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Antianxiety Activity of Fractions and Isolated Compounds of *Verbena officinalis* Aerial Parts

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ABSTRACT

Verbena officinalis L. (Vervain; family-Verbenaceae) has long been used in the treatment of mental disorders, but not validated systemically for traditional claims. The ethyl acetate fraction (rich in flavonoids) of *V. officinalis* aerial parts was subjected to bioactivity-guided fractionation to isolate main chemical constituents responsible for antianxiety activity. The column chromatography of ethyl acetate fraction yielded five fractions F_1-F_5, which were screened for antianxiety activity employing elevated plus maze model. F_2 produced significant antianxiety activity at the dose of 16 mg/kg, *p.o.*, upon acute administration in mice. F_2 was also subjected to column chromatography, and yielded four sub fractions ($F_{2.1}$ – $F_{2.4}$). Upon acute oral administration of sub fractions, antianxiety activity was evaluated. Only $F_{2.3}$ exhibited significant antianxiety activity, which was statistically equivalent to the standard drug (Diazepam, 2 mg/kg, *p.o.*). Three compounds (VO-1, VO-2 and VO-3) were isolated during the process of fractionation of bioactive fraction and sub fraction. After antianxiety evaluation of compounds, it was observed that VO-2 and VO-3 exhibit antianxiety activity.

Keywords: Antianxiety activity, Diazepam, *Verbena officinalis*, Verbenaceae, Elevated Plus maze.

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INTRODUCTION

Mental illness has profound impact on lives of persons living in developing and developed countries. The global cost for mental health problems is estimated to be US $2.5 trillions. [1] Mental illness costs the economy each year of Canada about US $51 billion [2]; UK about €100 billion [3] and US about $147. [4] WHO reported that four of ten leading causes of disability worldwide are neuropsychiatric disorders. These disorders account for 30.8% of total disability and 12.3% of the total burden of disease. [5] Twelve month prevalence of anxiety disorders in US is 18.1% adult population [6]; 20% of Australian population (2.3 million) [7] and 6.5% of Indian population. [8] Synthetic anxiolytic drugs especially benzodiazepines are frequently used in the treatment of anxiety disorders, but long term use of these drugs cause addiction and severe side effects. [9] Therefore, natural sources are being explored to get newer, efficacious and safer anxiolytic drugs.

Verbena officinalis L. (Vervain; family-Verbenaceae) has been traditionally used as nervine tonic, antidepressant, and anticonvulsant; prescribed in liver and gall bladder complaints (spasm of the bladder and strangury), nervous and menstrual disorders; also for bronchitis, asthma and febrile affections. [10-12] *V. officinalis* has been reported to contain secoiridoid glycosides [13]; triterpenoids [14]; flavonoids [15]; volatile oil [16] and phytosterol. [17] A close scrutiny of the available

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literature revealed a startling fact that the plant has not been explored pharmacologically for validation of its traditional claims of antianxiety activity. Previous studies showed that ethyl acetate fraction of methanol extract of *V. officinalis* exhibits significant antianxiety activity. [18] Thus, it was considered worthwhile to screen antianxiety activity of fractions / sub fractions and isolates of ethyl acetate fraction of *V. officinalis* aerial parts.

MATERIALS AND METHODS

Plant Material: The dried aerial parts of *V. officinalis* were procured from Himalaya Herbs Store, Madhav Nagar, Saharanpur, (Uttar Pradesh), India in September, 2012. The plant was identified by Dr. Avneet Pal Singh, Assistant Professor, Department of Botany, Punjabi University, Patiala, India (Reference No. SPL-101/Bot, dated 15-10-2013).

Solvents and Chemicals: Silica gel (# 60-120), solvents – *n*-hexane, ethyl acetate, methanol, chloroform, all of LR grade, were procured from Merck, New Delhi.

Preparation of Various Fractions / Sub fractions / Isolates: The ethyl acetate fraction prepared by standardized procedure [18] was purified by column chromatography. The ethyl acetate fraction (75 g) was dissolved in 250 ml of ethyl acetate to make a clear solution. The sample was impregnated on 150 g silica gel (#60-120), and the solvent was then removed under reduced pressure using rotary vacuum evaporator (BUCHI, Switzerland) to get free flowing powder. The slurry of 700 g silica gel (#60-120) was prepared in 1.5 L *n*-hexane in a beaker (2 L). The slurry was packed in a column (length – 100 cm, internal diameter – 5.8 cm). The column was run with *n*-hexane continuously for 24 h at the flow rate of 10 ml/min to get a compact bed of silica gel. The sample was then loaded over the silica gel bed. The fractionation of sample was done by eluting fractions using *n*-hexane, *n*-hexane-ethyl acetate and ethyl acetate-methanol in different concentrations as mobile phases. A total of 950 fractions, 500 ml each, were collected. These fractions were pooled based on similar thin layer chromatograms, to get 5 fractions (F_1-F_5). A compound (white amorphous powder) was separated while concentrating F_3. It was designated as VO-1. Thin layer chromatograms were obtained on pre-coated silica gel plates using different solvent systems and visualized in UV chamber (254/366 nm).

Twelve g of F_2 was dissolved in 50 ml of ethyl acetate to make a clear solution. The sample was impregnated on 50 g silica gel (#60-120), and the solvent was then removed under reduced pressure using rotary vacuum evaporator to get free flowing powder. The slurry of 250 g silica gel (#60-120) was prepared in 500 mL *n*-hexane in a beaker (2 L). The slurry was packed in a column (length – 100 cm, internal diameter – 3.8 cm). The column was run with *n*-hexane continuously for 24 h at the flow rate of 10 ml/min to get a compact bed of silica gel. A total of 300 fractions, 250 ml each, were collected. These fractions were pooled based on similar thin layer chromatograms, to get 4 sub fractions ($F_{2.1}$–$F_{2.4}$). Thin layer chromatograms were obtained on pre-coated silica gel plates using different solvent systems and visualized in UV chamber (254/366 nm). Two compounds were obtained from $F_{2.3}$ which was designated as VO-2 and VO-3.

Antianxiety Activity Studies of Fractions, Sub fractions and isolates of *V. officinalis*

Animals: Swiss albino mice (either sex) of body weight 20-25 g purchased from the Panacea Biotec Ltd., Ambala-Chandigarh highway, Lalru, Punjab, India were used for antianxiety activity studies. The animals studies were approved from Institutional Animal Ethics Committee of CT Institute of Pharmacy, Jalandhar [IAEC – CTIPS/2014/V/0029 (PCT - D), dated 15/12/2014]. The animals were fed with normal laboratory pellet diet (Shri Jagdamby Feed Industry, Moga) and water *ad libitum*. Groups of six animals were used in all sets of experiments.

Vehicle and Standard Drug: The test doses of crude extracts and fractions of the plant were prepared using vehicle {Distilled water + Tween 80 (2%)}. Diazepam (Triko Pharmaceuticals, Rohtak, Haryana) was used as standard antianxiety drug at the dose of 2 mg/kg, *p.o.*

Experimental Design: Fourteen groups of mice were prepared and each group comprised 6 mice.

Group 1 served as control group received vehicle (0.25 ml, *p.o.*); Group 2 served as standard group received diazepam (2 mg/kg, *p.o.*); Group 3 served as test group received 20 mg/kg dose of F_1; Group 4 served as test group received 16 mg/kg dose of F_2; Group 5 served as test group received 19 mg/kg dose of F_3; Group 6 served as test group received 11 mg/kg dose of F_4; Group 7 served as test group received 13 mg/kg dose of F_5; Group 8 served as test group received 1.5 mg/kg dose of $F_{2.1}$; Group 9 served as test group received 6 mg/kg dose of $F_{2.2}$; Group 10 served as test group received 1 mg/kg dose of $F_{2.3}$; Group 11 served as test group received 5 mg/kg dose of F2.4; Group 12 served as test group received 2 mg/kg dose of VO-1; Group 13 served as test group received 2 mg/kg dose of VO-2; Group 14 served as test group received 2 mg/kg dose of VO-3.

Evaluation of Antianxiety Activity Using Elevated Plus Maze Model: Antianxiety activity of various extracts and fractions was performed using elevated plus maze (EPM) model. [19]

Statistics: The results are presented as mean ± standard deviation (SD). The statistical significance was checked by comparing with standard drug and control using one way analysis of variance (ANOVA), which was followed by post hoc analysis – Student-Newman-Keul's test. [20]

RESULTS AND DISCUSSION

Verbena officinalis is traditionally used and medicinally promising plant. Previous studies have shown significant anxiolytic activity of ethyl acetate fraction (EAF) of methanol extract of the plant. The bioactive

EAF was subjected to antianxiety activity-guided fractionation to isolate main chemical constituents.

Table 1: Fractionation of EAF of *V. officinalis* aerial parts using column chromatography.

Fraction	Eluent	Yield (g)	Constituent isolated and yield
F_1	*n*-hexane *n*-hexane : ethyl acetate (4 : 1) *n*-hexane : ethyl acetate (7 : 3) *n*-hexane : ethyl acetate (3 : 2) *n*-hexane : ethyl acetate (1 : 1)	15.25	--
F_2	*n*-hexane : ethyl acetate (2 : 3) *n*-hexane : ethyl acetate (3 : 7) *n*-hexane : ethyl acetate (1 : 4) *n*-hexane : ethyl acetate (3 : 9) ethyl acetate	12.05	--
F_3	ethyl acetate : methanol (199 : 1) ethyl acetate : methanol (99 : 1) ethyl acetate : methanol (49 : 1)	14.25	Colorless amorphous powder (VO-1; 121 mg)
F_4	ethyl acetate : methanol (97 : 3) ethyl acetate : methanol (19 : 1)	7.98	--
F_5	ethyl acetate : methanol (9 : 1) ethyl acetate : methanol (4 : 1)	9.87	--

Table 2: Fractionation of F_2 obtained from fractionation of EAF using column chromatography.

Sub-fraction	Eluent	Yield (g)	Constituent isolated and yield
$F_{2.1}$	*n*-hexane *n*-hexane : ethyl acetate (4 : 1)	1.15	--
$F_{2.2}$	*n*-hexane : ethyl acetate (7 : 3) *n*-hexane : ethyl acetate (3 : 2) *n*-hexane : ethyl acetate (5 : 1)	4.23	--
$F_{2.3}$	*n*-hexane : ethyl acetate (2 : 3) *n*-hexane : ethyl acetate (3 : 7) *n*-hexane : ethyl acetate (1 : 4) *n*-hexane : ethyl acetate (1 : 9) ethyl acetate	0.75	Pale yellow crystalline powder (VO-2; 41 mg) Yellow colored crystalline powder (VO-3; 32 mg)
$F_{2.4}$	ethyl acetate : methanol (199 : 1) ethyl acetate : methanol (99 : 1) ethyl acetate : methanol (49 : 1)	3.72	--

Table 3: Antianxiety activity of various fractions, sub fractions and isolated compounds using EPM.

Treatment	Dose (mg/kg)	Number of entries in open arms (Mean^n ± S.D.)	Time spent in open arms (sec) (Mean^n ± S.D.)
Control	Vehicle	2.33 ± 0.74^a	3.40 ± 0.42^a
Diazepam	2	8.33 ± 1.10*	12.49 ± 0.89*
F_1	20	2.17 ± 0.75^a	3.45 ± 0.45^a
F_2	16	8.50 ± 1.25*	12.09 ± 1.09*
F_3	19	2.67 ± 0.94^a	3.27 ± 0.48^a
F_4	11	3.00 ± 0.89^a	3.05 ± 0.23^a
F_5	13	2.83 ± 0.68^a	3.25 ± 0.40^a
$F_{2.1}$	1.5	3.33 ± 0.52^a	3.11 ± 0.27^a
$F_{2.2}$	6	2.00 ± 0.58^a	3.23 ± 0.26^a
$F_{2.3}$	1	8.16 ± 1.06*	12.38 ± 0.82*
$F_{2.4}$	5	2.50 ± 0.84^a	2.98 ± 0.28^a
VO-1	2	3.17 ± 0.89^a	3.24 ± 0.44^a
VO-2	2	8.00 ± 1.29*	12.65 ± 0.98*
VO-3	2	7.83 ± 1.06*	13.10 ± 0.87*

n=6; $^*P<0.05$ vs. Control; $^aP<0.05$ vs. Diazepam (Standard drug); one way ANOVA followed by Student-Newman-Keul's test.

Elevated plus maze model of anxiety was chosen to assess antianxiety activity of various fractions, sub fractions and isolates of *V. officinalis*. The column chromatography of EAF yielded five pooled fractions (F_1-F_5) and a compound (VO-1), which was obtained from F_3 (Table 1). The fractions and isolated compound were screened for antianxiety activity using EPM in mice at different doses. The dose selection for fractions was made on the basis of their percentage yields in relation to EAF. Amongst various fractions, only F_2 exhibited significant antianxiety activity at the dose of 16 mg/kg. The activity was found to be statistically equivalent to the standard drug, diazepam (Table 3). The bioactive F_2 was further fractionated using column chromatography. It yielded four sub fractions ($F_{2.1}$-$F_{2.4}$) (Table 2), which were screened for antianxiety activity. $F_{2.3}$ exhibited significant antianxiety activity at the dose of 1 mg/kg. $F_{2.3}$ was kept for crystallization in the mixture of chloroform and methanol overnight. A pale yellow colored crystalline powder (VO-2) was obtained. The mother liquor was precipitated by adding excess of chloroform. The precipitates were separated and kept for crystallization in mixture of chloroform and methanol. It yielded yellow colored crystalline powder (VO-3). Finally, $F_{2.3}$ led to the isolation of two major compounds. Both compounds were screened for antianxiety activity. Interesting both compounds showed significant antianxiety activity at the dose of 2 mg/kg. Preliminary phytochemical screening showed that VO-2 and VO-3 are flavonoids. It is finally conclude that flavonoids are main chemical constituents responsible for antianxiety activity of *V. officinalis*.

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