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Anti-anxiety and CNS modulatory activities of *Vitex agnus-castus* Linn.

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Abstract

Vitex agnus-castus Linn. (Verbenaceae) has been traditionally used for the treatment of various ailments such as management of female reproductive disorders, menopausal symptoms, insufficient milk production, cyclical mastalgia and inflammatory conditions, diarrhea and flatulence. Despite a long tradition of use, no systematic phytochemical and pharmacological work has been carried out on this potential plant. Thus, *V. agnus-castus* was subjected to preliminary anti-anxiety screening studies, with a view to ascertain the verity of its traditional use as an anxiolytic. In the present investigation, fruits of the plant were extracted using solvents viz., petroleum ether (60–80^{o}C), chloroform, methanol and distilled water. All the crude extracts were evaluated for anti-anxiety activity in rats using elevated plus maze, light/dark box and hole board test models. Among all these extracts, only methanol and water extracts exhibited significant anti-anxiety activity at a dose of 200 mg/kg with respect to control as well as standard (diazepam, 2 mg/kg). Phytochemical screening showed presence of alkaloids, iridoid glycosides, tannins, carbohydrates and flavonoids in methanol and water extracts of *V. agnus-castus*.

Keywords: *Vitex agnus-castus;* anti-anxiety activity; Verbenaceae

Introduction

Vitex agnus-castus Linn. (Family Verbenaceae) is commonly known as chaste tree or chasteberry. Its leaves, flowers, and/or berries traditionally may be consumed as a decoction, traditional tincture, cider vinegar tincture, syrup, elixir, or simply eaten straight off the plant as a medicinal food. The berries are considered a tonic herb for both the male and female reproductive systems. The leaves are believed to have the same effect but to a lesser degree. (Hartung, 2000; Chevallier, 2000). This plant is commonly called *monk's pepper* because it was originally used as anti-libido medicine by monks to aid their attempts to remain celibate. It is believed to be an aphrodisiac, hence the name *chaste tree.* Clinical studies have shown its beneficial effects in the management of premenstrual stress syndrome (PMS) (Wuttke, *et al.,* 2003) and infertility and also treat disorders including corpus luteum insufficiency cyclic

mastalgia as well as to treat hormonally induced acne (Mahady, *et al.*,., 2005). Phytochemically, *V. agnus-castus* contains three new iridoids: 6' Ofoliamenthoylmussaenos-idic acid (agnucastoside A), 6'-O-(6,7-dihydrofoliamenthoyl)mussaenosidic acid (agnucasto-side B) and 7-O-trans-p-coumaroyl-6'-Otrans-caffeoyl-8-epiloganic acid (agnucastoside C) in addition to four known iridoids (aucubin, agnuside, mussaenosidic acid and 6'-O-p-hydrox-ybenzoylmussaenosidic acid) and one known phenylbutanone glucoside (myzodendrone) (Kuruuzum-Uz, *et al.,* 2003). The main compounds of the essential oil were 1, 8-cineole, sabinene, **α**-pinene, **α**-phellandrene and **α**-terpinyl acetate, trans-**α**-farnesene and bicycloger-macrene. **α**-Caryophyllene was the major sesquiterpene compounds (Novak, *et al.,* 2005). Hexadecanoic acid, heptadecanoic acid, 9, 12-octadecadienoic acid, 13-octadecanoic acid, octadecanoic acid, and 9-octadecenoic acid are the main fatty acids (Cengiz, *et al.,* 2003). An exhausted literature survey on *V. agnus-castu* revealed that sporadic phytochemical and pharmacological reports are available on this plant. As *V. agnus-castus* has been used traditionally for the treatment of various ailments, this plant holds great potential for in depth phytochemical and pharmacological evaluations.

Despite a long history of use of *V. agnus-castus* as a traditional medicine for the treatment of various ailments, the plant has never been subjected to central nervous system (CNS) activity studies. Thus, it was considered worthwhile to subject *V. agnus-castus* to antianxiety screening studies.

Materials and methods

Plant material

Dried fruits of *V. agnus-castus* were procured from Himalaya Herbs Stores, Saharanpur, India. Identity of the plant was confirmed through Dr. H. B. Singh, Scientist F, Head of Raw Material Herbarium and Museum (RHMD), National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India (Ref. No. NISCAIR/RHMD/Consult/-2008-09/1192/224, Dated 09- 04-2009).

Animals

Albino Wistar rats weighing between 150-200 gms were employed in the present study. The animals were maintained on standard environmental conditions and fed with standard rodent diet (Kissan Feeds Ltd,Mumbai, India) and tap water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee and care of the animals was carried out

Chemicals

Ammonia, acetic acid, chloroform, Draggendorff's reagent, ethyl acetate, Fehling's solutions, ferric chloride, Mayer's reagent, methanol, olive oil, Petroleum ether (60–80^{o}C), sulfuric acid, all of LR grade, distilled under normal atmospheric pressure were employed for extraction of the plant material. Solvents from extracts were recovered under reduced pressure using Rotary vacuum evaporator (Popular Traders Store, Ambala), and the dried extracts were preserved in a vacuum desiccators containing fused calcium chloride (S.D. Fine Chemicals).

Preparation of extracts

Dried, coarsely powdered fruits of *V. agnus-castus* (100 g) were successively extracted with petroleum ether, chloroform and methanol using a Soxhlet apparatus. The marc was air dried, and water extract was obtained by boiling with distilled water for 2 h, filtering, concentrating and drying in an oven at 40-50^{o}C. All the four extracts were dissolved in respective solvents, and were screened for different classes of phytoconstituents (Farnsworth, 1966).

Preliminary phytochemical screening

The tests have been done to find the presence of the active chemical constituents such as alkaloids, steroids, flavonoids, reducing sugar and tannin by the following procedure.

Test for alkaloids

0.5 g of extract was diluted to 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids. (Siddiqui and Ali, 1997).

Test for Steroids and terpenoids (Salkowski test)

0.2 g of the extract of the whole plant sample was mixed with 2ml of chloroform ($CHCl_3$) and concentrated H_2 $S0_4$ (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to indicate positive results for the presence of terpenoids. (Siddiqui and Ali, 1997)

Test for flavonoids

Three methods were used to test for flavonoids. First, dilute ammonia (5 ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 ml) was added. A yellow coloration that disappears on standing indicates the presence of flavonoids. Second, a few drops of 1% aluminium solution were added to a portion of the filtrate. A yellow coloration indicates the presence of flavonoids. Third, a portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration indicates the presence of flavonoids.

Test for Tannins

To 0.5 ml of extract solution 1ml of water and 1-2 drops of ferric chloride solution was added. Blue color was observed for gallic tannins and green black for catecholic tannins (Iyengar, 1995).

Test for Reducing Sugars

To 0.5 ml of extracts solution, 1ml of water and 5-8 drops of Fehling's solution was added at hot and observed for brick red precipitate.

Test for cardiac glycosides (Keller-Killiani test)

To 0.5 g of extract diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Test for saponins

To 0.5 g of extract was added 5 ml of distilled water in a test tube. The solution was shaken vigourously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigourously after which it was observed for the formation of an emulsion.

Test for anthraquinones

0.5 g of the extract was boiled with 10 ml of sulphuric acid (H2SO4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

Elevated plus maze (EPM) model

The plus-maze apparatus consisting of two open arms (30 cm x 5 cm x 0.2 cm) and two closed arms (30 cm x 5 cm x 15 cm) extending from a central platform and were elevated to a height of 45cm above the floor was used to observe anxiolytic behaviour in animals (Kulkarni *et al.*,., 1996; Vogel and Vogel, 1997). Each rat was placed at the centre of the elevated plus maze with its head facing the open arms. During this 5 minutes experiment, the behavior of the rat was recorded as: (a) the number of entries into the open arms, (b) average time spent by the rat in the open arms (average time = total time spent in open arms/number of entries in arms). Extracts of *V. agnus-castus* were administered orally using a tuberculin syringe fitted with oral canula. The dose administration schedule was so adjusted that each rat was having its turn on the elevated plus-maze apparatus 60 minutes after the administration of the dose. During the entire experiment, the animals were allowed to socialize. Every precaution was taken to ensure that no external stimuli, other than the height of plus-maze could invoke anxiety in the animals. Every time before placing each animal, the arena was washed with 5% alcohol to eliminate the possible bias due the odor left by the previous animal. Diazepam dose of 2 mg/kg, (p.o.) was used as a reference standard.

Light/ Dark Box Test

The light/dark box apparatus consisted of a light, open topped, opaque, Plexiglas box connected to a dark, closed topped, plexiglas box, each compartment measuring (30 cm X 40 cm X 40 cm). The boxes were connected by a small opening that allows the rat to cross between chambers. Each rat was placed individually in the center of the light compartment and observed for the next 5 minutes for the number of crossing between two compartments and time spent in the light and dark compartments. Diazepam dose of 2 mg/kg, i.p. was used as a reference standard (Amborgi and Giachetti, 1986; Crawley and Goodwin, 1981; Hascoet, *et al.*,2001; Walf and Frye, 2005). Light box entry was defined as the rat having all four paws into the light box.

Hole board test

The hole board apparatus consists of metal plate floor (40 cm X 40 cm) placed 25 cm above the ground. The metal plate consists six hole (1.5 cm in diameter), spaced symmetrically in a diamond pattern. Diazepam dose of 2 mg/kg, i.p. was used as a reference standard. Thirty minutes after the administration of the test drug, each rat was individually placed in the centre of the board (facing away from the observer). During 5 minutes test period, number of head dips was noted.

Statistical analysis

The results have been expressed as mean ± standard error mean (S.E.M.). The test doses were compared among themselves, and also with standard and control by analysis of variance (ANOVA) followed by Student Neumann Keuls test (Scheffer, 1980). Control group was also compared with the standard group.

Results

Plant material and extraction

Yield of Petroleum ether extract, Chloroform, Methanol, Water was found to be 3.8, 3.22, 15.95, and 7.14 % respectively. Table 1 shows results of phytochemical screening of various extracts of *V. agnus-castus* fruits.

Elevated plus maze

Administration of diazepam (2 mg/kg) significantly increased the amount of average time spent in the open arms and the number of open arm entries ($P < 0.05$) compared to control group Table 2. Methanol and water extracts of *V. agnus-castus* fruits at 200 mg/kg ($P < 0.05$) significantly increased the average time spent and number of entries in the open arms compared to control.

Light/Dark Box Test

Diazepam (2 mg/kg) significantly increased the time spent in light compartment ($P< 0.05$) compared to control-treated group Table 3. Significant increase in the time spent and number of entries in the light compartment ($P < 0.05$) was seen with administration of

Table 1: Results of phytochemical screening of various extracts of *V. agnus-castus* fruits

Phytochemical class	Petroleum ether extract	Chloroform extract	Methanol extract	Aqueous extract
Alkaloids	-	+	+	+
Anthraquinone glycosides	-	-	-	-
Saponin glycosides	-	-	-	-
Cardiac glycosides	-	-	-	-
Coumarin glycosides	-	-	-	-
Iridoid glycoside	-	+	+	+
Steroids/Triterpenonoids	-	-	-	-
Flavonoids	-	+	+	+
Tannins	-	+	+	+
Carbohydrates	-	+	+	+
Proteins	-	-	-	-

200 mg/kg of methanol and water extracts of *V. agnus-castus* fruits compared to control.

Hole-Board test

The number of head dipping was increased significantly (P<0.05) in case of Diazepam (2 mg/kg) treated animals as compared to the control Table 4 and Figure 3. The methanol and water extracts *V. agnus-castus* fruits at 200 mg/kg dose levels showed an increase in the head dipping significantly (P<0.05) as compared to the control animals. All other plant extracts had no significant effects on any of the parameters that were measured on these three models.

Discussion

Anti-anxiety activity of various extracts of *V. agnus-castus* fruits was evaluated employing a widely used models, elevated plus-maze, light/dark box test, hole board test. These models were chosen as it is effective, cheap, simple, less time consuming, requires no

Table 2: Anti-anxiety activity of various extracts of *V. agnus-castus* fruits using Elevated plus maze

Treatment	Dose (mg/kg)	Number of entries in open arms (Mean ± S.E.M.)	Average time spent in open arms (Mean ± S.E.M.)
Control	Vehicle	3.8 ± 0.37a	25.17 ± 0.60[a]
Diazepam (Standard)	2	7.2 ± 0.37*	35.17 ± 0.95*
Petroleum ether extract	100	3 ± 0.32[a]	26.17 ± 1.35[a]
	200	3.6 ± 0.24[a]	24.17 ± 1.28[a]
Methanol extract	100	5 ± 0.32a*	20 ± 0.58 a*
	200	7.4 ± 0.40*	35.83 ± 0.95*
Chloroform extract	100	4.6 ± 0.51[a]	26.83 ± 0.60 [a]
	200	4 ± 0.45[a]	25.85 ± 0.60[a]
Water extract	100	4.2 ± 0.37[a]	25 ± 0.52[a]
	200	7 ± 0.71*	35.15 ± 0.67*

n = 5; The data is expressed as Mean ± S.E.M.; *P<0.05 vs Control; [a]P<0.05 vs Standard; ANOVA followed by Student Newmann Keul's test.

Table 3. Anti-anxiety activity of various extracts of *V. agnus-castus* fruits using light/dark box test

Treatment	Dose (mg/kg)	Number of entries in the light compartment (Mean ± S.E.M.)	Average time spent in light compartment (Mean ± S.E.M.)
Control	Vehicle	3.8 ± 0.37[a]	25.17 ± 0.60[a]
Diazepam (Standard)	2	7.2 ± 0.37*	35.17 ± 0.95*
Petroleum ether extract	100	3 ± 0.32[a]	26.17 ± 1.35[a]
	200	3.6 ± 0.24[a]	24.17 ± 1.28[a]
Methanol extract	100	5 ± 0.32[a]*	20 ± 0.58 [a]*
	200	7.4 ± 0.40*	35.83 ± 0.95*
Chloroform extract	100	4.6 ± 0.51[a]	26.83 ± 0.60 [a]
	200	4 ± 0.45[a]	25.85 ± 0.60[a]
Water extract	100	4.2 ± 0.37[a]	25 ± 0.52[a]
	200	7 ± 0.71*	35.15 ± 0.67*

n = 5; The data is expressed as Mean ± S.E.M.; *P<0.05 vs Control; [a]P<0.05 vs Standard; ANOVA followed by Student Newmann Keul's test.

preliminary training to the rats and does not cause much discomfort to the animals while handling. Dried petroleum ether, chloroform, methanol and water extracts of *V. agnus-castus* fruits, separately suspended in a suitable vehicle, were administered orally to rats, and the activity was compared with that observed in the control group as well as with the group treated with the standard anxiolytic drug diazepam.

Elevated plus maze model is principally based on the observations that the exposure of animals to an elevated and open maze results in approach–avoidance conflict which is manifested as an exploratory-cum-fear drive. The fear due to height (acrophobia) induces anxiety in the animals when placed on the elevated plus-maze. The ultimate manifestation of anxiety and fear in the animals is exhibited by decrease in motor activity, which is measured by the time spent by the animal in the open arms. Complete manifestation of anxiety in rats of the control group is evident from the minimum average time spent in the open arms of elevated plus-maze by these animals. Among the extracts tested, maximum anxiolytic activity was observed in the methanol and water at the dose of 200 mg/kg which was at par

Table 4. Anti-anxiety activity of various extracts of *V. agnus-castus* fruits using hole board test.

Treatment	Dose (mg/kg)	Number of head dipping (Mean ± S.E.M.)
Control	Vehicle	25.17 ± 0.60[a]
Diazepam (Standard)	2	35.17 ± 0.95*
Petroleum ether extract	100	26.17 ± 1.35[a]
	200	24.17 ± 1.28[a]
Methanol extract	100	20 ± 0.58 [a]*
	200	35.83 ± 0.95*
Chloroform extract	100	26.83 ± 0.60 [a]
	200	25.85 ± 0.60[a]
Water extract	100	25 ± 0.52[a]
	200	35.15 ± 0.67*

n = 5; The data is expressed as Mean ± S.E.M.; *P<0.05 vs Control; [a]P<0.05 vs Standard; ANOVA followed by Student Newmann Keul's test.

that of diazepam as is evident from statistical equivalence between the results of this dose and that manifested by diazepam.

In the light/dark box test**,** a significant increase in the time spent in seconds and number of entries in the light compartment and also in hole board model**,** a significant increase in the exploratory head-dipping behavior were observed after treatment with 200 mg/kg of *V. agnus-castus* fruits extract of methanol and water, thus reinforcing the hypothesis that it has anxiolytic activity. Phytochemical screening showed presence of alkaloids, iridoid glycosides, tannins, carbohydrates and flavonoids in methanol and water extracts of *V. agnus-castus* fruits.

In conclusion, the action of extracts upon the anxiety models tested are in accord with the traditional use of *V. agnus-castus* and could be useful in primary medical care. In the same way, identification of compound(s) responsible for biological activity could be used as prototype(s) to design new substances with anxiolytic activity. Although further major active components and precise anxiolytic mechanisms need to be identified.

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Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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