results: Alkaloid extract at a dose of 1 g/kg significantly reduced the number of writhing responses in female, but not male mice; 0.5 g/kg of alkaloid extract had no effect in either sex. The LD₅₀ was 5.1 g/kg. Signs of abnormal behavior, including hypoactivity, unstable gait, and dizziness were seen in animals given a dose of 4 g/kg or higher; abnormal behavior lasted for several hours after administration of the alkaloids.

Conclusion: According to the classification of Loomis and Hayes, *M. fragrans* seed alkaloids have analgesic activity and are slightly toxic.

Keywords: analgesic, mice, LD₅₀, acetic acid, visceral pain, nutmeg

Introduction

Myristica fragrans Houtt (nutmeg) is an aromatic evergreen tree of the plant family Myristicaceae.¹ Nutmeg, the actual seed of the tree, is important in folk medicine, where it is used to treat colds, fever, catarrh, general respiratory ailments, and skin diseases like scabies. It is also used as an appetite stimulant, carminative, antiemetic, and abortifacient.^{2,3}

In controlled laboratory studies, *M. fragrans* has been shown to possess insulin-like,⁴ insecticidal,⁵⁻⁸ antibacterial,⁹⁻¹² and antioxidant activities.¹³ However, prolonged use of nutmeg can cause degenerative changes in the kidney, spleen, liver, heart, medial geniculate body, and superior colliculus.¹³⁻¹⁶

Alkaloids are any of a class of naturally occurring, organic nitrogen-containing bases. Traditionally isolated from plants, alkaloids have been increasingly found in animals, insects, marine invertebrates, and microorganisms.¹⁷⁻¹⁹ Plant-derived alkaloids elicit many biological effects, including analgesia. Previous studies have demonstrated that an acetone-soluble substance within the n-hexane extract of *M. fragrans* exerts analgesic activity;^{20,21} however, the identity of the active constituents responsible for the analgesic activity remains unknown. Thus, the present study was designed to study the analgesic effect of alkaloids extracted from *M. fragrans* seeds in mice subjected

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to acetic acid-induced visceral pain and to assess the acute toxicity of these alkaloids.

Materials and methods

Plant materials

Dried *M. fragrans* seeds were collected from Iraqi markets in Thi-Qar City, Iraq and authenticated as *M. fragrans* seeds by AA Malik Al-Saadi Sahar. Only the seed kernels (nutmeg) were used in this study.

Detection of alkaloids

Seed kernels were ground to a fine powder. Fifty mL of 4% HCl was added to 10 grams of nutmeg powder, heated to a boil, cooled, and filtered. Three drops each of Mayer's reagent/picric acid/Dragendorff's reagent were added to 0.5 mL of filtrate. The presence of alkaloid was indicated as a white precipitate (Mayer's reagent), a yellow precipitate (picric acid), or an orange precipitate (Dragendorff's reagent).²²

Alkaloid extraction

Crude alkaloid compounds were extracted according to the modified method of Harborne.²² Crushed seed kernels (20 grams) were suspended in 200 mL of hexane, and lipid soluble impurities were removed by continuous extraction using a Quickfit® Soxhlet apparatus (Sigma-Aldrich, St Louis, MO, USA). Kernels were dried under laboratory conditions and subjected to an extraction process with 200 mL of 10% acetic acid in 95% ethyl alcohol for 24 hours in the Soxhlet apparatus. The extract was filtered through No 1 Whatman filter paper (BDH Pharmaceuticals, London, UK) and concentrated by a rotary evaporator (Bibby Scientific Ltd, Staffordshire, UK) at 45°C to 20 mL. The pH was adjusted to 9 by adding concentrated ammonium hydroxide solution, and the solution was partitioned three times with 50 mL of chloroform in a separation funnel, which was shaken vigorously and left to stand each time. The extract separated into two layers. The lower (chloroform) layer contained the alkaloids, which was confirmed with Mayer's reagent, picric acid, or Dragendorff's reagent. The chloroform layer was concentrated using a rotary evaporator and left to dry under laboratory conditions. Dried alkaloids were stored in a clean, dark vial at 4°C.

Experimental animals

Two-month-old BALB/c mice of both sexes, weighing 20–25 grams, were obtained from the animal house, College of Science, Thi-Qar University. The animals were housed in a vivarium maintained under standard hygienic conditions at

20°C ± 2°C with a 12-hour day/night cycle and access to food and water ad libitum. Animals were treated in accordance with the Ethical Guidelines for the Investigation of Experimental Pain in Conscious Animals issued by the International Association for the Study of Pain, and were approved by the local animal care ethics committee.²³

Acute toxicity study

Male and female mice were divided into six groups of twelve mice each (six males and six females). Animals were matched for weight and size and allowed to acclimate for 3 days. Groups were given vehicle or 2, 3, 4, 5, or 6 g/kg of nutmeg alkaloids suspended in 0.4 mL of 70% ethanol:distilled water (1:3 by volume) by oral gavage. Animals were observed for 72 hours for behavioral changes or mortality. The median lethal dose (LD₅₀) was determined by probit analysis.

Analgesic activity in the acetic acid-induced writhing model

Mice were randomly divided into four groups of twelve animals each (six males and six females) and set to receive: (1) vehicle (0.4 mL 70% ethanol:distilled water [1:3 by volume]) delivered orally; (2) diclofenac sodium (20 mg/kg) delivered by intraperitoneal injection; (3) *M. fragrans* seed alkaloids (0.5 g/kg) delivered orally in 0.4 mL of vehicle; or (4) *M. fragrans* seed alkaloids (1 g/kg) delivered orally in 0.4 mL of vehicle.

Animals were placed separately into a cage and allowed to acclimate for at least 10 minutes. Mice were given vehicle,

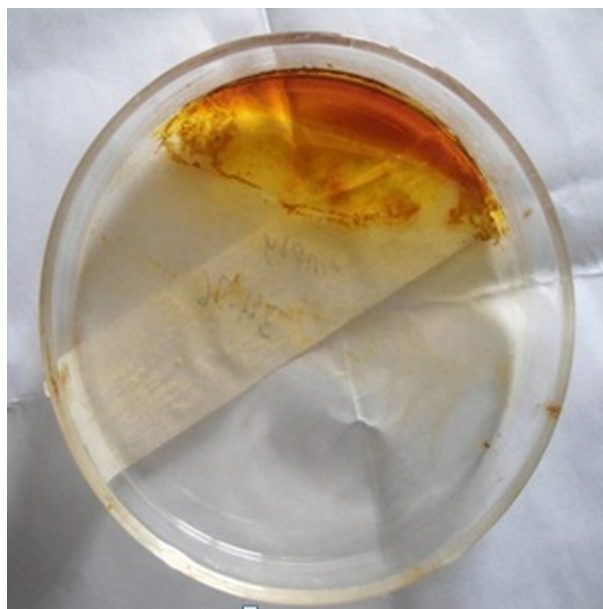


Figure 1 Alkaloids extracted from *Myristica fragrans* seeds.

Table 1 Acute toxicity of *Myristica fragrans* alkaloids

Dose g/kg	Male				Female				Total number of dead animals	Mortality %
	Total number	24 hours	48 hours	72 hours	Total number	24 hours	48 hours	72 hours		
2	6	0	0	0	6	0	0	0	0	0
3	6	0	0	0	6	0	0	0	0	0
4	6	1	0	0	6	2	0	0	3	25
5	6	3	0	0	6	2	0	0	5	41.6
6	6	3	0	0	6	6	0	0	9	75

diclofenac, or alkaloid suspensions 1 hour prior to induction of visceral pain. Visceral pain was induced by intraperitoneal injection of a 0.6% volume per volume (10 mL/kg) glacial acetic acid solution in normal saline. Animals were returned to their cages and observed for writhing behavior, indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb.²⁴ The number of writhing responses was counted for 15 minutes, starting directly after the acid injection.

Statistical analysis

Data are expressed as mean \pm standard error of the mean and were analyzed by two-way analysis of variance. Differences among means were considered significant at $P < 0.01$ using the Fisher's Least Significant Difference. Analyses were performed using the Statistical Product and Service Solutions, 2006 (IBM Corporation, Armonk, NY, USA).

Results

Detection of alkaloids

The appearance of white, yellow, or orange precipitate using Mayer's reagent, picric acid, or Dragendorff's reagent, respectively, revealed the presence of alkaloids. Figure 1 is a representative picture, showing alkaloids extracted from nutmeg as a brown sticky material.

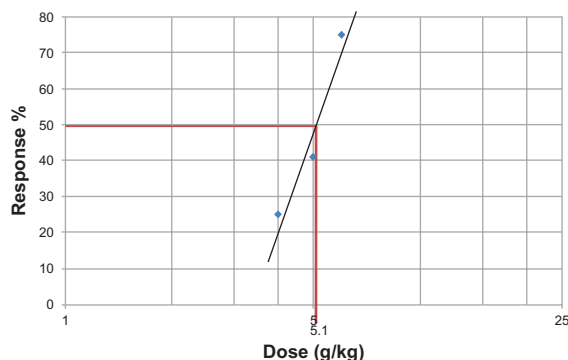


Figure 2 Probit plot of deaths caused by acute treatment with *Myristica fragrans* alkaloids.

Acute toxicity study

The number of deaths resulting from administration of *M. fragrans* crude alkaloids is given in Table 1; LD₅₀ was 5.1 g/kg (Figure 2). Animals given a dose of 4 g/kg or greater exhibited abnormal behavior, including hypoactivity, unstable gait, or dizziness lasting for several hours, doses of 3 g/kg or less did not elicit any abnormal behavior.

Analgesic activity of the alkaloids

The analgesic effect of nutmeg crude alkaloids in the acetic acid-induced writhing model is shown in Table 2. The number of writhing responses was significantly reduced in female, but not in male mice treated with alkaloids at a dose 1 g/kg of alkaloids; 0.5 g/kg had no effect in either sex. Diclofenac caused comparable decreases in the number of writhing responses in male and female mice; the effect of diclofenac was significantly greater than that of nutmeg alkaloids.

Discussion

Acute toxicity study

In this study, the LD₅₀ of alkaloids extracted from *M. fragrans* seeds was calculated to be 5.1 g/kg. According to classifications put forth by Pascoe²⁵ and by Loomis and Hayes,²⁶ this corresponds to ratings of slightly toxic and practically nontoxic, respectively. Our results agree with those from previous studies examining the effects of other extracts derived

Table 2 Number of writhing responses induced by acetic acid

Treatment	Female	Male	Mean treatment
Control	22 \pm 3 ^{ab}	22 \pm 2 ^{ab}	22 \pm 2 ^a
0.5 g/kg	23 \pm 3 ^a	21 \pm 3 ^b	22 \pm 2 ^a
1 g/kg	17 \pm 4 ^c	21 \pm 4 ^b	19 \pm 3 ^b
Diclofenac	4 \pm 1 ^d	4 \pm 1 ^d	4 \pm 1 ^c
Mean	17 \pm 2	17 \pm 2	
LSD	Treatment = 2.53	Interaction = 1.49	Sex NS

Note: Values represent mean \pm SEM. Values with nonidentical superscripted letters (a–d) are considered significantly different ($P < 0.01$).

Abbreviations: SEM, standard error of the mean; LSD, least significant difference; NS, non significant.

from *M. fragrans*. In those studies, the acetone-soluble component of the n-hexane extract²¹ and a 50% ethanolic extract²⁷ caused no deaths and had no effect on behavior up to doses 3 and 4 g/kg, respectively.

Analgesic activity

The acetic acid-induced writhing model is used to evaluate the effect of analgesics such as nonsteroidal anti-inflammatory drugs on visceral pain.^{28,29} We found that alkaloids extracted from *M. fragrans* seeds caused a modest, but significant, reduction in writhing behavior in female, but not male mice.

Acetic acid causes pain by releasing endogenous mediators that stimulate nociceptive neurons. These include cytokines, such as interleukin-1 β and interleukin-8 released by resident peritoneal macrophage and mast cells, and prostaglandins and lipoxygenase products released into the peritoneum.^{29–31} Nonsteroidal anti-inflammatory drugs induce analgesia by inhibiting prostaglandin synthesis via the cyclooxygenase pathway.³² Chemical substance extracts from Myristicaceae plants have been shown to inhibit phospholipase A₂, thus lowering the availability of arachidonic acid precursor for prostaglandin synthesis.³³ Other investigators have postulated that the antinociceptive activity of plant extracts may be due to inhibition of interleukin-1 β and interleukin-8 release by resident peritoneal cells or to suppression of prostaglandins and bradykinin;^{32,34,35} however, direct evidence for these actions is lacking.

There is some variability in the literature regarding the criteria applied to evaluate writhing behavior. Some investigators have divided these into complete (or full) and half responses; two half responses are considered to be one complete response. Others have evaluated the response similar to the method used in the present study.^{24,36}

We conclude that alkaloids derived from *M. fragrans* seeds possess analgesic activity. However, further study is warranted to identify the active constituent.

Disclosure

The authors report no conflicts of interest in this work.

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