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Isolation, characterization and quantification of an anxiolytic constituent - mahanimbine, from *Murraya koenigii* Linn. Spreng Leaves

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ABSTRACT

Ethnopharmacological relevance: Leaves of *M. koenigii* Linn. Spreng (Rutaceae) have been used as traditional medicine for anxiety disorders. Aim of the study was to isolate antianxiety principle(s) from the leaves of *M. koenigii* using bioactivity guided approach.

Material and methods: Hydroalcoholic extract of *M. koenigii* leaves was prepared using soxhlet apparatus, and the same was evaluated for antianxiety activity at 250, 500 and 750 mg/kg, po, using Elevated plus-maze (EPM). The extract was further partitioned successively with pet ether, chloroform, ethyl acetate and 1-butanol. All the fractions were evaluated for antianxiety activity. The bioactive ethyl acetate fraction was column chromatographed to get 5 fractions (F_1-F_5). All the fractions were evaluated for antianxiety activity using EPM. A pure compound, separated out from F_2, was characterized using standard spectroscopic techniques, and its anxiolytic activity was evaluated using EPM. Antianxiety activity of isolated compound was further evaluated using Actophotometer and m-CPP induced anxiety model. TLC-densitometric method was developed to quantify mahanimbine in the plant.

Results: The present study resulted in the isolation of mahanimbine, which exhibits potent antianxiety activity at 3 mg/kg, and the activity was statistically comparable to that of diazepam (2 mg/kg). The developed TLC-densitometric method is specific, linear, precise, accurate, repeatable and robust.

Conclusions: This study validates the ethnopharmacological use of *M. koenigii* leaves in the management of anxiety disorders. Mahanimbine is responsible for the antianxiety effect of *M. koenigii* leaves.

1. Introduction

Murraya koenigii Linn. Spreng (Syn. *Bergera koenigii* Linn., *Chalcas koenigii* Linn. Kurz), family Rutaceae, is an ancient Indian medicinal plant (Adebajao et al., 2006) native to Indo-China (Rao et al., 2011; Bandyopadhyay et al., 2012). It is commonly known as curry leaves, and is widely used as condiment. The plant has been used to treat various ailments in Indian traditional system of medicine. *M. koenigii* is highly valued for its leaves which are widely used in Indian cookery for centuries to promote appetite and digestion (Handral et al., 2012). Traditionally, the leaves, bark and roots of the plant are used as tonic, stomachic and to purify blood (Tembhurne and Sakarkar, 2011). Green leaves are eaten raw for curing dysentery, diarrhoea, depression, inflammation, kidney pain, and vomiting (Jain et al., 2012). Water extract of the plant has been traditionally used in anxiety disorders by Ayurvedic physicians in Sri Lanka (Ratnasooriya et al., 1994).

M. koenigii has been reported to possess diverse biological activities. Leaf of this plant is reported to exhibit analgesic (Gupta et al., 2010), antiamnesic (Tembhurne and Sakarkar, 2011), antiasthmatic (Parmar et al., 2010), antiinflammatory (Gupta et al., 2010; Mathur, 2011), antileukemial (Nakahara et al., 2004; Itoigawa et al., 2006), antioxidant (Nakatani et al., 2001), antitumour (Vairappan et al., 2011), anxiolytic (Ratnasooriya et al., 1994), heptoprotective (Sathaye et al., 2011; Ramachandran et al., 2012), hypoglycemic (Watal et al., 2005), *etc.* activities.

Mahanimbine, a carbazole alkaloid, is reported to be present in leaf, stem bark and root of *M. koenigii*. Most of the carbazole alkaloids have been isolated from taxanonomically related plants of the genus *Murraya*, *Glycosmic* and *Clausena* from the family Rutaceae (Knolker and Reddy, 2002). *M. koeinigii* has been regarded as the richest source of carbazole alkaloids. Further, carbazole alkaloids have been reported to exhibit phramacological properties such as anti-tumor, anti-viral, anti-inflammatory, anti-convulsant, diuretic and anti-oxidant activities (Knolker and Reddy, 2008).

Based on traditional use of *M. koenigii* leaves in anxiety disorders, it was considered worthwhile to carry out detailed investigation on the leaves employing bioactivity guided isolation with a view to isolate its anxiolytic constituent(s).

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2. Materials and methods

2.1. Plant material

M. koenigii leaves were collected from Panjab University campus, Chandigarh, India, in the month of August 2013 and dried in shade. Authors confirmed the identity of collected leaves by comparing their morphology, microscopy and TLC fingerprint profile with the authentic leaves of *M. koenigii* collected from the Medicinal Plant Garden of the University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, and a voucher specimen (No. 31, dated 26.8.2013) has been deposited in the Herbarium-cum-Museum of this institute for future reference.

2.2. General

Solvents used included petroleum ether (40–60 °C), chloroform, methanol, ethanol, 1-butanol and ethyl acetate (Merck specialities Limited, Mumbai). Chromatograms were obtained on 20×10 cm pre-coated aluminium-based TLC sheets (Merck, Silica gel G 60, 0.2 mm). Silica gel (# 60-120, s.d. fine-chem Ltd, Mumbai) was used as stationary phase for column chromatography. One µl standard capillary tubes (CAMAG) were used for loading the sample on TLC plates. Thin layer chromatograms were visualized initially under UV light at 254 nm (DESAGA, Heidelberg), and also after spraying with 0.5% anisaldehyde reagent followed by heating at 110 °C for 10 min.

UV and IR spectra were obtained on Perkin Elmer Hitachi 330 (Lambda 15 UV/VIS) spectrophotometer and IR spectrophotometer (Multispoke FT-IR synthesis monitoring system, Perkin Elmer, Germany), respectively. ^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker spectrophotometer (Avane, Germany) at 400 MHz using $CDCl_3$ as solvent. Shifts were expressed in parts per million (ppm) relative to tetramethylsilane (TMS) as the internal standard. Mass spectra were obtained in the positive ion mode on mass spectrometer (Finnigan, USA) equipped with a pneumatically-assisted atmospheric-pressure chemical ionization (APCI). Waters HPLC system (Waters 2695 separations module) equipped with Waters 2996 photodiode array (PDA) detector, a column oven and a quaternary pump system controlled by the Empower 2 software and SunFire C_{18} analytical column (4.6×250 mm, 5 µm; Water Corporation, MA, USA) were used.

Lacca mice (either sex), bred at the Central Animal House, Panjab University, were allowed standard pellet diet and water *ad libitum*. Groups of 6 mice (20–30 g) were used in all sets of experiments. The animals were fasted overnight before use. Approval from the Institutional Animal Ethical Committee of Panjab University, Chandigarh was taken before carrying out biological studies (Approval No. IAEC/411, dated 11.09.2013).

Aqueous carboxy methyl cellulose (0.5% w/v): Tween 80 (5%)::95:5 was used as vehicle for preparing the suspension of various test doses of extracts, fractions and bioactive constituent of *M. koenigii*. 1-(3-chlorophenyl)piperazine (mCPP; Sigma Aldrich) and diazepam (Java Pharmaceuticals, Gurugram) were used respectively as standard anxiogenic and antianxiety agents. All doses were prepared by suspending appropriate quantities in the vehicle so as to administer these to mice in volumes ranging between 0.20 and 0.30 ml per oral route.

2.3. Extraction and isolation

Coarsely powdered dried leaves of *M. koenigii* (500 g) were extracted with ethanol-water (70:30) using soxhlet apparatus, and the solvent was recovered under reduced pressure using rotary evaporator (Eyela, N1100) to get hydroalcoholic extract (HAE, 5%). HAE was suspended in water and solvent-partitioned to obtain petroleum ether (11.44% w/w), chloroform (17.60% w/w), ethyl acetate (1.84% w/w), 1-butanol (1.12% w/w) and remaining hydro-alcoholic fraction (67% w/w). Ethyl acetate fraction (10 g) was separated over column chromatography using silica gel (# 60–120) using pet ether, pet ether-chloroform (1:1), pet ether-chloroform (2:8), chloroform, chloroform-methanol (95:5) and chloroform-methanol (92:8) as eluants to obtain 160 fractions, each of 500 ml. These were pooled, based on similar thin layer chromatograms, to get 5 fractions (F_1-F_5). Concentrated solutions of fractions were kept in refrigerator. Precipitates observed in fraction F_2, were separated by filtration, and washed (×3) with cold pet ether to get a white solid compound A (yield: 0.0122%). TLC of this compound showed a single spot using pet ether-chloroform (1:1) as mobile phase.

2.4. Characterization of compound A

Compound A was characterized based on melting point, UV, IR, ^{1}H NMR, ^{13}C NMR and Mass spectra.

2.5. Purity determination of mahanimbine using HPLC

A concentration of 20 µg/ml was prepared by appropriate dilution of a methanolic stock solution (100 µg/ml) of mahanimbine. The sample was degassed and filtered through 0.22 µm syringe filter before analysis. Injection volume was kept 10 µl and run time was 20 min. Separation was achieved over a C_{18} column using isocratic elution with methanol–0.5% acetic acid in water (90:10 v/v) as the mobile phase at a flow rate of 0.8 ml/min, oven temperature 40 °C and PDA detection at 290 nm. The column was equilibrated with mobile phase prior to chromatographic analysis (Pandit et al., 2011).

2.6. Phytochemical screening

HAE and its ethyl acetate fraction were screened for different classes of phytoconstituents using standard procedures (Farnsworth, 1966).

2.7. Pharmacological evaluations

2.7.1. Acute oral toxicity studies

Acute toxicity studies were conducted following OECD 423 guidelines (OECD, 2001). After 12 h of fasting, different groups of mice were administered single oral dose (500, 1000 or 2000 mg/kg) of HAE of *M. koenigii* leaves. Immediately after the dose, animals were observed for signs of toxicity during the first 0.5, 1, 2, 4, 8 and 12 h, and at every 24 h for 14 days. Behavior parameters, weight, amount of water and feed consumed and mortality were observed.

2.7.2. Antianxiety activity

Antianxiety activity of HAE and its various fractions was evaluated using elevated plus-maze (EPM). Activity of compound A was evaluated using EPM, actophotometer and mCPP-induced antianxiety model (Pellow et al., 1985; Vogel et al., 2002).

2.7.2.1. EPM. Antianxiety activity was evaluated using the modified EPM (Vogel et al., 2002). The apparatus comprising two open arms (16×5 cm) and two closed arms (16×5×12 cm) having an open roof was kept at a height of 25 cm from the floor. Mice were allowed to socialize during the entire course of experiment. Every precaution was taken to ensure that no external stimuli, other than the height of the plus-maze, could invoke anxiety in mice. Doses were administered orally using tuberculin syringe fitted with an oral canula. The dose administration schedule was so adjusted that each mouse was having its turn on the elevated plus-maze apparatus 60 min after the administration of the test extract/fraction/pure isolate, diazepam or vehicle. Every mouse was placed at the center of EPM with its head facing towards the open arm, and following parameters were recorded for a period of 5 min: a) the number of entries into the open arms; b) mean time spent by the mouse in open arms.