Bioactivity-guided isolation of potent anxiolytic compounds from leaves of *Citrus paradisi*

Vikas Gupta, Ravinder Sharma[1], Parveen Bansal, Gunpreet Kaur

Department of Herbal Drug Technology, University Centre of Excellence in Research, Baba Farid University of Health Sciences, [1]Department of Pharmaceutical Chemistry, University Institute of Pharmaceutical Sciences and Research, Faridkot, Punjab, India

Abstract

Context: *Citrus* fragrances have been attributed with mood-enhancing properties by aromatherapists. Leaves of this plant have been reported to exert anti-anxiety activity. Till date, no specific phytoconstituent responsible for this has been identified. **Objective:** Isolation of anxiolytic constituent of *Citrus paradisi* using bioactivity-guided fractionation. **Materials and Methods:** Leaf extracts of four varieties of *C. paradisi* in petroleum ether, chloroform, methanol and water were evaluated for anti-anxiety activity in mice using elevated plus-maze apparatus. Because of activity in methanol extract, it was used for safety evaluation/acute toxicity studies in animals. Bioactive fraction of methanol extract was subjected to column chromatography and structure of the isolated compound was elucidated by melting point, ultraviolet, infrared, nuclear mass reactor and mass spectroscopy. The isolated constituents were further evaluated for anti-anxiety activity using light/dark model and hole-board model of anxiety. **Results:** Results showed no mortality at a dose up to 2000 mg/kg body weight that indirectly reflects the safety profile of the leaf extracts. Fractionation of methanol extract led to the isolation of four flavonoids (rutin, quercetin, kaempferol and myricetin). The isolated compounds exhibited significant anxiolytic activity in different animal models. **Conclusion:** The study confirms the presence of four flavonoids responsible for anti-anxiety activity.

Keywords: Anti-anxiety, bioactivity, *Citrus paradisi*, flavonoids

Introduction

Stress and anxiety disorders are one of the most common mental disorders of modern world experienced by children and adolescents. In some circumstances, stress and anxiety are beneficial as they can motivate and help one to become more productive, but when it becomes excessive, it impairs day-to-day life and leads to physical or psychological illnesses. Anxiety is a central nervous system disorder with emotional state, unpleasant in nature, associated with uneasiness, discomfort and concern or fear about some defined or undefined future threat.[1,2] Studies showed that a lifetime prevalence rate of anxiety is between 13.6% and 28.8% in Western countries and in 4.5% of the world population. It has been reported that the prevalence of anxiety in adult males in India is 24.4%.[3,4] Conventional pharmacotherapy is associated with side effects such as psychomotor impairment, sexual dysfunction and dependence liability. A number of studies have been done on anti-anxiety activity of medicinal plants, but none of isolated compound has been conceived in the form of a medicine due to lack of marker compound responsible for anti-anxiety activity and nonavailability of plant materials. Hence, this study has been planned to isolate a biomarker having anti-anxiety activity.

Citrus plants belonging to Rutaceae originated in Asia and are now cultivated all over the world. *Citrus* fruits are rich sources of bioactive compounds which show pharmacological activities such as antioxidant, antimicrobial, antitumor and anti-inflammatory activity[5] and thus *Citrus* family is one of the most commercially important horticultural plants. The volatile oils obtained from genus *Citrus* have been recommended and used for the treatment of anxiety. A quantum of literature reflects that *Citrus paradisi* has been widely employed in herbal medicine as well as aromatherapy and a significant preliminary

Address for correspondence: Dr. Vikas Gupta, University Centre of Excellence in Research, Baba Farid University of Health Sciences, Faridkot, Punjab, India.
E-mail: vikas_4308@rediffmail.com

Access this article online

Quick Response Code:

Website: www.ayujournal.org

DOI: 10.4103/ayu.AYU_173_17

How to cite this article: Gupta V, Sharma R, Bansal P, Kaur G. Bioactivity-guided isolation of potent anxiolytic compounds from leaves of *Citrus paradisi*. Ayu 2018;39:21-8.

work has already been carried out by authors and other researchers on the anxiolytic effects of the plant extracts.[6-11]

In this study, the authors intended to select four varieties of plant *C. paradisi* available worldwide and test the anti-anxiety activity of leaf extracts. This particular species has been selected because a negligible amount of work has been done on this species as compared to other species. Leaf has been selected for isolation of compounds because it can be made available at commercial scale. Hence, the present study was designed to isolate the biomarker compound responsible for anti-anxiety activity from leaves of different varieties of *C. paradisi*.

Materials and Methods

Collection and identification of plant material

The leaves of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* were procured and identified from cultivated source of Punjab Agricultural University Regional Centre, Abohar (Punjab, India) in the month of March 2012 and March 2013. The plant material was dried in shade.

Solvents

Chloroform, methanol, toluene, ethyl acetate and formic acid (Merck specialties limited, Mumbai) were employed for thin-layer chromatography. All solvents were of guaranteed reagent grade except chloroform and methanol (laboratory reagents grade) which were used for column chromatography after distilling under normal atmospheric pressure. Petroleum ether (60°C–80°C), chloroform and methanol (Merck specialties limited) all of LR grade, were employed for the extraction of plant material. Diazepam (Ranbaxy) was used as standard drug.

Animals

The experimental 216 animals (Swiss albino mice (20–25 gm) of either sex were procured from the Central Animal House, Akal College of Pharmacy and Technical Education, Sangrur. The animals were given standard laboratory feed and water *ad libitum*, both being withdrawn 12 h before experimentation. The experiments were performed between 6.00 am and 12.00 noon h. The experiments were conducted in a semi-soundproof laboratory. The biological studies were carried out as per the guidelines of Institutional animal ethical committee. The approval from the Institutional animal ethical committee was taken before carrying out biological studies vide letter number ATRC/05/13 dated 04/05/13.

Acute toxicity study

The acute toxicity studies were performed according to the OECD guidelines.[12] The selected animals were given methanol extract orally at a dose of 400, 800, 1200, 1600 and 2000 mg/kg body weight after overnight fast. The animals were observed for 7 days for morbidity and presentation of toxicity.

Preparation of extracts and fractions

Leaves of *C. paradisi* var. foster, *duncan*, *marsh seedless* and *star ruby* were dried in shade and powdered. One kilogram of powdered leaves were subjected to successive Soxhlet extraction by solvents in increasing order of polarity, namely, petroleum ether (60°C–80°C), chloroform, methanol and water. Before each extraction, the powdered material was dried in hot air-oven below 50°C. Each extract was concentrated by distilling off the solvent using rotary evaporator and then evaporating to dryness on water-bath and the dried extracts were preserved in vacuum desiccator.

Group I (vehicle)

Syrup + carboxymethyl cellouse (2%) was used as a vehicle for preparation of suspensions of various test doses of different extracts, fractions and bioactive constituents of all varieties.

Group II (standard drug)

Diazepam (2 mg/kg orally).

Group III–VI (test solutions)

The dried plant extracts of all varieties were separately suspended in the vehicle. Different doses, namely 50, 100, 200 and 400 mg/kg of each extract (petroleum ether, chloroform, methanol and water) were administered for 5-day per oral (p. o.) once daily and the last dose was given on the 5th day, 60 min prior to start the experiment. A similar protocol was followed for standard and control groups. The isolated compounds at the dose of 2, 5 and 10 mg/kg were tested using hole-board model and light dark model.

Preparation of ethyl acetate fraction and the residual methanol fraction

The active methanol extract of leaves of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* were suspended in water, placed in three-necked round bottom flask connected with Teflon stirrer and partitioned with ethyl acetate by heating (50°C) for 30 min with continuous stirring. The procedure was repeated five times. All ethyl acetate fractions (EAFs) were pooled and solvent was recovered using Buchi 461 rotary vacuum evaporator. Thus, two fractions were obtained – EAF and the residual methanol fraction (RMF) and each fraction was evaluated for anxiolytic activity using elevated plus maze (EPM).

Fractionation of ethyl acetate fraction and anti-anxiety activity of fractions

The bioactive EAF (35 g) of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* was loaded on a column packed with silica gel (#60–120) and eluted using chloroform/chloroform-methanol/methanol as the mobile phase. A total of 189, 201, 187 and 211 fractions of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* respectively, 200 ml each were collected. These were pooled based on similar thin-layer chromatograms, to get 8 (EAF_{1F}–EAF_{8F}), 9 (EAF_{1D}–EAF_{9D}), 12 (EAF_{1M}–EAF_{12M}) and 10 (EAF_{1S}–EAF_{10S}) subfractions which were evaluated for antianxiety activity at various doses (5, 10 and 20 mg/kg PO) using EPM apparatus.

Bioactive EAF_{5F} (6 g), EAF_{5D} (6 g), EAF_{5M} (6 g) and EAF_{5S} (6 g) of *foster*, *duncan*, *marsh seedles* and *star ruby*

fractions were subjected to column chromatography using silica gel (#230–400) and elution was done with petroleum ether, chloroform, and chloroform-methanol to get a total of 53, 65, 57, 63 fractions of EAF5 of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby*, respectively, 200 ml each were collected. These were pooled based on similar thin-layer chromatograms, to get 3, 4, 3 and 3 subfractions which were evaluated for anti-anxiety activity at various doses (5, 10 and 20 mg/kg PO) using EPM apparatus.

Bioactive fractions $EAF_{5F.3}$ (3 g), $EAF_{5D.3}$ (3 g), $EAF_{5M.3}$ (3 g), and $EAF_{5S.3}$ (3 g) of *foster*, *duncan*, *marsh seedles* and *star ruby* were again column chromatographed over silica gel (#230–400). Elution was done with petroleum ether, chloroform and chloroform-methanol. A total of 121, 103, 153 and 93 fractions of $EAF_{5.3}$ of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby*, respectively, 100 ml each were collected. These were pooled based on similar thin-layer chromatograms, to get 3, 3, 2, 2 subfractions which were evaluated for anti-anxiety activity at various doses (2, 5 and 10 mg/kg PO) using EPM apparatus.

$EAF_{5F.3.2}$, $EAF_{5D.3.2}$, $EAF_{5M.3.2}$, and $EAF_{5S.3.2}$ of *C. paradisi foster*, *duncan*, *marsh seedless* and *star ruby* were subjected to flash column chromatography and isolated the pure compound (Z1, Z2, Z3 and Z4) from all the varieties which was further evaluated for anxiolytic effect using hole-board model and light-dark model.

Characterization of isolated compounds

The melting point of Z1, Z2, Z3 and Z4 was determined and they were then subjected to spectroscopic analysis (ultraviolet [UV], infrared [IR], ^{1}H nuclear mass reactor [NMR] and mass spectroscopy) for structural elucidation. UV spectra were obtained on Perkin Elmer Hitachi 330 (lambda 15 UV/visible spectroscopy) spectroscopy. IR spectra of test sample as neat film was obtained on an IR spectrophotometer (multispoke fourier transform IR synthesis monitoring system, Perkin Elmer, Germany). ^{1}HNMR spectra were obtained on a Bruker spectrometer (Avane, Germany). Mass spectra was acquired in the positive ion mode on a mass spectrometer (Finnigan, USA) equipped with a pneumatically assisted atmospheric pressure chemical ionization.

Statistics

The anxiolytic activities of the fractions, diazepam and control were analyzed using analysis of variance (ANOVA) followed by Tukey's multiple range test/Dunnett's test. Difference was considered statistically significant at $P < 0.05$.

Results

Acute toxicity study

Acute oral toxicity studies revealed the nontoxic nature of different extracts of different varieties of *C. paradisi*. There was no morbidity observed or any profound toxic reactions found at a dose of up to 2000 mg/kg body weight, which indirectly reflects the safety profile of the plant extract.

Anti-anxiety activity of various extracts

The anxiolytic activity of various leaf extracts was evaluated using elevated plus maze model. Extracts of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* were screened for anxiolytic potential using EPM. Methanol extract of all the four varieties exhibited significant anxiolytic activity in mice at the dose of 100 mg/kg PO and the activity was comparable to that of diazepam. There was marked increased in time spent in open arms of the EPM with the administration of 100 mg/kg PO methanolic leaves extract of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* [Table 1].

The methanolic extract of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* was selected for bioactivity-guided fractionation in view to isolate anxiolytic compound(s). The fractionation of bioactive methanol extract of *C. paradisi var. foster*, *duncan*, *marsh seedless* and *star ruby* leaves yielded two fractions, that is, EAF and RMF which were further evaluated for anti-anxiety activity in mice using EPM apparatus. EAF of *C. paradisi* var. *foster*, *duncan*, *star ruby* and *marsh seedless* at the dose of 20 mg/kg PO using EPM showed anxiolytic activity comparable to diazepam [Table 2].

Column chromatography of ethyl acetate fraction

Bioactive EAF_{5F}, EAF_{5D}, EAF_{5M} and EAF_{5S} of *foster*, *duncan*, *marsh seedles* and *star ruby* at the dose of 20 mg/kg PO showed anxiolytic activity using EPM as comparable to diazepam [Table 3].

Table 1: Anti-anxiety activity of various extracts of leaves of *Citrus paradisi* using elevated plus maze apparatus

Group	Treatment	Average time spent in open arms (s) Methanol extract (mean±SEM)			
		Citrus paradisi var. *foster*	*Citrus paradisi* var. *duncan*	*Citrus paradisi* var. *marsh seedless*	*Citrus paradisi* var. *star ruby*
I	Vehicle	7.987±0.613	8.642±0.351	10.023±0.850	5.837±0.585
II	Diazepam	24.567±0.683*	25.502±0.684*	26.133±0.692*	22.158±0.963*
III	50 mg/kg	18.382±0.321*	18.560±0.549*	19.150±0.571*	18.527±0.729*
IV	100 mg/kg	21.750±0.243*	23.143±0.520*	25.050±0.369*	20.948±0.651*
V	200 mg/kg	18.218±0.226*	18.900±0.655*	18.155±0.326*	15.857±0.525*
VI	400 mg/kg	13.988±1.056*	13.470±0.951*	13.298±0.522*	11.492±0.628*

Values are mean±SEM (n=6); one-way ANOVA and Dunnett's multiple range test. ANOVA: Analysis of variance, SEM: Standard error of mean; *$P \leq 0.05$, *=Significant

Table 2: Anti-anxiety activity of ethyl acetate fraction and residual methanol fraction of different species of *Citrus paradisi* using elevated plus-maze

Group	Treatment	Average time spent in open arms (s)			
		Extracts		Control, mean±SEM	
		EAF	RMF	Negative	Positive
Citrus paradisi var. *foster*	Vehicle	-	-	7.987±0.613	-
	Diazepam	-	-	-	24.567±0.683*
	20 mg/kg	23.353±0.211*	18.743±0.219*	-	-
	40 mg/kg	22.891±0.112*	18.871±0.157*	-	-
Citrus paradisi var. *Duncan*	Vehicle	-	-	8.642±0.315	-
	Diazepam	-	-	-	25.502±0.684*
	20 mg/kg	25.153±0.613*	17.413±0.344*	-	-
	40 mg/kg	25.094±0.144*	18.432±0.195*	-	-
Citrus paradisi var. *marsh seedless*	Vehicle	-	-	10.023±0.850	-
	Diazepam	-	-	-	26.133±0.692*
	20 mg/kg	24.953±1.117*	18.321±0.193*	-	-
	40 mg/kg	24.876±0.214*	17.343±0.331*	-	-
Citrus paradisi var. *star ruby*	Vehicle	-	-	5.837±0.585	-
	Diazepam	-	-	-	22.158±0.963*
	20 mg/kg	21.976±0.613*	15.329±0.429*	-	-
	40 mg/kg	20.988±0.218*	17.343±1.331*	-	-

Values are mean±SEM (n=6); One-way ANOVA and Dunnett's multiple range test. EAF: Ethyl acetate fraction, RMF: Residual methanol fraction, SEM: Standard error of mean, ANOVA: Analysis of variance; *$P \leq 0.05$

Bioactive fraction $EAF_{5F.3}$, $EAF_{5D.3}$, $EAF_{5M.3}$ and $EAF_{5S.3}$ of *C. paradisi foster*, *duncan*, *marsh seedless* and *star ruby* at the dose of 20 mg/kg PO showed anxiolytic activity using EPM as comparable to diazepam [Table 4].

Bioactive fractions $EAF_{5F.3.2}$, $EAF_{5D.3.2}$, $EAF_{5M.3.2}$, and $EAF_{5S.3.2}$ of *C. paradisi foster*, *duncan*, *marsh seedless* and *star ruby* at the dose of 10 mg/kg PO at the dose of 10 mg/kg PO showed anxiolytic activity using EPM as comparable to diazepam [Table 5].

$EAF_{5F.3.2}$, $EAF_{5D.3.2}$, $EAF_{5M.3.2}$, and $EAF_{5S.3.2}$ of *C. paradisi foster*, *duncan*, *marsh seedless* and *star ruby* were subjected to flash column chromatography. This resulted in separation of 4 fractions which were then analyzed by TLC using chloroform: methanol (7:3). All four fractions were having different Rf values (F1, F2, F3, F4 of *C. paradisi foster*; D1, D2, D3, D4 of *C. paradisi Duncan*; M1, M2, M3, M4 of *C. paradisi marsh seedless*; and S1, S2, S3, S4 of *C. paradisi star ruby*). It was found out that F1, D1, M1, and S1 were having same Rf values, therefore, they were pooled together. Similarly F2, D2, M2, S2; F3, D3, M3, S3; and F4, D4, M4, S4 were pooled together. All fractions were run again in five random mobile phases and gave single spot, so they were deemed to be pure compounds and named as Z1, Z2, Z3 and Z4.

Characterization of isolated compounds

Z1: Color: Pale yellow powder; Melting Point: 241°C–242°C; Yield: 252 mg; UV_{max} (MeOH): 359 nm; IR: 3483 (O-H stretch), 2931 cm^{-1} (C-H stretch), 1669 cm^{-1} (C = O), 1504 cm^{-1} (C = C aromatic stretch), 1348 cm^{-1}, 1141 cm^{-1} (C-O-C); ^{1}H-NMR (400 MHz, DMSO solvent): δ 12.5 (C5-OH), 10.9 (C7-OH), 6.4 (d, 1H, J = 1.4, C8-H), 6.2 (m, 1H, J = 1.4, C6-H), 5.3 (s, 1H, J = 7.0, C1"-H), 4.3 (brs, 1H, C1'"-H), 3.0–3.7 (m, sugar protons), 1.0 (s, 3H, J = 6.0, C6'"-H); MS: [M + H] +=611, [M + H] +-Rhamnose=465, [M + H] +-Rhamnose-Glucose=303. This corresponds to $C_{27}H_{30}O_{16}$. The compound in fraction F1, D1, M1, S1 is identified as rutin and named as Z1.

Z2: Colour: Yellow powder; Melting Point: 314°C–315°C; Yield: 330 mg; UV_{max} (MeOH): 375 nm; IR: 3349 cm^{-1} (O-H stretch), 2899 cm^{-1} (C-H stretch), 1673 cm^{-1} (C = O), 1489 cm^{-1} (C = C stretch); ^{1}H-NMR (400 MHz, DMSO solvent): δ 6.85 (s, 1H, OH), δ 6.79 (s, 1H, OH), δ 6.54 (s, 1H, OH); MS: [M + H] +=302, [M + H-H2O] +=285, [M + H-CO] +=275, [M + H-H2O-CO] +=257.[M + H-H2O-2CO] +=229. This corresponds to $C_{15}H_{10}O_7$. The compound in fraction F2, D2, M2, S2 is identified as quercetin and named as Z2.

Z3: Colour: Yellow powder; Melting Point: 272°C–274°C; Yield: 267 mg; UV_{max} (MeOH): 260 nm; IR: 3467 cm^{-1} (O-H stretch), 2835 cm^{-1} (C-H stretch), 1679 cm^{-1} (C = O), 1573 and 1412 cm^{-1} (Aromatic ring); ^{1}H-NMR (400 MHz, DMSO solvent): 10.76 (s, 1H, 7-OH), 7.78 (s, 2H, H-2'), 7.45 (d, 1H, H-8), 7.24 (d, 2H, H-3' and H-5'), 5.35 (s, 1H, H-6); MS: [M + H] +=286. This corresponds to $C_{15}H_{10}O_6$. The compound in fraction F3, D3, M3, S3 is identified as Kaempferol and named as Z3.

Z4: Colour: Yellow crystals; Melting Point: 353°C–355°C; Yield: 264 mg; UV_{max} (MeOH): 375 nm; IR: 3384 cm^{-1} (O-H stretch), 1678 and 1631 cm^{-1} (C-O stretch), 1593 cm^{-1} (C-C stretch), 1536 cm^{-1} (Aromatic ring), 1359 and 1165 cm^{-1} (C-O-C stretch); ^{1}H-NMR (400 MHz, DMSO solvent): 6.3 (s, 2H, H-2′, 6′), 5.8 (s, 2H, H-8), 4.98 (m, 5H, H); MS: M+=318, [M + H-H2O] =301, [M + H-H2O-CO] +=273. This corresponds to $C_{15}H_{10}O_8$. The

Table 3: Antianxiety activity profile on elevated plus-maze apparatus of various subfractions obtained after column chromatography of ethyl acetate fraction

Group	Treatment	Dose (mg/kg, p.o.)	Average time spent in open arms (s), mean±SEM
Citrus paradisi var. *foster*	Control	Vehicle	7.987±0.613
	Diazepam	2	24.567±0.683*
	EAF5F	5	19.745±0.864*
		10	23.545±0.452*
		20	23.857±0.398*
Citrus paradisi var. *duncan*	Control	Vehicle	8.642±0.351
	Diazepam	2	25.502±0.684*
	EAF5D	5	19.214±0.655*
		10	22.942±0.880*
		20	24.989±0.132*
Citrus paradisi var. *marsh seedless*	Control	Vehicle	10.023±0.850
	Diazepam	2	26.133±0.692*
	EAF5M	5	22.340±0.139*
		10	24.878±0.712*
		20	25.712±0.534*
Citrus paradisi var. *star ruby*	Control	Vehicle	5.837±0.585
	Diazepam	2	22.158±0.963*
	EAF5S	5	18.426±0.491*
		10	21.103±0.012*
		20	21.988±0.311*

Values are mean±SEM (n=6); one-way ANOVA and Dunnett's multiple range test. SEM: Standard error of mean, ANOVA: Analysis of variance, EAF: Ethyl acetate fraction, p.o.: per oral ; *P≤0.05

Table 4: Anti-anxiety activity profile on elevated plus maze apparatus of various subfractions obtained after column chromatography of ethyl acetate fraction

Group	Treatment	Dose (mg/kg, p.o.)	Average time spent in open arms (s), mean±SEM
Citrus paradisi var. *foster*	Control	Vehicle	7.987±0.613
	Diazepam	2	24.567±0.683*
	EAF5F.3	5	20.482±0.351*
		10	22.343±0.543*
		20	24.063±0.661*
Citrus paradisi var. *duncan*	Control	Vehicle	8.642±0.351
	Diazepam	2	25.502±0.684*
	EAF5D.3	5	22.892±0.373*
		10	23.839±0.763*
		20	24.331±0.836*
Citrus paradisi var. *marsh seedless*	Control	Vehicle	10.023±0.850
	Diazepam	2	26.133±0.692*
	EAF5M.3	5	22.944±0.196*
		10	23.937±0.714*
		20	24.984±0.999*
Citrus paradisi var. *star ruby*	Control	Vehicle	5.837±0.585
	Diazepam	2	22.158±0.963*
	EAF5S.3	5	18.347±0.453*
		10	20.987±0.446*
		20	22.033±0.345*

Values are mean±SEM (n=6); one-way ANOVA and Dunnett's multiple range test. SEM: Standard error of mean, ANOVA: Analysis of variance, EAF: Ethyl acetate fraction; p.o.: per oral; *P≤0.05

compound in fraction F4, D4, M4, S4 is identified as Myricetin and named as Z4 [Figure 1].

Confirmation of anti-anxiety activity of isolated compounds

The anti-anxiety activity of isolated compounds was further confirmed using hole-board model and light dark test.

Hole-board model

The number of line crossing and head dipping was increased significantly in case of diazepam-treated animals as compared to the control animals. All the isolated compounds Z1, Z2, Z3 and Z4 showed an increase in the number of line crossing and head dipping significantly and the result were similar as that of standard drug [Tables 6 and 7].

Light-dark model

The time spent in lit box was increased significantly in case of 2, 5 and 10 mg/kg dose of isolated compounds Z1, Z2, Z3 and Z4 and the result was comparable to the standard drug, diazepam [Table 8].

Discussion

Anxiety disorders are serious medical illnesses that have affected one-eighth of the total population worldwide

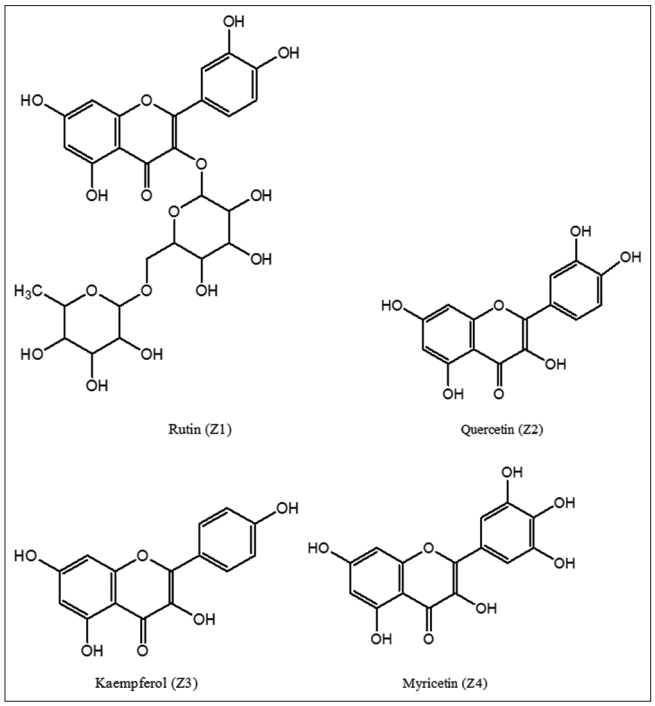

Figure 1: Structure of the isolated compounds

irrespective of gender, age, religion, nationality and profession.[13] Mental disorder has attracted the attention of researchers toward various pharmacotherapeutic approaches.[14] Benzodiazepines (BZDS) are used as a first line of treatment. Today, at least 20 million people worldwide are prescribed these "minor tranquilizers." Regular use of BZDs causes deterioration of cognitive functioning, addiction, physical dependence and tolerance.[15] Due to adverse effects associated with the synthetic drugs, researchers have been exploring natural resources based on traditional systems of medicine to come across safer and effective drugs.[16]

Selection of plants, that is, *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* have a long history of use in alternative and traditional system of medicine and similar species in Ayurvedic pharmaco-therapeutics for the treatment of mental disorders. Leaves of different varieties of *C. paradisi* have never been evaluated for their anti-anxiety potential using standard protocols. Hence, it was considered worthwhile to investigate anti-anxiety potential of the *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* and to isolate the components responsible for this activity.

Various extracts of leaves of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* were obtained by successive exhaustive extraction of the plant material using solvents in increasing order of polarity, namely, petroleum ether, chloroform, methanol and water. The rationale for using these solvents was to separate plant constituents on the basis of polarity.

The EPM is a novel, effective, cheap and simple method, requires no preliminary training of experimental animals and

Table 5: Anti-anxiety activity profile on elevated plus maze apparatus of various subfractions obtained through column chromatography of ethyl acetate fraction

Group	Treatment	Dose (mg/kg, p.o.)	Average time spent in open arms (s), mean±SEM
Citrus paradisi var. *foster*	Control	Vehicle	7.987±0.613
	Diazepam	2	24.567±0.683*
	EAF5F.3.2	2	18.933±0.113*
		5	20.328±0.361*
		10	23.842±0.111*
Citrus paradisi var. *duncan*	Control	Vehicle	8.642±0.351
	Diazepam	2	25.502±0.684*
	EAF5D.3.2	2	19.334±0.357*
		5	21.364±0.466*
		10	23.974±0.713*
Citrus paradisi var. *marsh seedless*	Control	Vehicle	10.023±0.850
	Diazepam	2	26.133±0.692*
	EAF5M.3.2	2	19.484±0.376*
		5	22.494±0.931*
		10	25.776±0.472*
Citrus paradisi var. *star ruby*	Control	Vehicle	5.837±0.585
	Diazepam	2	22.158±0.963*
	EAF5S.3.2	2	16.946±0.469*
		5	19.848±0.887*
		10	22.073±0.487*

Values are mean±SEM (n=6); one-way ANOVA and Dunnett's multiple range test. SEM: Standard error of mean, ANOVA: Analysis of variance, EAF: Ethyl acetate fraction; p.o.: per oral; *$P \leq 0.05$

does not cause much discomfort to them while handling. The fear due to height (acrophobia) induces anxiety, measured by time spent by mice in open arms of the EPM. Anxiolytic compounds, by decreasing anxiety, increasing the open-arm exploration time; anxiogenic compounds have the opposite effect.[17] Mice constitute an invaluable tool for modeling human anxiety in its various forms as these display remarkable similarities on anatomical, physiological, biochemical, molecular and behavioral levels.[18] In the study, standard values, for mean time spent in open arms of EPM, of the control (vehicle) and

Table 6: Anti-anxiety activity of isolated compounds in hole-board model (head dipping)

Groups	Treatment	Number of head dipping					
		Isolated compound				Controls	
		Z1	Z2	Z3	Z4	Negative	Positive
I	Vehicle					22.3±1.05	
II	Diazepam 2 mg/kg PO						42.6±2.43*
III	2 mg/kg	30.7±0.54*	24.9±0.12	31.4±0.42	29.4±0.47		
IV	5 mg/kg	34.2±0.73*	36.4±0.32*	35.4±0.98*	36.8±0.72*		
V	10 mg/kg	42.1±0.74*	41.4±0.45*	40.2±0.56*	42.4±0.48*		

Values are mean±SEM (n=6); one-way ANOVA and Dunnett's multiple range test. SEM: Standard error of mean, ANOVA: Analysis of variance; *P≤0.05

Table 7: Anti-anxiety activity of isolated compounds in hole-board model (line crossing)

Groups	Treatment	Number of line crossing					
		Isolated compound				Controls	
		Z1	Z2	Z3	Z4	Negative	Positive
I	Vehicle					129.3±2.06	
II	Diazepam 2 mg/kg PO						181.0±3.65*
III	2 mg/kg	154.4±0.42*	140.3±0.73	143.0±0.37	149.4±0.74*		
IV	5 mg/kg	167.8±0.32*	156.8±0.93*	149.3±0.98*	163.8±0.30*		
V	10 mg/kg	180.3±0.73*	178.5±1.37*	176.8±1.34*	182.8±1.43*		

Values are mean±SEM (n=6); one-way ANOVA and Dunnett's multiple range test. SEM: Standard error of mean, ANOVA: Analysis of variance; *P≤0.05

Table 8: Anti-anxiety activity of isolated compounds in light-dark model

Groups	Treatment	Time spent in lit box (s/5 min)					
		Isolated compound				Controls	
		Z1	Z2	Z3	Z4	Negative	Positive
I	Vehicle					2.8±1.69	
II	Diazepam 2 mg/kg PO						22.3±2.69*
III	2 mg/kg	12.4±0.71*	13.4±0.47*	15.7±0.73*	10.8±0.48*		
IV	5 mg/kg	19.4±0.53*	15.8±0.48*	16.1±0.81*	13.3±0.23*		
V	10 mg/kg	23.4±0.45*	24.4±0.77*	19.9±0.46*	20.4±0.63*		

Values are mean±SEM (n=6); one-way ANOVA and Dunnett's multiple range test. SEM: Standard error of mean, ANOVA: Analysis of variance; *P≤0.05

the standard (diazepam) groups of mice were initially generated using 12 mice in each group – a number of two times the number (6 mice) used for the test group. These values were then used as reference in all the subsequent anti-anxiety evaluations of the test samples. It is also observed that even at the dose of 2000 mg/kg, PO, no apparent toxic/adverse effect was observed with any of the leaves extracts in the study. Methanol extract of *C. paradisi* exhibited significant anxiolytic activity in mice at the dose of 100 mg/kg PO and the activity was comparable to that of diazepam. However, the activity decreased at higher dose which might be due to mild sedation. The methanol extract of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* was selected for bioactivity-guided fractionation with a view to isolate anxiolytic compound(s).

In an attempt to separate the contents of bioactive methanol extract of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* exhibiting significant anxiolytic activity, it was fractionated by shaking with ethyl acetate. Among the two fractions, namely, EAF and the RMF, only EAF of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby* at a dose of 20 mg/kg PO exhibited significant anxiolytic activity using EPM model.

The bioactive EAF of *C. paradisi var. foster*, *duncan*, *marsh seedless* and *star ruby* was subjected to column chromatography which yielded different fractions. $EAF_{5F.3.2}$, $EAF_{5D.3.2}$, $EAF_{5M.3.2}$ and $EAF_{5S.3.2}$ of *C. paradisi foster*, *duncan*, *marsh seedless* and *star ruby* were subjected to flash column chromatography using chloroform: methanol (80:20) yielded four pure isolates Z1, Z2, Z3 and Z4 named rutin, quercetin, kaempferol, and myricetin, respectively. The anti-anxiety activity of isolated compounds Z1, Z2, Z3 and Z4 was further confirmed using hole-board model and light-dark test.

Flavonoids and terpenoids act as antianxiety agents by modulating the gamma-aminobutyric acid (GABA) receptors similar to that of benzodiazepines, thereby increasing the frequency of chloride channel opening and resulting in the neuronal hyperpolarization.[19-21] In addition to the receptor modulating activity, flavonoids also act as antioxidant agents because of their hydrogen donating ability, which ultimately result in neuroprotective role.[22,23] As the phytochemical, chromatographic and spectroscopic investigation has shown the presence of various flavonoids in different varieties of *C. paradisi*, therefore, they are potential candidates for the treatment of anxiety disorders, as justified by the results of various animal models of anxiety.

Conclusion

In light of the above findings, it is concluded that rutin, quercetin, kaempferol and myricetin are responsible for the antianxiety activity of leaves of *C. paradisi* var. *foster*, *duncan*, *marsh seedless* and *star ruby*. However, further studies are needed to determine the exact mechanism by which these phytochemical constituents isolated from *C. paradisi* interact with GABA or other receptors to exert the antianxiety activity.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Evans DL, Charney DS, Lewis L, Golden RN, Gorman JM, Krishnan KR, *et al.* Mood disorders in the medically ill: Scientific review and recommendations. Biol Psychiatry 2005;58:175-89.
2. Gureje O, Von Korff M, Simon GE, Gater R. Persistent pain and well-being: A World health organization study in primary care. JAMA 1998;280:147-51.
3. Raakhee AS, Aparna N. A study on the prevalence of anxiety disorders among higher secondary students. GESJ Educ Sci Psychol 2011;1:33-7.
4. Sahoo S, Khess CR. Prevalence of depression, anxiety, and stress among young male adults in India: A dimensional and categorical diagnoses-based study. J Nerv Ment Dis 2010;198:901-4.
5. Mabberley DJ. *Citrus* (Rutaceae): A review of recent advances in etymology, systematics and medical applications. Blumea 2004;49:481-98.
6. Gupta V, Bansal P, Kumar P, Shri R. Anxiolytic and antidepressant activities of different extracts from *Citrus paradisi* var. foster. J Pharm Res 2009;2:1864-6.
7. Gupta V, Bansal P, Kumar P, Shri R. Anxiolytic and antidepressant activities of different extracts from *Citrus paradisi* var. duncan. Asian J Pharm Clin Res 2010;3:98-100.
8. Gupta V, Bansal P, Kumar P, Kaur G. Pharmacopoeial standards and pharmacognostical studies of leaves of *Citrus paradisi* var. foster. Res J Pharmacognosy Phytochem 2010;2:140-3.
9. Gupta V, Bansal P, Niazi J, Kaur G. Anti-anxiety activity of *Citrus paradisi* var. star ruby extracts. Int J Pharm Tech Res 2011;2:1655-7.
10. Gupta V, Ghaiye P, Bansal P, Shri R. Pharmacopoeial standards and pharmacognostical studies of leaves of *Citrus paradisi* var. duncan. J Pharm Res 2011;4:1084-6.
11. Gupta V, Bansal P, Kohli K, Ghaiye P. Development of economic herbal based drug substitute from *Citrus paradisi* (Grape fruit) for existing anti-anxiety drug modules. Nat Prod Chem Res 2014; S1:001. [doi: 10.4172/2329-6836.S1-001].
12. OECD. OECD Guidelines for Testing of Chemicals. Health effects: Test No. 425: Acute Oral Toxicity: Up and Down Procedure. Sec. 4. France: OECD Publishing; 2006. [Last accessed on 2017 Jun 25].
13. NIMH. Statistics. National Institute of Mental Health, Office of Communications and Public Liaison. Bethesda, Maryland; 2011. Available from: http://www.nimh.nih.gov/statistics/index.shtml. [Last accessed on 2016 Jun 25].
14. Riaza Bermudo-Soriano C, Perez-Rodriguez MM, Vaquero-Lorenzo C, Baca-Garcia E. New perspectives in glutamate and anxiety. Pharmacol Biochem Behav 2012;100:752-74.
15. Magnani P, Conforti A, Zanolin E, Marzotto M, Bellavite P. Dose-effect study of *Gelsemium sempervirens* in high dilutions on anxiety-related responses in mice. Psychopharmacology (Berl) 2010;210:533-45.
16. Dhawan BN. Centrally acting agents from Indian plants. In: Koslow SH, Murthy RS, Coelho GV, editors. Decade of the Brain. Rockville, MD: National Institute of Mental Health; 1995. p. 197-202.
17. Vogel HG. Drug Discovery and Evaluation Pharmacological Assays. 2nd ed. Germany: Springer-Verlag; 2002.
18. Hohoff C. Anxiety in mice and men: A comparison. J Neural Transm (Vienna) 2009;116:679-87.
19. Hanrahan JR, Chebib M, Johnston GA. Flavonoid modulation of GABA(A) receptors. Br J Pharmacol 2011;163:234-45.
20. Johnston GA. GABA(A) receptor channel pharmacology. Curr Pharm Des 2005;11:1867-85.
21. Edewor-Kuponiyi TI. Plant-derived compounds with potential sedative and anxiolytic activities. Int J Basic Appl Sci 2013;2:63-78.
22. Sandhar HK, Kumar B, Prasher S, Tiwari P, Salhan M, Sharma P. A review of phytochemistry and pharmacology of flavonoids. Int Pharm Sci 2011;1:25-41.
23. Sen S, Chakraborty R. Revival, modernization and integration of Indian traditional herbal medicine in clinical practice: Importance, challenges and future. J Tradit Complement Med 2017;7:234-44.