Pharmacological and biological evaluation of extracts from *Gratiola officinalis* L. (Scrophulariaceae)

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Abstract: The crude extract of *Gratiola officinalis* and its *n*-hexane, chloroform, ethyl acetate, *n*-butanol and aqueous fractions were subjected to biological (Brine Shrimp Bioassay, Insecticidal and Phytotoxicity/Cytotoxic) and neuropharmacological (Head dip, Open field Forced swimming test, Sodium pentothal induced sleep) activities. Results obtained in this study indicated that at high concentration dose (1000µg/ml), all test samples showed 60-95% phytotoxicity. In crude extract, *n*-butanol and aqueous fractions produced more than 85% phytotoxicity. While low concentration (10µg/ml) dose showed 25-28% phytotoxicity in all test samples. The crude extract was devoid of any effect against the growth of C*allosbruchus analis* and *Tribolium castaneum* and caused 10 mortality of *Rhyzopertha dominica*. *n*-Hexane, chloroform, ethylacetate, *n*-butanol and aqueous fractions caused 50, 30, 40, 10 and 20% mortality respectively of *C. analis* where as chloroform, ethyl-acetate, aqueous and crude extract, *n*-hexane, ethyl-acetate fractions also caused low mortality (10%) of *Tribolium castaneum* and *Rhyzopertha dominica* respectively. In cytotoxic assay at 1000µg/ml concentration, *n*-butanol fraction produced 36.7% and the crude extract produced 13.3% mortality of brine shrimp, its aqueous fraction was inactive at all concentrations. The results of head dip, open field, mobility time and Pentothal Na induced sleep indicated that crude extract, *n*-butanol and ethylacetate fractions of *G. officinalis* had mild sedative effect. However aqueous fraction was found to produce a significant decrease in motor activities and potentiated the duration of sleep.

Keywords: *Gratiola officinalis* Linn., Scrophulariaceae, phytotoxic, cytotoxic insecticidal, antimicrobial and motor activities.

INTRODUCTION

Gratiola officinalis Linn is (Scrophulariaceae) locally known as Hedge Hyssop (Hooker 1982) and grows abundantly in northern part of Pakistan especially in lower Dir and Sawat. The dried top of it is reported as diuretic and emetic. In folk medicines it is used for the treatment of a variety of ailments such as scrofula, cystitis, colic, stomach and menstrual disorders, skin and liver diseases as well as enlargement of the spleen, dropsy, jaundice and intestinal worms. *G. officinalis* is also used as a biostimulating tablet in hematopoietic, liver and respiratory disorders in human. The root and the flowering herb are cardiac tonic, diuretic, violently purgative and vermifuge (Bown, 1995; Launert, 1981; Nasir and Ali, 1974; Grieve, 1983; Lust, 1983; Graves, 1996).

Toth *et al*. (1977) stated that the Schrophulariaceae species are rich in lysine, isoleucine and phenylalanine and valine. Therefore, an interest was developed to carry out study on biological and neuropharmacological aspects of *G. officinalis* because no data was available on this species regarding biological and pharmacological aspects. Present study deals with the *in vitro* evaluation of the phytotoxic, cytotoxic and insecticidal activities and *in vivo* neuropharmacological activities of the *G. officinalis*.

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EXPERIMENTAL

Plant material

The plant material was collected from Dir District, Kyber Pakhtoon Khua, Pakistan. After identification of whole plant, a voucher specimen OG-01/2002 was deposited in the herbarium of Department of Pharmacognosy, University of Karachi, Pakistan.

Extraction and Phytochemical analysis

Shade-dried *G. officinalis* (25 kg) was ground and extracted by maceration with methanol at room temperature (3×23L). The combined methanol macerate was filtered and evaporated under vacuum at 40°C to obtain a thick gummy mass, the crude extract (500g). Later it was fractionated with classical method and the following fractions were collected i.e. *n*-hexane, chloroform, ethyl acetate, *n*-butanol and aqueous fractions.

FTIR and *FTNIR* were performed on these fractions and crude extract according to the modified methods described by Andrei *et al* (2006).

UV spectra of crude extract: 500 mg of crude was taken in 500 ml round bottom flask, after adding 50 ml methanol it was refluxed for 30 minutes and then cooled to room temperature. It was filtered in 100 ml volumetric flask using Whatman filter paper 40 and volume was made up by methanol. A Lambda-20 Perkin Elmer apparatus was used for UV light absorption record.

HPLC analysis crude extract: 2 g of *G. officinalis* crude extract was dissolved in 10 ml of methanol, with the help of sonicator at 40^{o}C for 30 min. The sample was filtered through the 0.45 mm filter paper with the help of Sunnix filtering assembly. HPLC was performed using a Shimadzu HPLC (Detector: SPD 20A, Pump: LC 20AT, Auto-sampler: SIL 20A, System Controller: CBM 20A and HPLC Column Manufacture: Waters µBondapak C18 3.9 x 300mm). The system was operated at room temperature (20^{o}C), the injection volume was 20 µliters, and the detection wavelength was 225 and 325 nm (Munir, 2006).

Phytotoxic activity

The Phytotoxic activity was performed according to the modified protocol used described by khan *et al.,* 2008 and Ahmad *et al*. (2012). The crude extract and fractions were tested against the *Lemna minor* at different concentrations. For positive control Paraquat was used as a standard drug while volatile solvent was used as negative control.

Insecticidal activity

All the extracts of *G. officinalis* were subjected to insecticidal activity according to the method described by Ahmad *et al*., 2011 and Atta-ur-Rahman *et al.* (2001). For this purpose test insects *Tribolium castaneum*, C*allosbruchus analis,* and *Rhyzopertha dominica* were used. The concentration of test samples were 200mg each and dissolved in 3 ml acetone to impregnate the filter paper in Petri Dishes. The Permethrin and acetone were used as a positive and negative control respectively. The results were calculated by percentage of mortality according to the given formula: % of M=Control-Test/Control x 100 (control: no. of insects in negative control, Test: No. of survived insects in test sample.

Brine shrimp lethality bioassay

Crude extract and fractions of *G. officinalis* were examined for Brine Shrimp Bioassay according to the method described by Meyer (Meyer *et al*., 1982; Santos *et al*., 2003). Etoposide and DMSO were used for positive and negative control respectively.

Neuropharmacological activities (Head dip test, Open field test, Forced swimming test, Pentothal Na induced sleeping test)

Neuropharmacological activities were studied by head dip, open field, swimming induced depression and Pentothal Na induced sleep test. In each test, animals (mice and rats) were divided into 8 groups (each group comprised of 5 animals). In head dip, open field, and forced swimming test mice were treated with crude extract and fractions orally at the dose of 300mg/kg where as Diazepam was used as a standard drug. The control animals were treated orally with the same volume of saline as the crude extract and fractions. In all the tests observations were made after 30 to 40 minutes of oral dose administration of test drugs.

In head dip study observation was made to count the number of head dips by the animal through the holes head dip apparatus (Kasture *et al.,* 2002). The open field was performed in a quiet room under white light as described by Kennett *et al.,* 1985). Forced swimming test was performed according to Porsolt *et al.* (1978). Pentothal Na induced sleep activity was performed by the modified method as described by Kasture *et al* (2002). In this test rats (180-200 gm) were divided in to eight groups (each group contained 5 animals). They received 40 mg/kg (i.p.) Pentothal sodium, 30 min after the oral administration of 300 mg/kg of test extracts. The sleeping time was recorded and measured as the time interval between the loss and regaining of the rightining reflex (Kasture *et al.,* 2002).

STATISTICAL ANALYSIS

The results were expressed as mean ± S.E.M. All statistical comparisons were made by means of Student's *t*-test and a *P* value smaller than 0.05 was regarded as significant.

RESULTS

Results of spectral analysis were presented in figs. 1-5. UV analysis indicated the presence of peaks at 206.022, 262.920, 273.3 nm while HPLC analysis was made at 225 nm and 325 nm indicated presence of peaks at 1.023, 1.920, 2.626, 3.383 and 4.448 minutes.

G. officinalis crude extract and its different fractions were screened for their phytotoxic, insecticidal and cytotoxic (Brine Shrimps Bioassay) activities (table 1, 2 and 3 respectively).

The results of head dip, open field and forced swimming test in mice are given in table 4 and graph 1. Among these results crude extract showed significant decrease (49±1.98, 35±2.02, 3.52±1.55) in the response; ethyl acetate (45±3.01, 39±1.75, 3.44±2.01) and aqueous fraction (36±2.04, 29±2.31, 2.98±1.05) also produced significant decrease in head dip, open field and forced swimming activities respectively at $p<0.05$. Other extracts also showed comparable results with control (less significant results).

Table 1: *In vitro* phytotoxic bioassay of *G. officinalis* samples

Samples	Concentration µg/ml	Number of fronds samples	Negative control	% Growth regulation	Conc. of std. drug (µg/ml)
Crude extract		29		27.5	
n-hexane		30		25.0	
Chloroform		28		30.0	
Ethyl acetate	10	30	40	25.0	0.015
n-butanol		29		27.5	
Aqueous		27		32.5	
Crude extract		23		42.5	
n-hexane		25		37.5	
Chloroform		27		32.5	
Ethyl acetate	100	23	40	42.5	0.015
n-butanol		24		40.0	
Aqueous		20		50.0	
Crude extract		05		87.5	
n-hexane		15		62.5	
Chloroform		08		80.0	
Ethyl acetate	1000	09	40	77.5	0.015
n-butanol		04		90.0	
Aqueous		02		95.0	

Table 2: Insecticidal activity of *Gratiola officinalis* samples

Insect species	% mortality(Concentration 200mg/3ml)							
	Positive control	Negative control	*n*-hexane	Chloroform	Ethyl acetate	*n*-butanol	Aqueous	CE
C.analis	100	00	50	30	40	10	20	00
T.castaneum	100	00	00	10	10	00	10	00
R.dominica	100	00	10	00	10	00	00	10

CE: Crude Extract, positive control: Permethrin, Negative control: Distilled water

Table 3: *In vitro* cytotoxic activity of G. *officinalis* samples on brine shrimp growth

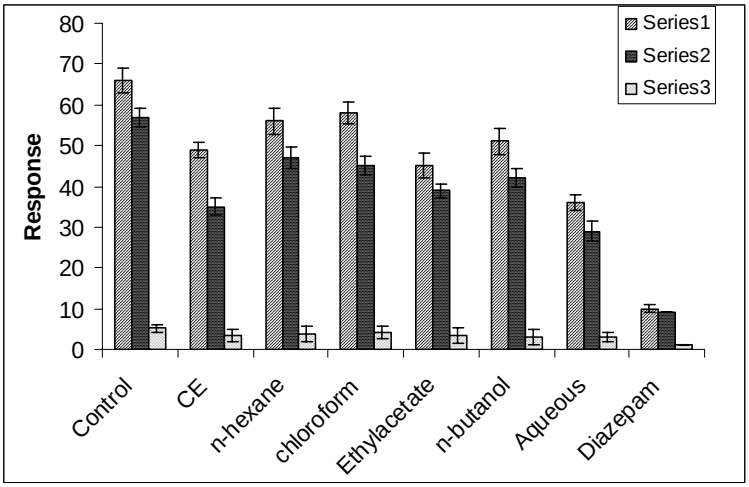
Samples	Concentration µg/ml	Negative control	Number of survivors	% mortality
Crude extract			30	0
n-hexane	10	30	30	0
Chloroform			30	0
Ethyl acetate			30	0
n-butanol			30	0
Aqueous			30	0
Crude extract			25	16.7
n-hexane	100	30	30	0
Chloroform			28	6.6
Ethyl acetate			29	3.3
n-butanol			27	10
Aqueous			27	10
Crude extract			20	33.3
n-hexane	1000	30	28	6.6
Chloroform			25	16.7
Ethyl acetate			25	16.7
n-butanol			23	23.3
Aqueous			24	20

Standard drug: Etoposide; LD_{50} µg/ml is 7.463

Table 4: Head dip, Open field and Mobility time of *G. officinalis* crude extract and fractions

Treatment	Head dip	Open field	Mobility time
		Mean no. of observation ±SEM, Dose 300 mg/kg	
Control	66 ± 3.01	57 ± 2.32	5.2 ± 1.02
CE	49 ± 1.98*	35 ± 2.02*	3.52 ± 1.55*
n-hexane	56 ± 3.22	47 ± 2.67	3.76 ± 1.95
chloroform	58 ± 2.55	45 ± 2.11	4.2 ± 1.55
Ethyl acetate	45 ± 3.01*	39 ± 1.75*	3.44 ± 2.01*
n-butanol	51 ± 3.25	42 ± 2.32	3.12 ± 1.95*
Aqueous	36 ± 2.04*	29 ± 2.31*	2.98 ± 1.05*
Diazepam	10 ± 0.95**	09 ± 0.05**	1.02 ± 0.03**

* significant; ** highly significant at P < 0.05 with ± S.E.M



Graph 1: Effect of Crude extract and fractions of *G. officinalis* on head dip, open field and Forced swimming test.
Series1: Head dip test, Series 2: Open field test, Series 3: Mobility time in min.

Table 5: Pentothal Na induced sleeping time of *G. officinalis* crude extract and fractions

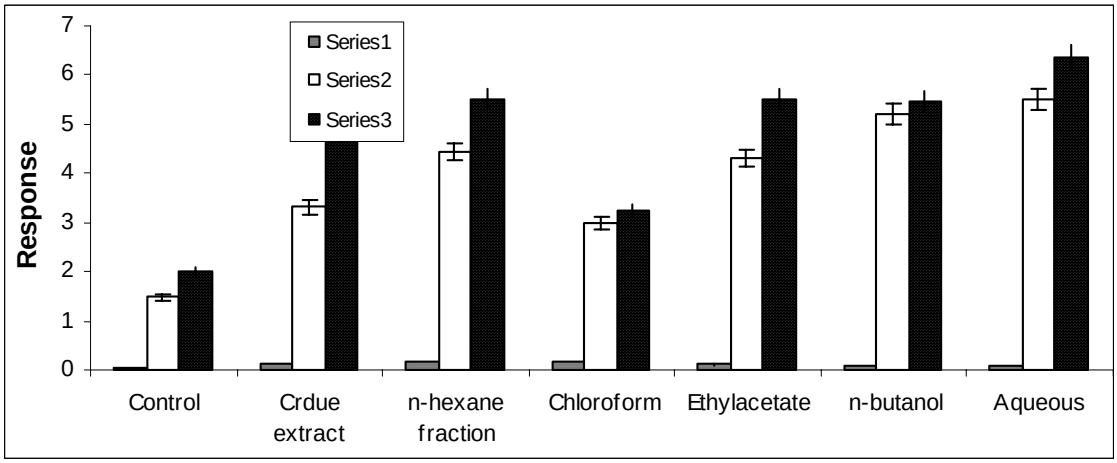
Treatment	Onset of action	Duration of action	Recovery time
	minutes	hours	hours
Control	2.5±1.45	1.48± 1.67	5.01± 1.93
Pentothal Na 40mg/kg			
Crude extract	7.5 ± 2.01*	3.31 ± 2.10*	5.02± 2.15*
n-hexane fraction	9.3 ± 2.01	4.45±2.12	5.51± 2.02*
Chloroform	10 ± 2.02	2.98 ± 1.71	3.25 ±3.19*
Ethyl acetate	6.5± 1.98*	4.32± 2.12*	5.51± 2.22*
n-butanol	5.5 ± 1.12*	5.20 ± 2.35*	5.45 ± 2.52
Aqueous	5.2±1.26*	5.52±2.48*	6.35 ± 2.25

* significant, ** highly significant at P < 0.05 with ± S.E.M

The results of Pentothal sodium induced sleeping time are given in table 5 and graph 2. Crude extract and fractions of *G. officinalis* (at the dose of 300 mg/kg) affect the onset duration and recovery time in rats. The onset, duration and recovery of sleep time for control group is 2.5±1.45, 1.48±1.67, 5.01±1.93 respectively, which is comparable with all test samples. The aqueous and *n*-butanol fractions were significantly increased the duration of sleep (5.20 ± 2.35 and 5.52±2.48).

DISCUSSION

Basic phytochemical screening by UV, FTIR, FTNIR and HPLC analysis indicated some further elaboration of



Graph 2: Effect of Crude extract and fractions of *G. officinalis* on sleeping time induced by Pentothal Na.
Series1: Onset of action in hours, Series 2: Duration of action in hours, Series 3: Recovery time in hours

presence of different chemical constituents in crude extract of *G. officinalis* (figs. 1-5).

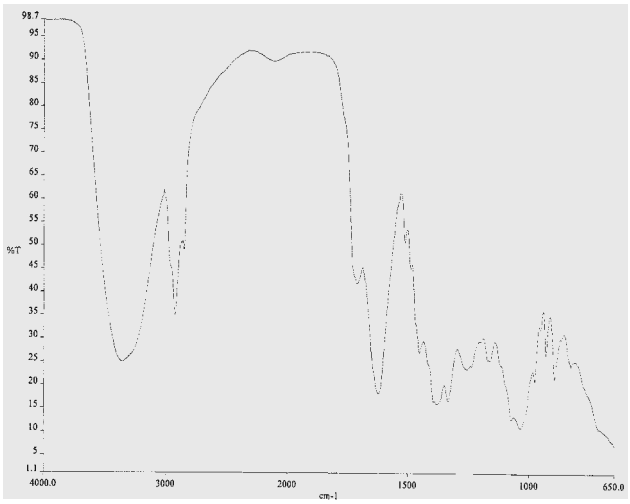


Fig. 1: FTIR of *Gratiola officinalis* crude extract

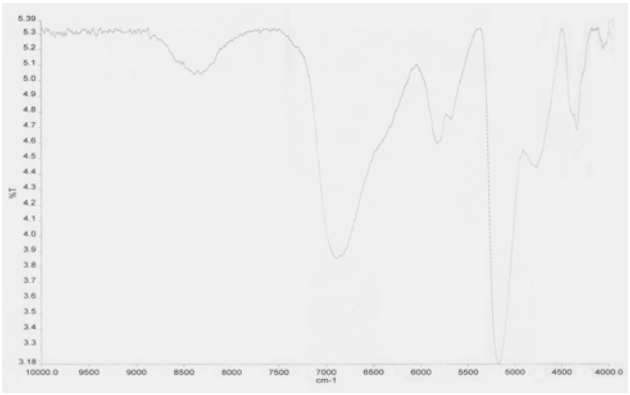


Fig. 2: FTNIR of *Gratiola officinalis* extract

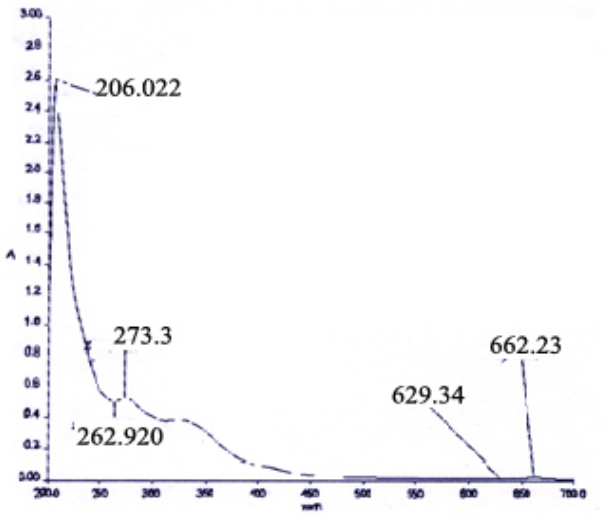


Fig. 3: UV analysis of *Gratiola officinalis* extract

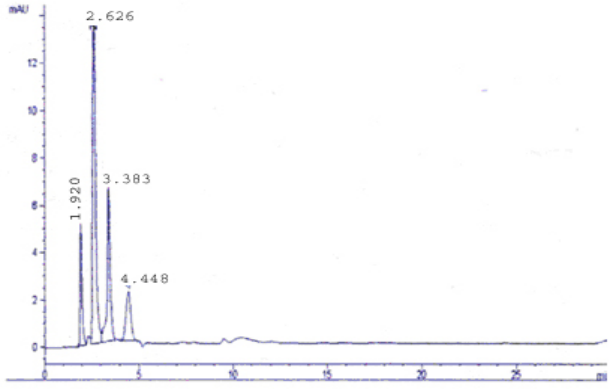


Fig. 4: HPLC of *Gratiola officinalis* at 225 nm

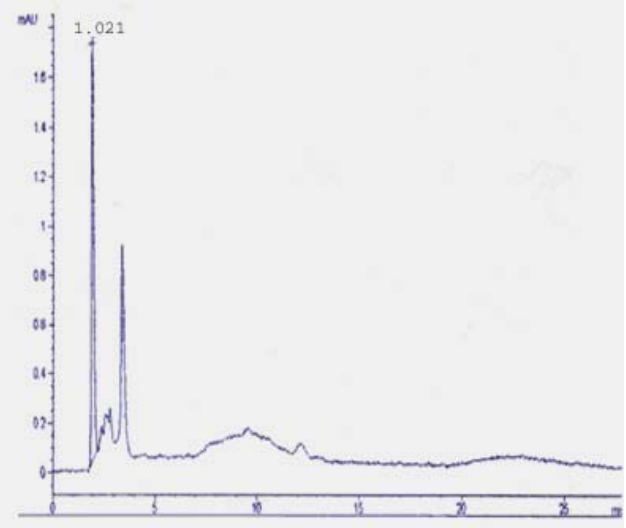


Fig. 5: HPLC of *Gratiola officinalis* at 325 nm

In the phytotoxic assay, at the highest test concentration of 1000 µg/ml, they produced more than 60% growth regulation of *Lemna minor*. The most active samples are the crude extract, chloroform, *n*-butanol and aqueous fractions (≥ 80% growth regulation), where as the ethyl acetate fraction exhibited 75% to < 80% growth regulation). The *n*-hexane fraction was the less active fraction (62.5% growth regulation). However, the phytotoxic activity of all tested samples was low than 50% when tested at concentrations ranging from 10 to 100µg/ml, but significant compared to the negative control (table 1). These results also demonstrated that the inhibition activity of all tested samples is concentration dependent. As it was already reported by Rashid *et al*. (2009), we could speculate that this activity in *G. officinalis* samples may be due to the occurrence of herbicidal compounds. The presence of some polyphenolic compounds identified in the crude extract such as tannins and flavonoids or terpenes (cucurbitacin) may be implicated in the death of the host tissues (Waterman and Mole, 1994; Rashid *et al*., 2009).

In present study the crude ethanol extract did not show significant activity (0% mortality) on the growth of *Callosbruchus analis* and *Ttribolium casaneum*, but only caused 10% mortality of *Rhyzopertha dominica*. Its *n*-hexane, chloroform, ethylacetate, *n*-butanol and aqueous fractions produced 50, 30, 40, 10 and 20% lethality respectively of *C. analis*. Some fractions were found to cause low lethality (10% mortality) of *T. castaneum* and *R. dominica* and other were inactive (table 2). The aqueous fraction had maximum insecticidal activity against *C. analis*. Similarly all fractions had less or no activity against *T. castaneum* and *R. dominica*.

Brine shrimp bioassay is well known easy method which requires relatively small amount of samples. This assay was used to discover new classes of natural pesticides and active antitumor agents since this bioassay reveals good correlation between the cytotoxic activity and the antitumoral or pesticidal activity (McLauglin *et al*., 1998; Santos *et al.,* 2003). In present study, at highest test concentration of 1000 µg/ml, the crude extract and its *n*-hexane, ethyl acetate and *n*-butanol fraction caused 13.3, 30.3, 26.6 and 36.7% mortality of Brine Shrimps. At concentrations of 10 and 100 µg/ml, all samples caused less than 50% mortality, but significant when compared to negative controls. It was concluded that these tested samples from *G. officinalis* were non-toxic against brine shrimp in our experimental conditions.

The crude extract and fractions of *G. officinalis* were subjected to basic neuropharmacological activities which possessed mild to moderate decreased in motor activities and increased the duration of sleep. From these results anxiolytic and mild sedative effects were observed with *G. officinalis* crude, ethylacetate, *n*-butanol and aqueous fractions. It is reported that *G. officinalis* have tetra cyclic triterpene, flavonoids and alkaloids (Kaya and Melzig 2008), therefore, the present sedative/anxiolytic effect is most likely due to the occurrence of chemical constituents mentioned above (Carlini, 2003). Therefore, this beneficial effect of the *G. officinalis* of Scrophulariaceae family may be categorized as a potent agent like Digitalis of the same family.

CONCLUSION

In conclusion, *G. officinalis* can be considered as a new source of phytotoxic agents, but not for pesticidal or antitumor agents because the cytotoxic activity found in our experimental condition is less than 50%. Its sedative and anxiolytic properties also provide potential therapeutic role in CNS disorders.

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