Journal of Pharmacognosy and Phytotherapy Vol. 2(2), pp. 017-023, March 2010
Available online at http://www.academicjournals.org/JPP

Full Length Research Paper

Bioactivity-directed separation of an anxiolytic fraction from *Aethusa cynapium* L.

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Accepted 16 July, 2009

The present study has been designed to evaluate the putative anxiolytic activity of petroleum ether, chloroform, methanol and water extracts of the aerial parts of *Aethusa cynapium* using the widely accepted elevated plus-maze model in mice. An attempt has been made to isolate the bioactive fraction by resorting to bioactivity directed fractionation and chromatographic procedures like column and flash chromatography. Fraction 3.1.3.2, which was derived from the methanol extract of the plant, showed significant anxiolytic activity at a dose of 50 mg/kg as compared to the standard drug, diazepam (2 mg/kg p.o.). This subfraction comprises of two flavonoid components. The study validates the traditional use of *A. cynapium* for the treatment of anxiety.

Key words: *Aethusa cynapium* L., anxiolytic effect, elevated plus-maze model, column and flash chromatography.

INTRODUCTION

Aethusa cynapium L. (Apiaceae) is commonly called Fool's Parsley, dog's parsley or lesser hemlock. The plant is an annual (rarely biennial) well-known garden weed native to the United Kingdom (Bond and Turner, 2004).The plant has been used in folk medicine for gastrointestinal complaints in children, infantile cholera, summer diarrhea, convulsions, anxiety, sleep disorders, delirium, and as stomachic (Fleming, 2000; Vikramaditya and Joshi, 1997). The plant contains a volatile alkaloid named cynopine which resembles coniine in its physical and chemical characters, as well as physiological actions (Tutin, 1980; Clapham et al., 1987; Vikramaditya and Joshi, 1997); polyacetylenes(Andreev et al., 2001) including aethusin, aethusanol A and B; essential oil; flavone glycosides such as rutoside, narcissine, and ascorbic acid (Fleming, 2000). Fool's Parsley is poisonous when fresh, but is not harmful when dried (Salisbury, 1961). The toxins like cynopine are destroyed by drying, and hay containing the plant is not poisonous (Tutin, 1980; Clapham et al, 1987). The anti-anxiety activity of this plant has not been investigated. The present study has been designed to investigate the anti- anxiety activity of different extracts of *A. cynapium* using Elevated Plus Maze (EPM) model. The bioactivity directed fractionation to isolate bioactive fraction having anxiolytic effect is described.

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MATERIALS AND METHODS

Plant materials

Aeriel parts of *A. cynapium*, were collected from a cultivated source (Rati Ram Nursery) at village Khurrammpur via Kalsia, district Saharanpur (U. P., India) in March 2006. The identity of the plant was confirmed by Dr H. B. Singh, Head, Raw Materials, Herbarium and Museum at National Institute of Science Communication and Information Resources, (CSIR), New Delhi 110067). A voucher specimen no: NISCAIR/RHM/F-3/3/2005/Consult/698//15 is deposited in same herbarium.

Animals

Swiss albino mice of either sex, weighing 20 - 24 g were procured from the Central Animal House of Panjab University, Chandigarh. The mice were allowed to take standard laboratory feed and water *ad libitum*. The animals were fasted 18 h prior to the biological study (Vogel and Vogel, 1997). Groups of five mice were used in all sets of experiments. All animals used in the study were naive to the

elevated plus-maze test. The experiments were conducted in a semi-sound proof laboratory. The biological studies were carried out as per the guidelines of the Institutional Ethical Committee (Reg. no.107/1999/CPCSEA) of Department of Pharmacuetical Sciences and Drug Research, Punjabi University, Patiala, India.

Chemicals and instruments

Solvents namely petroleum ether (60 - 80°C), methanol, chloroform and 1-butanol (S.D. Fine-Chem Ltd., Mumbai), all of LR grade were employed for the extraction of the plant material. Silica gel (#60 - 120, S.D. Fine- Chem Ltd., Mumbai) was used for column chromatography. For Flash chromatography, Büchi pump module C-601 and Büchi pump controller C-610, silica gel (#250 - 400) were used. Pre-coated TLC sheets (Macherey-Nagel D-5160 DUREN, 0.25 mm, Polygram® SiLG) and 2 µl capillary tubes (CAMAG) were used for thin layer chromatography (TLC). The chromatograms were visualized under 254/366 nm UV light (DESAGA, Heildberg, Min. UVIS) and also by spraying with 60% v/v aqueous sulfuric acid (BDH). Diazepam I.P. was procured from Triko Pharmaceuticals, Rohtak, Haryana (India).

Preparation of extracts and evaluation of anxiolytic activity

Preparation of extracts: Aerial parts of *A. cynapium* were dried in shade and powdered (#60). One kg of the plant was successively extracted in the Soxhlet apparatus with petroleum ether, chloroform, methanol and water. Exhaustive extraction with each solvent was ensured. The four extracts were dried using Buchi 461 Rotary Vacuum Evaporator and the dried extracts were preserved in vacuum desicator containing anhydrous silica gel.

Elevated plus-maze model of anxiety: Anxiolytic activity was evaluated using the modified elevated plus-maze (Montgomery, 1958; Pellow et al. 1985; Lister, 1987). The plus-maze apparatus consisted of two open arms (16 × 5 cm) and two closed arms (16 × 5 × 12 cm) having an open roof, with the plus-maze elevated (25 cm) from the floor was used to evaluate anxiolytic behavior in animals (Kulkarni and Reddy, 1996). Vehicle [5% Tween 80 in Simple Syrup I.P. (66.7% w/w sucrose in water)] (0.25 ml), extracts of *A. cynapium* (50, 100, 200 and 400 mg/kg) and reference drug (diazepam, 2 mg/kg), both suspended in vehicle, were administered orally using a tuberculin syringe fitted with oral canula. The dose administration schedule was adjusted so that each mouse was having its turn on the elevated plus-maze apparatus 45 min after the administration of the test extract, diazepam or vehicle.

Each mouse was placed at the center of the elevated plus-maze with its head facing towards the open arms. During the 5 min duration of the experiment, the behavior of the mouse was recorded as (a) the number of entries into the open or closed arms, (b) mean time spent by the mouse in each of the arms. During the entire experiment, the animals were allowed to socialize. Every precaution was taken to ensure that no external stimuli could invoke anxiety in the animals.

Fractionation of bioactive methanol extract (F-3) and evaluation of anxiolytic activity

The methanol extract (30 g) was shaken successively with chloroform and butanol (4 x 10 ml each). Thus three fractions were obtained namely chloroform fraction, butanol fraction, and the remaining methanol soluble fraction and each fraction was evaluated for the anxiolytic activity using the elevated plus maze model.

Column chromatography of chloroform fraction (F-3.1) and evaluation of anxiolytic activity

The bioactive chloroform fraction (F-3.1, 7 g) was subjected to column chromatography using silica gel. Elution was done with chloroform and chloroform-methanol in increasing order of polarity. The different collected fractions were pooled on the basis of similar TLC profiles. Four fractions were generated. The anxiolytic activity of each fraction was evaluated using the elevated plus maze model.

Flash chromatography of F-3.1.3 and evaluation of anxiolytic activity

The bioactive fraction F-3.1.3 (1.8 g) was subjected to flash chromatography using silica gel (#250 - 400). Elution was done with chloroform and chloroform-methanol in increasing order of polarity. The eluants were driven through the column by pressurized air (10 psi). The different collected fractions were pooled on the basis of similar TLC profiles. Five subfractions were collected. The anxiolytic activity of each fraction was evaluated using the elevated plus maze model.

Phytochemical screening and TLC profiles

Extracts and different fractions were subjected to phytochemical screening (Farnsworth, 1966) and their TLC profiles were examined (Wagner et al., 1984).

Statistics

The anxiolytic activities of test substances, diazepam (standard) and control were analyzed using one-way analysis of variance (ANOVA) and post hoc analysis was done using Tukey's multiple range test.

RESULTS

Anxiolytic activity of various extracts of *A. cynapium*: The dried aerial parts of *A. cynapium* were subjected to successive extractions with solvents in increasing order of polarity yielding four extracts. The weight of various extracts after exhaustive extraction was: petroleum ether (2.2% w/w), chloroform (1.8% w/w), methanol (3.8% w/w) and aqueous (2.9% w/w). The methanol extract (F-3) demonstrated the highest level of anxiolytic activity (Table 1) in mice at a dose of 400 mg/kg, as it significantly increased both the time spent and number of open in the arm entries of EPM. The activity was comparable to the effect of the standard drug, diazepam. Methanol extract was fractionated using chloroform and 1-butanol resulting in three fractions: chloroform fraction F-3.1 (36.6% w/w), butanol fraction F-3.2 (34.7% w/w) and remaining methanol soluble fraction F-3.3 (27.4% w/w). Relative anxiolytic activity profile of the three fractions (Figures 1 and 2) showed that significant anxiolytic activity resided in F-3.1; it was subjected to column chromatography. Four fractions (F-3.1.1 to F-3.1.4) (Table 2) were obtained and F-3.1.3 was observed to be the only fraction with significant anxiolytic activity at doses 50 and 100 mg/kg (Figures 3 and 4). F-3.1.3 was subjected to flash chromatography

Table 1. Anxiolytic effect of different extracts of *A. cynapium* using the EPM.

Treatment	Dose (mg/kg), p.o	Mean number of entries in open arms [a]	Mean time spent in open arms (seconds)[b]
Vehicle (control)	0.25 ml/kg	4.8 ± 1.3	3.2 ± 1.3
Diazepam (standard)	2	11.2 ± 2.3	12.2 ± 1.2
Pet. ether extract (F-1)	50	6.2 ± 0.8*,**	7.0 ± 1.0*,**
	100	6.8 ± 1.3*	7.9 ± 0.4*,**
	200	6.6 ± 1.1*	7.88 ± 0.8*,**
	400	5.2 ± 1.6*	5.9 ± 0.9*, **
Chloroform extract (F-2)	50	7.4 ± 1.1*,**	5.1 ± 1.2*,**
	100	7.0 ± 2.6*	8.1 ± 1.2*,**
	200	5.8 ± 1.14*	7.6 ± 1.0*,**
	400	7.2 ± 1.3*	7.0 ± 1.3*, **
Methanol extract (F-3)	50	5.8 ± 2.3*,**	7.7 ± 0.9*, **
	100	6.4 ± 1.7*,**	8.2 ± 0.9*, **
	200	7.8 ± 2.9**	9.5 ± 1.1*, **
	400	9.4 ± 2.6**	12.7 ± 1.5**
Water extract (F-4)	50	4.4 ± 1.1*	5.2 ± 0.6*, **
	100	5.0 ± 1.6*	8.2 ± 1.7*, **
	200	8.2 ± 0.5**	8.4 ± 1.5*,**
	400	7.6 ± 2.7*	7.9 ± 1.4*, **

a: Values are expressed as mean ± S.D, b: Values are expressed as mean ± S.D. (n = 5) ANOVA followed by Tukey's multiple range tests. $p < 0.05$. * = significant with respect to standard, ** = significant with respect to control.

Table 2. Column chromatography of chloroform fraction F-3.1.

Fraction	Obtained by pooling fractions	Eluants	Yield (g)
F-3.1.1	1 - 10	Chloroform	1.21
F-3.1.2	11 - 26	Chloroform: methanol (70:30)	0.96
F-3.1.3	27 - 32	Chloroform: methanol (60:40)	2.64
F-3.1.4	33 - 50	Chloroform: methanol (40:60)	0.88

Table 3. Flash chromatography of fraction F-3.1.3.

Fraction (g)	Obtained by pooling fractions	Eluants	Yield
F-3.1.3.1	1 - 3	Chloroform: methanol (97.5:2.5)	0.435
F-3.1.3.2	4	Chloroform: methanol (92.5:7.5)	0.524
F-3.1.3.3	5	Chloroform: methanol (90:10)	0.300
F-3.1.3.4	6 - 7	Chloroform: methanol (50:50)	0.281
F-3.1.3.5	8 - 10	Chloroform: methanol (20:80)	0.215

(Table 3) and subfraction F-3.1.3.2 showed significant anxiolytic effect at doses of 50 and 75 mg/kg (Figures 5 and 6).

Phytochemical screening and TLC profiles: Phytochemical screening of the fraction F-3.1.3.2 showed the absence of alkaloids and the presence of flavonoids. TLC of F-3.1.3.2 using chloroform: methanol (9:1) as the mobile phase showed two distinct spots (Rf 0.50, green; Rf 0.35, yellow green), when sprayed with natural products reagent (Wagner et al., 1984) indicating the presence of flavonoids.

DISCUSSION

Traditionally *A. cynapium* has been used as a sedative

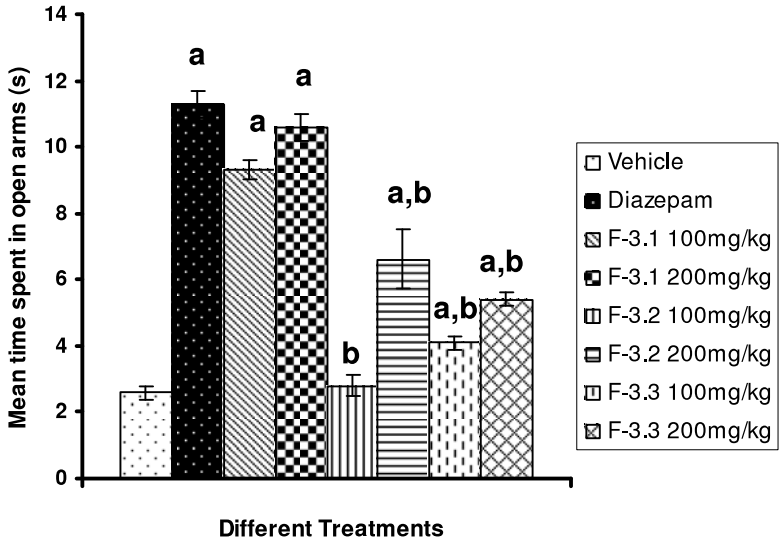

Figure 1. Effect of different fractions of the methanol extract of *A. cynapium* on the average time spent in open arms of EPM. The data was analyzed by one way ANOVA and *post hoc* Tukey's multiple range test. a = $p < 0.05$ vs. Control (Vehicle); b = $p < 0.05$ vs. diazepam (standard drug, 2 mg/kg).

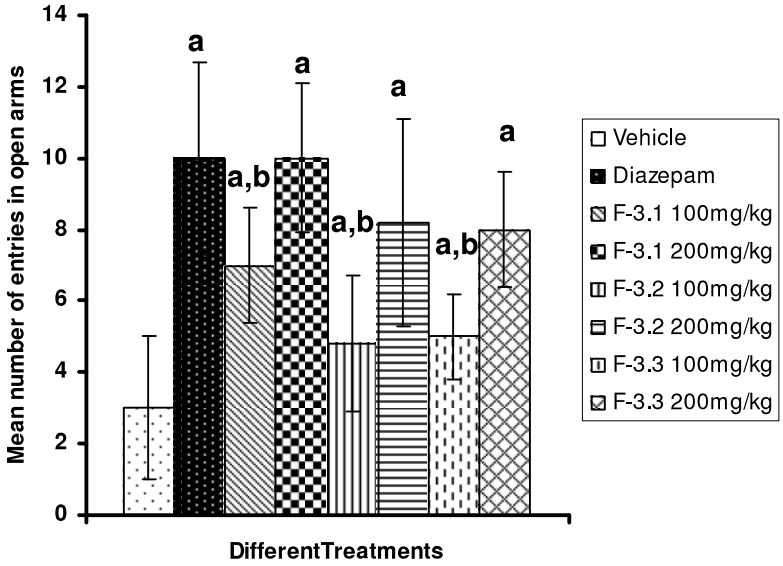

Figure 2. Effect of different fractions of the methanol extract of *A. cynapium* on number of entries in open arms of EPM. The data was analyzed by one way ANOVA and *post hoc* Tukey's multiple range tests. a = $p < 0.05$ vs. Control (Vehicle); b = $p < 0.05$ vs. Diazepam (Standard Drug, 2 mg/kg).

sedative (Fleming, 2000; Vikramaditya and Joshi, 1997). How-ever, no study has investigated the anxiolytic effect of this plant. In this study bioactivity-directed fractionation of *A. cynapium* aerial parts was followed with to isolate the an-xiolytic fraction. The Elevated plus Maze model employed in this study is a valid animal model because natural stimuli have been used in this model (Dawson and Tricklebank, 1995). In this study, treatment with subfrac-tion F-3.1.3.2, obtained from the methanol extract of *A. cynapium*, significantly increased the time spent in open arms and the frequency of open arm entries in EPM, thus suggesting an anxiolytic effect (Fleming, 2000). *A. cynapium* is reported to contain flavone glycosides. Phytochemical screening of F-3.1.3.2

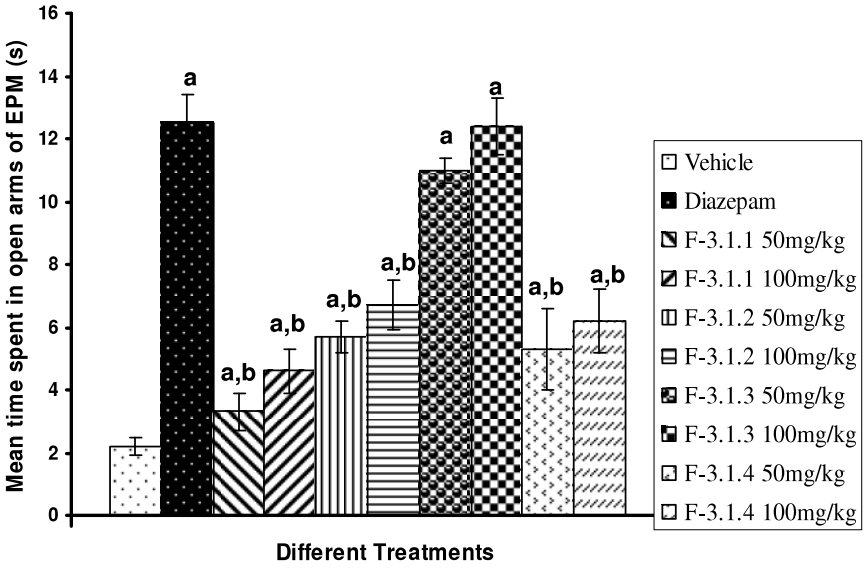

Figure 3. Effect of different fractions obtained from column chromatography of fraction F-3.1 on average time spent in open arms of EPM. The data was analyzed by one way ANOVA and *post hoc* Tukey's multiple range tests. a = $p < 0.05$ vs. control (vehicle); b = $p < 0.05$ vs. diazepam (standard drug, 2 mg/kg)

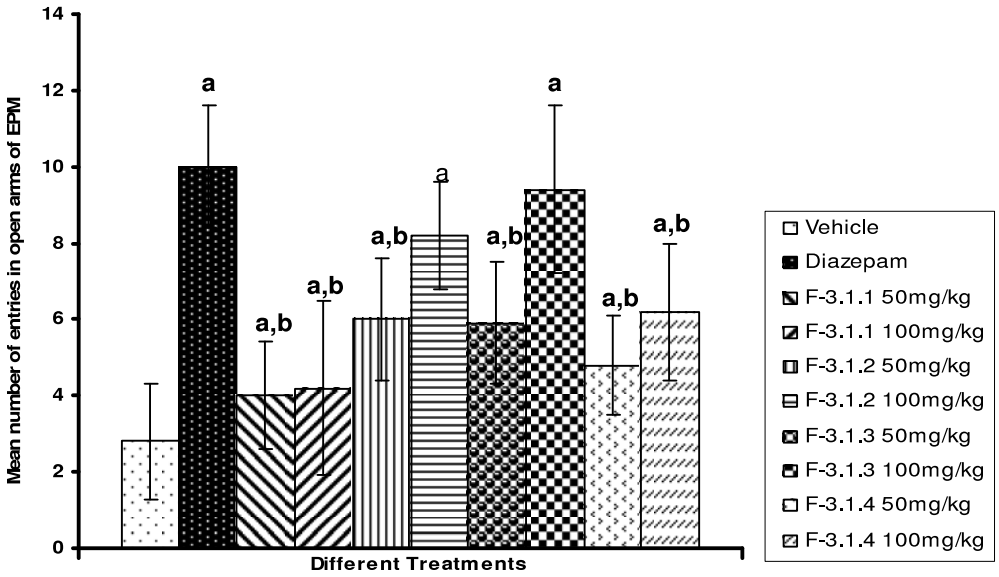

Figure 4. Effect of different fractions obtained from column chromatography of fraction F-3.1 on number of entries in open arms of EPM. The data was analyzed by one way ANOVA and *post hoc* Tukey's multiple range tests. a = $p < 0.05$ vs. control (vehicle); b = $p < 0.05$ vs. diazepam (standard drug, 2 mg/kg).

has shown presence of flavonoids and TLC profile of F-3.1.3.2 demonstrated presence of two components which may be flavonoids. Some natural and synthetic flavonoids have been found to bind specifically and competitively to benzodiazepine receptors and exhibit anxiolytic effects in the EPM test in rodents (Salgueiro et al., 1997). Synthetic flavonoids like 6-bro-moflavanone, 5-methoxy-6, 8-dibromoflavanone and 6-bromo-3′-nitroflavone possess anxiolytic-like properties (Griebel et al., 1999; Ognibene et al., 2008). Natural flavonoids like apigenin, 6-methylapigenin, chrysin, hesperidin, luteolin, orientin, iso orientin and wogonin possess antianxiety activity (Viola et

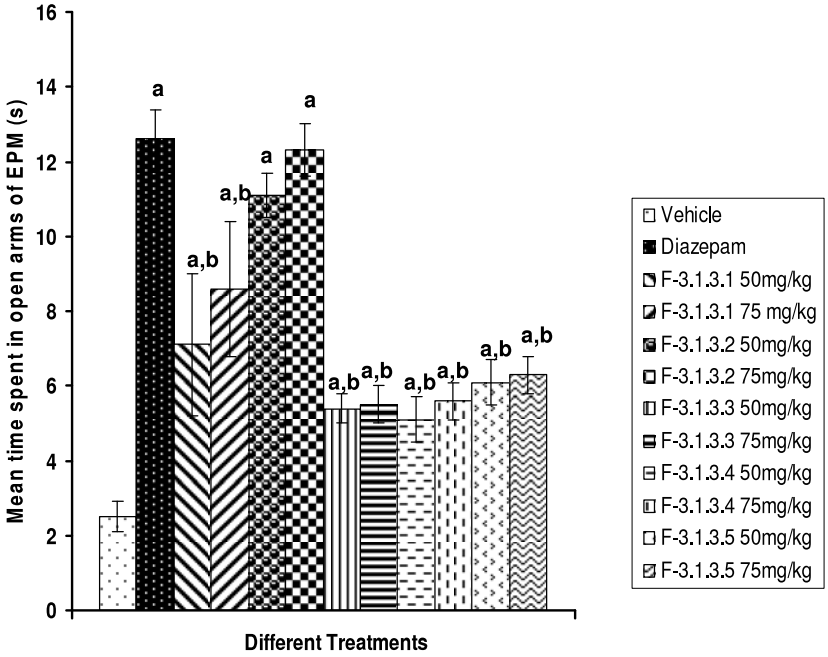

Figure 5. Effect of different subfractions obtained by flash chromatography of fraction F-3.1.3 on the time spent by mice in the open arms of the EPM. The data was analyzed by one way ANOVA and *post hoc* Tukey's multiple range tests. a = p < 0.05 vs. control (vehicle); b = p < 0.05 vs. diazepam (standard drug, 2 mg/kg).

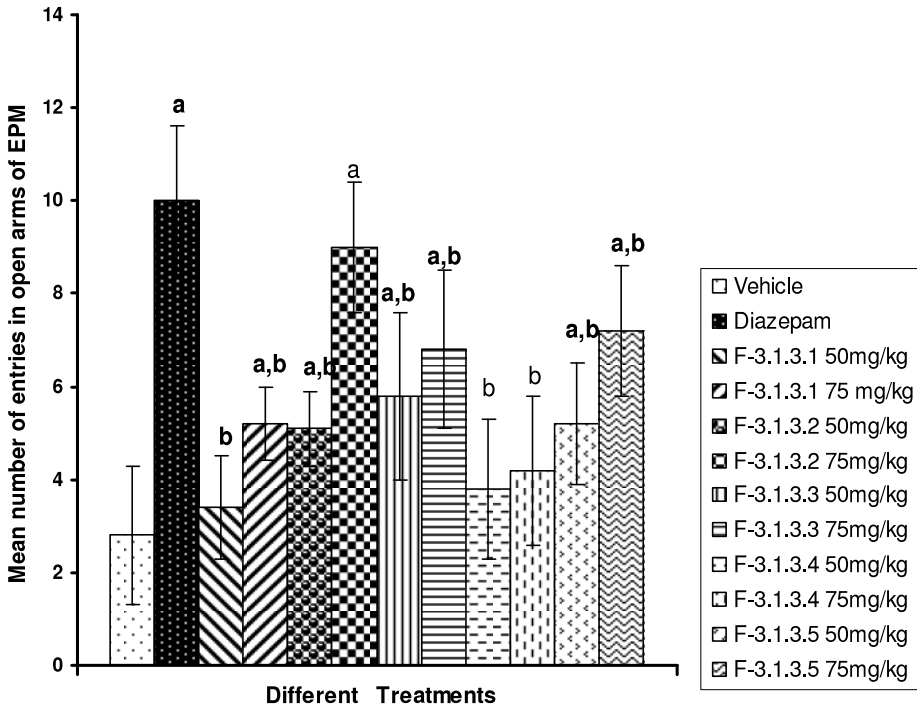

Figure 6. Effect of different subfractions obtained by flash chromatography of fraction F-3.1.3 on the time spent by mice in the open arms of the EPM. The data was analyzed by one way ANOVA and *post hoc* Tukey's multiple range tests. a = p < 0.05 vs. control (vehicle); b = p < 0.05 vs. diazepam (standard drug, 2 mg/kg).

(Viola et al., 1994; Wolfman et al., 1994; Zanoli et al., 2000; Hui et al., 2002; Rocha et al., 2002; Marder et al., 2003; Ferna´ndez et al., 2005; Coleta et al., 2008). Thus, flavonoid constituents present in the plant may be responsible for its noted anxiolytic activity. The authors are presently involved in characterising the constituent/s responsible for the anxiolytic activity.

ACKNOWLEDGMENTS

The authors wish to acknowledge the facilities of flash chromatography provided by Dr K.K. Bhutani, Prof and Head, Department of Natural Products, NIPER, Mohali, Punjab, INDIA.

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