Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2016; 8(3); 179-186

ISSN: 0975-5160

Research Article

Screening of Neuropharmacological Activities of *Viscum album* and Estimation of Major Flavonoid Constituents Using TLC Densitometry

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Available Online:10th June, 2016

ABSTRACT

Despite a long tradition of use in the treatment of mental disorders, *Viscum album* L. (Ujjral; family – Viscaceae) has not been systematically investigated to validate its traditional claims. The methanol extract of *V. album* aerial parts, its ethyl acetate fraction (EAF) and 1-butanol fraction (BF) were screened for various neuropharmacological activities. The methanol extract, EAF and BF exhibited significant antianxiety activity comparable to the standard drug, diazepam (2 mg/kg, *i.p.*) at the dose of 400, 10 and 10 mg/kg, respectively. Only BF exhibited specific antidepressant activity using despair swim test at the dose of 50 mg/kg. The methanol extract (200 or 400 mg/kg), EAF (25 or 50 mg/kg) and BF (25 or 50 mg/kg) exerted mild antistress and analgesic effects, and exhibited significant hypnotic activity. The content of quercetin and apigenin was found to be 0.00452% and 0.00058% w/w, respectively, in *V. album* aerial parts.

Keywords: Antianxiety, Antidepressant, Antistress, Hypnotic, Ujjral, Viscaceae.

INTRODUCTION

The mental disorders such as anxiety, depression, stress, seizures and schizophrenia have become serious problem in health management[1]. People suffering with these disorders are often subject to social isolation, poor quality of life and increased mortality[2]. During the last two decades, synthetic drugs are frequently prescribed for the treatment of mental disorders but these drugs are associated with severe side effects such as memory impairment, addiction and dependence[3], sexual dysfunction, headaches, dizziness and abnormal vision[4]. Researchers are exploring natural resources to find out newer, efficacious and safer drugs. Traditional plants seem viable alternatives for the development of newer and safer psychotropic drugs[5]. *V. album*, commonly known as Ujjral, belongs to family Viscaceae. It is widely distributed throughout the Europe and Asia. Traditionally, the plant has been used in the treatment of nervous disorders[6]. The plant has been reported to exhibit anti-inflammatory, anticancer, antimicrobial, anticonvulsant, antidiabetic, hepatoprotective and antipsychotic activities[7]. *V. album* has been reported to contain flavonoids, alkaloids, fatty acids, steroids and terpenoids. A survey of literature revealed that no systematic pharmacological work has been carried out on *V. album* to validate its traditional claims for neuropharmacological activities. Thus, it was considered worthwhile to investigate detailed neuropharmacological activities of *V. album* aerial parts, and to standardize the plant on the basis of marker compounds.

MATERIALS AND METHODS

Collection and identification of plant material: *Viscum album* aerial parts were procured from Himalaya Herb Stores, Madhav Nagar, Saharanpur, (Uttar Pradesh), India in September, 2014. The plant was identified by Dr. Avneet Pal Singh, Assistant Professor, Department of Botany, Punjabi University, Patiala, India (Reference No. SPL-104/Bot, dated 15-10-2014).

Solvents: Methanol (S.D. Fine Chemicals, Mumbai, India), *n*-hexane, 1-butanol, and ethyl acetate (E Merck, Delhi, India), of LR grade, were used for the preparation of various crude extracts and fractions of *V. album* aerial parts. Toluene, ethyl acetate (E Merck, Delhi, India), and glacial acetic acid (Ranbaxy Laboratory Chemicals, Mumbai, India), of AR grade, were used for thin layer chromatographic studies.

Animals: Laca mice (either sex) of body weight 20-25 g purchased from the Central Research Institute, Kasauli, India were used for pharmacological studies. The animals were fed with normal laboratory pellet diet and water *ad libitum*. The approval was taken from Institutional Animal Ethics Committee of Punjabi University, Patiala before carrying out animal studies (107/99/CPCSEA/2014-01, dated 11/10/2014). Groups of six animals were used in all sets of experiments.

Preparation of extracts and fractions: Dried and powdered plant material (2 kg) was successively extracted in a Soxhlet apparatus using solvents in increasing order of polarity *viz.*, *n*-hexane and methanol. The methanol extract (25 g) of plant material was suspended uniformly in water, and partitioned successively with ethyl acetate and 1-butanol as per standard procedure[8]. Vehicle and standard drugs: Distilled water + Tween 80 (2%) was

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Table 1: Antianxiety, antidepressant, locomotor, hypnotic and antistress activities of methanol extract, EAF and BF of *V. album* aerial parts.

Treatment	Dose (mg/kg)	Antianxiety activity		Antidepressant activity	Locomotor activity		Hypnotic activity[#]		Antistress activity
		Mean[n] number of entries in open arms ± S.D.	Mean[n] time spent in open arms (sec) ± S.D.	Mean[n] immobility time (sec) ± S.D.	Mean[n] number of squares crossed ± S.D.	Mean[n] number of rearings ± S.D.	Mean[n] latency time (min) ± S.D.	Mean[n] duration of sleep (min) ± S.D.	Mean[n] immobility time (sec) ± S.D.
Control	Vehicle	2.16 ± 0.80[a]	4.23 ± 0.79[a]	204.16 ± 9.70[a]	55.05 ± 5.90[a]	15.19 ± 3.20[a]	1.99 ± 0.54	63.33 ± 3.77[a]	145.00±5.44[a]
Diazepam	2	7.83 ± 1.00[*]	11.03 ± 1.15[*]	-------	-------	-------	-------	-------	-------
Imipramine	15	-------	-------	51.50 ± 7.00[*]	-------	-------	-------	-------	-------
Diazepam	2	-------	-------	-------	40.30 ± 5.45[*]	7.94 ± 2.28[*]	-------	-------	-------
Diazepam	5	-------	-------	-------	-------	-------	1.85 ± 0.25	144.95 ± 4.13[*]	-------
Diazepam	1	-------	-------	-------	-------	-------	-------	-------	48.33 ± 3.93[*]
Methanol extract	50	5.50± 1.32[*a]	6.92 ± 1.74[*a]	-------	-------	-------	-------	-------	-------
	100			-------	-------	-------	-------	-------	-------
	200	6.66 ± 1.92[*]	10.02 ± 1.64[*]	178.66 ± 8.23[*a]	47.97 ± 5.29[*a]	10.15 ± 2.35[*a]	1.87 ± 0.60	111.83 ± 5.19[*a]	74.00 ± 4.42[*a]
	400	------- -------	------- -------	170.50 ± 4.32[*a]	42.75 ± 4.35[*]	7.59 ± 1.50[*]	1.70 ± 0.29	138.50 ± 3.01[*]	60.33 ± 3.55[*a]
EAF	5	4.33± 0.98[*a]	7.38 ± 0.98[*a]	-------	-------	-------	-------	-------	-------
	10			-------	-------	-------	-------	-------	-------
	25	6.83 ± 1.11[*]	9.50 ± 1.05[*]	180.50 ± 10.74[*a]	48.90 ± 6.04[*a]	8.90 ± 1.77[*a]	1.80 ± 0.23	106.00 ± 4.60[*a]	76.16 ± 4.87[*a]
	50	------- -------	------- -------	168.16 ± 7.08[*a]	42.35 ± 4.22[*]	8.14 ± 1.99[*]	1.85 ± 0.54	140.50 ± 6.28[*]	59.33 ± 5.27[*a]
BF	5	4.83± 0.98[*a]	7.55 ± 1.02[*a]	-------	-------	-------	-------	-------	-------
	10			-------	-------	-------	-------	-------	-------
	25	7.16 ± 1.02[*]	10.04 ± 1.16[*]	94.16 ± 7.11[*a]	48.60 ± 5.10[*a]	9.10 ± 1.88[*a]	1.69 ± 0.45	120.83 ± 3.18[*a]	76.33 ± 4.76[*a]
	50	------- -------	------- -------	55.16 ± 6.24[*]	41.29 ± 3.58[*]	8.20 ± 1.90[*]	1.46 ± 0.33	136.66 ± 5.27[*]	57.83 ± 5.70[*a]

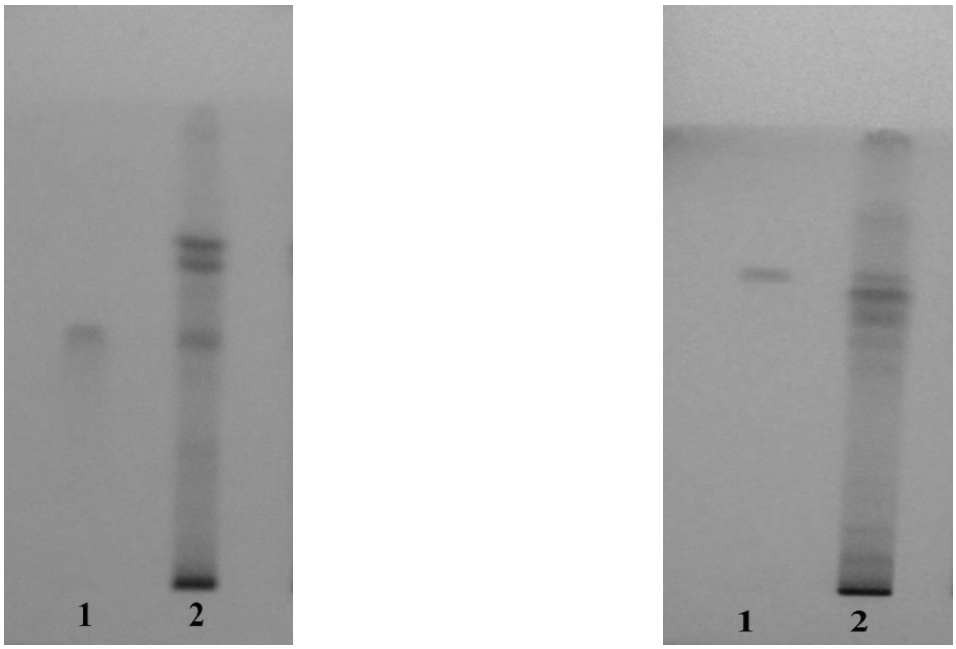
n = 6; The data is expressed as Mean ± S.D.; **P<0.05* vs. Control; [a]*P< 0.05* vs. Standard; one way ANOVA followed by Student-Newman-Keul's test. # Thiopentone sodium (80 mg/kg, *i.p.*) was administered to all mice treated with control, diazepam, methanol extract, EAF and BF.

used as vehicle for preparing various test doses in such a concentration as to administer a volume ranging 0.2 to 0.25 ml to the mice. Diazepam (2 mg/kg, *i.p.*), imipramine (15 mg/kg, *i.p.*), diazepam (5 mg/kg, *i.p.*), diazepam (2 mg/kg, *i.p.*), diazepam (1 mg/kg, *i.p.*) and morphine (5 mg/kg, *i.p.*), were used as standard antianxiety, antidepressant, hypnotic, CNS depressant, antistress and anlgesic drugs respectively. Thiopentone sodium (80 mg/kg, *i.p.*), a standard hypnotic drug, was administered to each mice for the assessment of hypnotic activity. Experimental design: Animals were divided into eight (I-VIII) groups. Group I - Control group received vehicle; Group II - Standard group received respective standard drug; Group III and IV - Test groups received different doses of methanol extract; Group V and VI - Test groups received different doses of EAF; Group VII and VIII - Test groups received different doses of BF. Neuropharmacological activities: Antianxiety, antidepressant, hypnotic, antistress and analgesic activities of test samples were performed using EPM, FST, thiopentone sodium-induced sleeping assay, cold swim test and tail immersion test, respectively, as per methods described in our laboratory[8]. Locomotor activity of test samples was preformed using open field test [9]. Statistical analysis: The results were expressed as mean ± standard deviation (S.D.). The test drugs were compared with standard drug and control by one way analysis of variance (ANOVA) followed by Student-Newman-Keul's test[10].

Table 2: Analgesic activity of methanol extract, EAF and BF of *V. album* aerial parts.

Treatmet	Dose (mg/kg)	Mean[n] basal reading (sec) ± S.D.	Mean[n] Reaction time (sec) ± S.D. 30 min	1 h	2h	3h	% MPE 30 min	1 h	2h	3h
Control	Vehicle	2.22 ± 0.45	2.75 ± 0.05[a]	2.70 ± 0.04[a]	2.60 ± 0.20[a]	2.55 ± 0.15[a]	4.14	3.75	2.97	2.58
Morphine	5.00	2.20 ± 0.11	9.90 ± 0.87[*]	8.40 ± 0.31[*]	6.22 ± 0.44[*]	5.11 ± 0.32[*]	60.15	48.44	31.41	22.73
Methanol extract	200	2.30 ± 0.06	6.30 ± 0.10[*a]	5.52 ± 0.24[*a]	3.12 ± 0.11[*a]	2.50 ± 0.21[a]	31.49	25.35	6.45	1.57
	400	2.70 ± 0.14	7.94 ± 0.22[*a]	6.55 ± 0.11[*a]	4.75± 0.36[*a]	3.90± 0.14[*a]	42.60	31.30	16.66	9.75
EAF	25	2.10 ± 0.21	7.02 ± 0.30[*a]	6.21 ± 0.55[*a]	4.22 ± 0.56[*a]	3.69± 0.33[*a]	38.14	31.86	16.43	12.33
	50	2.05 ± 0.14	8.02 ± 0.24[*a]	6.48 ± 0.32[*a]	4.58± 0.10[*a]	4.01± 0.25[*a]	46.10	33.59	19.54	15.14
BF	25	2.15 ± 0.10	6.90 ± 0.16[*a]	6.40 ± 0.12[*a]	5.35 ± 0.13[*a]	4.12± 0.27[*a]	36.96	33.07	24.90	15.33
	50	2.26 ± 0.33	7.80 ± 0.20[*a]	5.96 ± 0.20[*a]	4.90 ± 0.15[*a]	3.80± 0.29[*a]	43.48	29.04	20.72	12.09

n = 6; The data is expressed as Mean ± S.D.; **P<0.05* vs. Control; [a]*P<0.05* vs. Standard; one way ANOVA followed by Student-Newman-Keul's test.

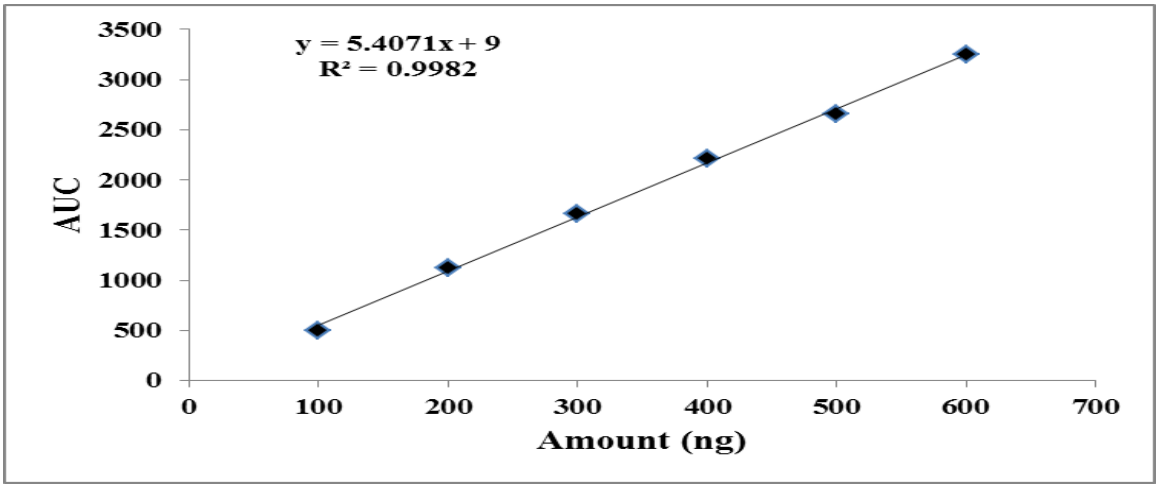


A **B**

Figure 1: Comparative TLC fingerprint profiles of marker compounds and methanol extract of *V. album* aerial parts under UV chamber at 254 nm. 1: Marker compound; 2: Methanol extract. (A) Quercetin and methanol extract, Solvent system – toluene:ethyl acetate:glacial acetic acid (15:11:2); (B) Apigenin and methanol extract, Solvent system – toulene:ethyl acetate (1:4).

TLC densitometric method development and validation studies

Preparation of standard plot: The stock solutions of apigenin and quercetin (5 mg/ml each) were diluted with methanol to get dilutions of different concentrations for apigenin (5, 7.5, 10, 12.5, 15 and 17.5 µg/ml) and



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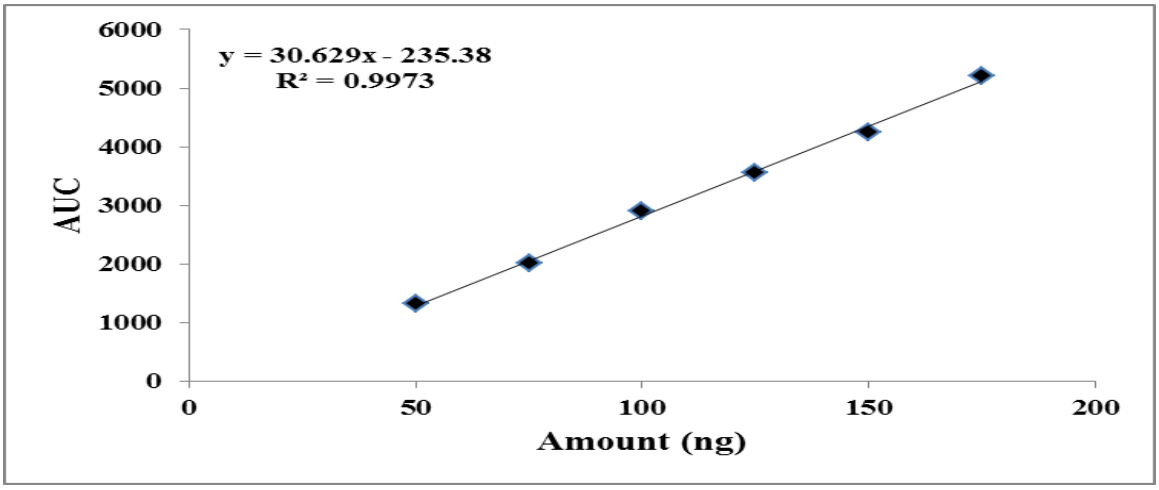

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Figure 2: Standard plots of marker compounds in TLC densitometric analysis. (A) Quercetin; (B) Apigenin. AUC – Area under the curve.

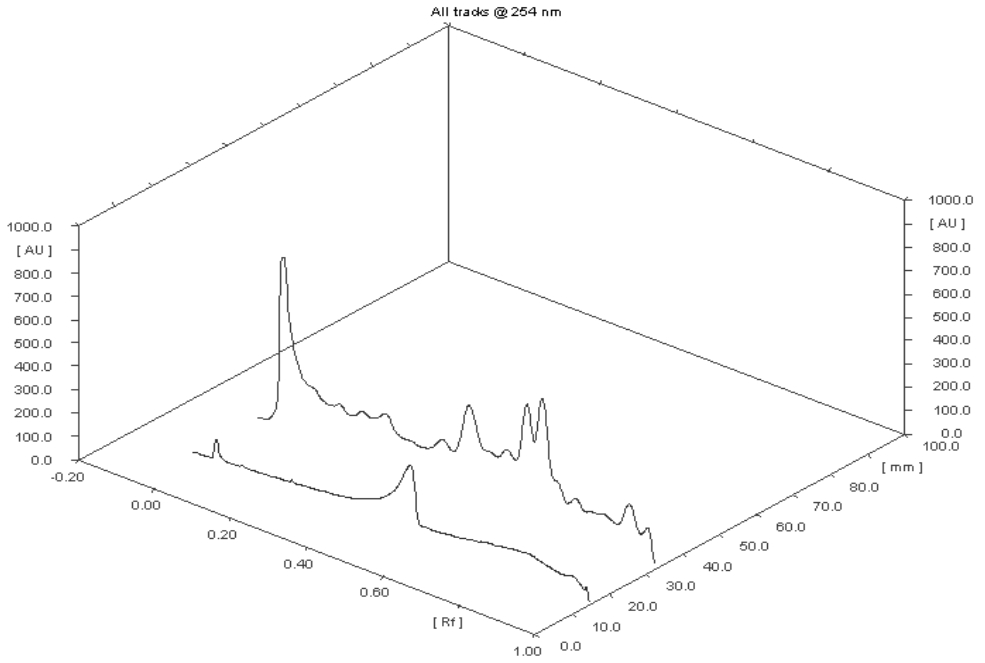
quercetin (10, 20, 30, 40, 50 and 60 µg/ml). A volume of 10 µl from each dilution was applied in triplicate on pre-coated TLC plate (E Merck, Mumbai, India; 0.2 mm; aluminum base) using CAMAG LINOMAT 5. The plates for apigenin and quercetin samples were developed using toulene: ethyl acetate (1:4) and toulene: ethyl acetate: glacial acetic acid (15:11:2), respectively, as solvent systems to a distance of 8 cm. The developed plates of apigenin and quercetin were scanned in TLC scanner at 326 and 254 nm respectively. The area under the curve (AUC) of the peak corresponding to marker compound was noted in each track. Preparation of test samples: The coarsely powdered plant material, 10 g of aerial parts, was exhaustively extracted with methanol in a Soxhlet apparatus. The extraction was continued till 2 ml of solvent collected, during siphoning process of Soxhlation, on a watch glass did not leave any residue after evaporation. The methanol extract was filtered, concentrated under reduced pressure and the volume was adjusted to 5 ml with methanol.

Estimation of marker in methanol extract: Test solutions (10 µl) of methanol extract was applied in triplicate on pre-coated TLC plate (5 × 10 cm). The plate was developed and scanned following the same procedure as used for the preparation of standard plot. The average AUC of the peaks corresponding to quercetin and apigenin were noted at 254 and 366 nm, respectively. The content of each marker compound was calculated from the regression equation of the standard plot.

TLC densitometric method validation studies: The developed methods were validated for the parameters described in ICH guidelines such as linearity, range, LOD, LOQ, inter day precision, intra-day precision, accuracy, repeatability and specificity[11].

RESULTS AND DISCUSSION

The methanol extract of *V. album* aerial parts was prepared by extracting properly identified plant in a Soxhlet apparatus with methanol, after defatting with *n*-hexane. Yield of methanol extract was found to be 9.96%



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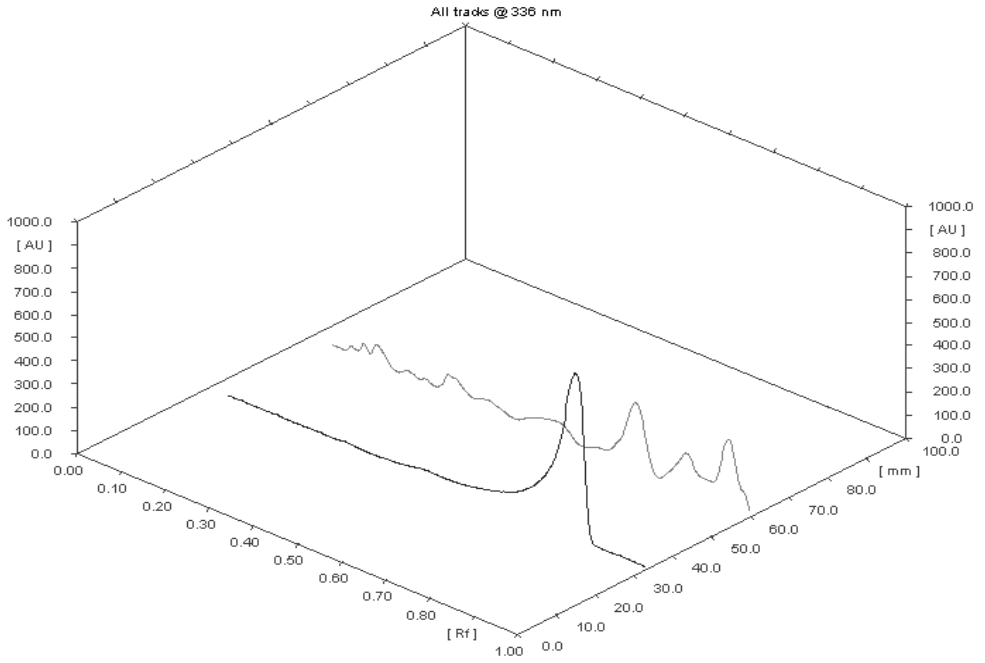
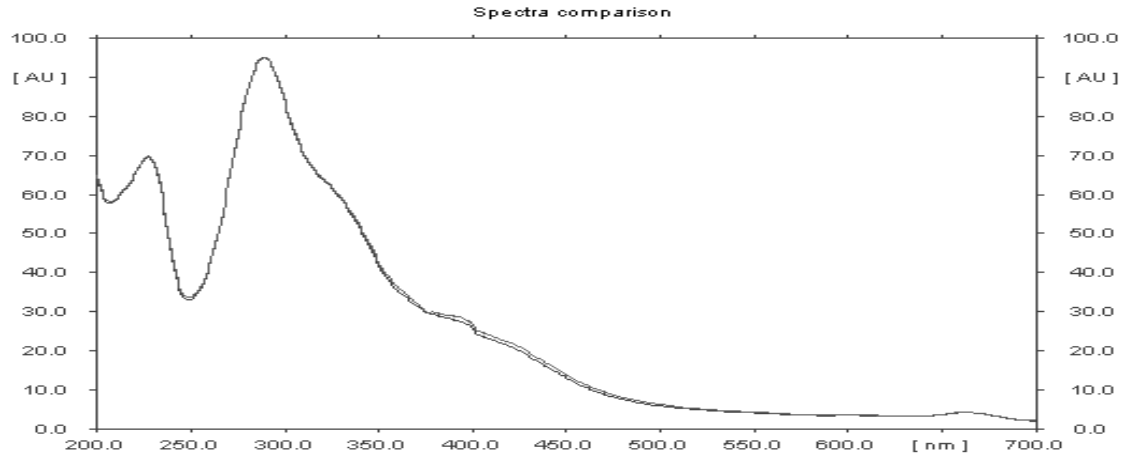


B

Figure 3: TLC densitometric chromatograms of marker compounds and methanol extract of *V. album* aerial parts. (A) Quercetin; (B) Apigenin.

w/w. Phytochemical screening showed presence of alkaloids, steroids, cardiac glycosides, flavonoids, triterpenoids and tannins in methanol extract. The methanol extract was further fractionated successively using ethyl acetate and 1-butanol. Yields of ethyl acetate fraction (EAF) and 1-butanol fraction (BF) were found to be 7.60 and 9.56%, respectively, in relation to the methanol extract. The methanol extract, EAF and BF of *V. album* aerial parts were screened for antianxiety activity in mice using EPM model. The mean number of entries and average time spent in open arms of EPM apparatus after the treatment of methanol extract (50 or 100 mg/kg, *p.o.*), EAF (5 or 10 mg/kg, *p.o.*), BF (5 or 10 mg/kg, *p.o.*), diazepam (2 mg/kg, *i.p.*) and the control (vehicle, *p.o.*) have been shown in table 1. The methanol extract, EAF and BF exhibited significant antianxiety



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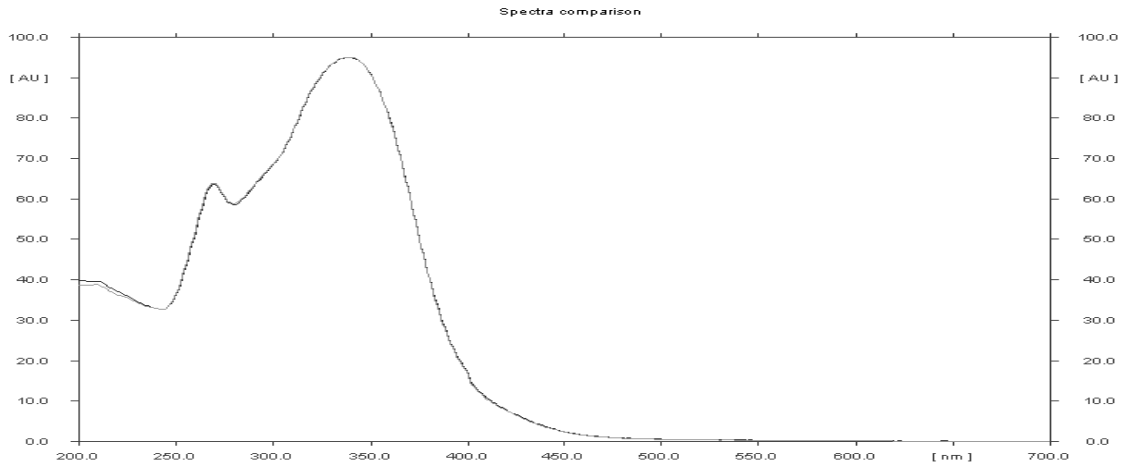

B

Figure 4: Spectra overlays of marker compounds with corresponding peak in methanol extract of *V. album* aerial parts. (A) Quercetin; (B) Apigenin.

activity with respect to control at all tested doses, but therapeutic level equivalent to the standard drug was not achieved at lower doses. The methanol extract (100 mg/kg), EAF (10 mg/kg) and BF (10 mg/kg) significantly increased number of entries and time spent in open arms of EPM in mice, statistically equivalent to the standard drug. The antidepressant activity was evaluated using despair swim test after acute administration of methanol extract (200 or 400 mg/kg, *p.o.*), EAF (25 or 50 mg/kg, *p.o.*), BF (25 or 50 mg/kg, *p.o.*), imipramine (15 mg/kg, *i.p.*) and the control (vehicle, *p.o.*). The methanol extract and EAF significantly reduced duration of immobility in mice at all doses with respect to control but none of the doses showed activity statistically equivalent to the standard drug (Table 1). Only BF elicited antidepressant effects in similar manner to the standard drug at the dose of 50 mg/kg. The reduction in duration of immobility in mice treated with test extract and fractions was not associated with increased locomotor activity in open field test (Table 1). These observations confirmed specific antidepressant activity of extract and fractions of the plant. The methanol extract, EAF and BF significantly reduced rearing and crossings with respect to control in open field test, thus, suggested to possess CNS depressant activity. Potentiation of thiopentone sodium induced-sleeping time assay was employed to assess hypnotic activity of the methanol extract, EAF and BF of *V. album* aerial parts. The mean latency time and duration of sleep after acute oral administration of methanol extract (200 or 400 mg/kg), EAF (25 or 50 mg/kg), BF (25 or 50 mg/kg), diazepam (5 mg/kg, *i.p.*) and the control (vehicle) have been shown in table 1. At higher doses, the methanol extract, EAF and BF significantly increased duration of sleep in mice. The antistress activity was evaluated in mice using cold swim test after acute treatment with methanol extract (200 or 400 mg/kg, *p.o.*), EAF (25 or 50 mg/kg, *p.o.*), BF (25 or 50 mg/kg, *p.o.*), diazepam (1 mg/kg, *i.p.*) and the control (vehicle, *p.o.*). The methanol extract, EAF and BF significantly reduced time spent by mice in immobile state with respect to control but the

activity was not equivalent to the standard drug. These observations inferred their mild antistress activity (Table 1). The methanol extract, EAF and BF of *V. album* aerial parts were subjected to analgesic activity in mice using tail immersion method. The tail withdrawal from the heat (flicking response) was recorded in the mice after administration of methanol extract (200 or 400 mg/kg, *p.o.*), EAF (25 or 50 mg/kg, *p.o.*), BF (25 or 50 mg/kg, *p.o.*) and morphine sulphate (5 mg/kg, *i.p.*). The methanol extract, EAF and BF exhibited significant analgesic activity at the tested doses with respect to control group during whole period of study (Table 2). But none of test doses of methanol extract, EAF and BF could show analgesic effects comparable to the standard drug. Comparative TLC fingerprint studies confirmed the presence of quercetin and apigenin in methanol extract (Fig. 1). Thus, quercetin and apigenin were taken as chemical markers to standardize *V. album* aerial parts using validated TLC densitometric method. Standard plots were prepared between different concentrations of quercetin and apigenin versus their peak areas after scanning at 254 and 336 nm respectively (Fig. 2). The estimation of marker compounds and their accuracy (recovery) studies were done in triplicate, and the values were expressed in percent w/w (mean ± S.D.). The content of quercetin and apigenin in *V. album* aerial parts was found to be 0.00452 ± 0.00001% and 0.00058 ± 0.00000% w/w, respectively. TLC densitometric methods were validated as per ICH guidelines. The instrumental precision, repeatability, linearity range, correlation coefficient, intra-day precision, inter-day precision, limit of detection (LOD), limit of quantification (LOQ) and accuracy of quercetin were found to be 0.71% CV, 0.19% CV, 100-600 ng, 0.998, 0.99% CV, 0.59% CV, 19 ng/spot, 59 ng/spot and 98.76 ± 0.26% respectively. In case of apigenin, instrumental precision, repeatability, linearity range, correlation coefficient, intra-day precision, inter-day precision, LOD, LOQ and accuracy were found to be 0.49% CV, 0.21% CV, 50-175 ng, 0.997, 0.83% CV, 0.46% CV, 6 ng/spot, 21 ng/spot and 98.84 ± 0.80% respectively. It is clearly evident from thin layer chromatogram (Fig. 3) and ultraviolet spectra (Fig. 4) overlay that there is no interference in quantitative analysis, thus, confirming specificity of the developed TLC densitometric method for each standard marker. The well established experimental models were selected for assessing neuropharma- cological activities of *V. album* extract and fractions. In EPM, the animal prefers to stay in enclosed arms due to acrophobia (anxiety due to height). Antianxiety drugs increase number of entries and time spent by mice in open arms. It is evident from the results of present investigation that the methanol extract, EAF and BF of the plant possess anxiolytic activity. Antianxiety drugs at higher doses show sedation in animals by acting on $GABA_A$ receptors[12]. Test extract and fractions elicited hypnotic effects at 5 times higher dose than anxiolytic dose, suggesting their anxiolytic and hypnotic activity through modulation of $GABA_A$ receptors. In forced swim test (FST), antidepressant drugs reduce immobility time without affecting locomotor activity. Psychostimulants reduce duration of immobility in FST but in contrast stimulate locomotor activity [13]. CNS depressant drugs do not affect immobility in FST but reduce motor activity. Test substances significantly reduced motor activity in open field test, and also immobility time in FST, thus, confirming their specific CNS depressant and antidepressant effects without psychostimulation. Preliminary TLC studies of methanol extract confirmed presence of bioactive flavonoids, i.e., quercetin and apigenin. It is well documented that quercetin and apigenin exhibit neuropharmacological activities. Quercetin has been reported to exhibit anxiolytic activity at the concentration of 41 μg/mg, using *in vitro* test[14]; antidepressant activity at the dose of 50 mg/kg, *p.o.*, using FST[15]; antistress activity at the dose of 100 mg/kg, *p.o.*, using FST[16]; analgesic activity at the dose of 10 or 60 mg/kg, *i.p.*, using acetic acid induced pain test[17] and sedative activity at the dose of 100 mg/kg, *p.o.*, using pentobarbital induced sleeping assay[18]. Apigenin has been reported to exhibit anxiolytic activity at the dose of 2 mg/kg, *p.o.*, using EPM[19]; antidepressant activity at the dose of 12.5, 25 or 200 mg/kg, *i.p.*, using FST[20]; analgesic activity at the dose of 10 mg/kg, *p.o.*, using tail immersion test[21] and sedative activity at the dose of 0.6 mg/kg, *p.o.*, using ethyl ether-induced hypnosis test[22]. In the light of these reports, it is suggested that neuropharmacological activities of *V. album* may be due to presence of bioactive flavonoids – quercetin and apigenin. Thus, these bioactive constituents were used as markers to standardize the plant. The contents of quercetin and apigenin were determined using validated TLC densitometric method. The validation parameters complied with the prescribed limits.

CONCLUSION

The present investigations have validated traditional claims of the plant for various neuropharmacological activities. *V. album* aerial parts possess anxiolytic, hypnotic, antidepressant, analgesic and antistress activities. Bioactive flavonoids – quercetin and apigenin have been suggested to play role in neuropharmacological activities of the plant.

ACKNOWLEDGEMENTS

Authors duly acknowledge Prof R.C. Gupta, Co-ordinator of DBT-IPLS project for providing access to instrumentation facilities at Punjabi University, Patiala.

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