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Development of Economic Herbal Based Drug Substitute from *Citrus paradisi* (Grape fruit) for Existing Anti-anxiety Drug Modules

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Abstract

Persistent and unrelenting stress often leads to anxiety and unhealthy behavior. Benzodiazepines are used as a first line of treatment however difficulties with pharmacotherapy of anxiety disorders such as dependence and low response rate encourage researchers to find new approaches. A number of studies have been done on anti-anxiety activity of medicinal plants but major constraint is non suitability of the tested plant material for human use and non availability of plant materials in bulk at economical rates. This study entails to development of safe anti-anxiety economic drug of easy availability. In normal course aromatic oils from plant are being used however authors selected four varieties of plant *Citrus paradisi* available worldwide and tested the anti-anxiety activity of leaf extracts as leaf extracts can be made available at commercial scale. All the selected varieties have demonstrated a potential diazepam like effect in methanolic extracts at a dose of 100 mg/kg body weight using elevated plus maze model. The results strongly justify the use of *Citrus paradisi* leaf extracts for treatment of anxiety. Further studies are in progress to find out active component involved in the anti-anxiety effect.

Keywords: Anxiety; *Citrus paradise;* Diazepam; Elevated plus maze

Introduction

Stress and anxiety are common psychiatric manifestations of the modern world and lifestyles. In small quantities, stress and anxiety are good; they can motivate and help one be more productive. However, too much stress, or a strong response to stress, is harmful. It can set up for general poor health as well as specific physical or psychological illnesses like infection, heart disease, or depression. Persistent and unrelenting stress often leads to anxiety and unhealthy behaviors. 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].

Therapies available and limitations

Benzodiazepines are used as a first line of treatment. Today, at least 20 million people worldwide are prescribed these "minor tranquilizers." Meanwhile, Western European and North American countries are facing epidemic levels of citizens hooked on these drugs. While benzodiazepines are a disaster in terms of the public health, pharmaceutical companies rake in a whopping $21 (€ 14 billion) billion a year selling them. So, difficulties with pharmacotherapy of anxiety disorders such as dependence and low response rate encourage researchers to find new approaches [3].

Alternative therapies

From the past, the role of medicinal plants has been a subject of intense interest. A number of studies have been done on anti-anxiety activity of medicinal plants but major constraint towards development of a marketable formulation is non suitability of the tested plant material for human use and non availability of plant materials in bulk at economical rates. This study entails to development of safe anti-anxiety economic drug of easy availability. In normal course aromatic oils from plant are being used for antianxiety activity but the cost of aromatic oils and its availability and cumbersome administration procedure creates a limitation for a market product. At the same time aromatic oils can be used topically or for inhalation purposes whereas leaf extracts can be a better medium for formulation development which can be easily administered.

Citrus fragrances have been particularly attributed with mood enhancing properties by aroma therapists. The volatile oils obtained from genus *Citrus* (*Citrus paradisi*) have been recommended and used for the treatment of anxiety. A review of literature also reflects that *Citrus paradisi* is widely employed in herbal medicine and aromatherapy and significant work has already been reviewed and carried out by authors on the anxiolytic effects of the plant extracts [4-10].

In this study authors intend to select four varieties of plant *Citrus paradisi* available worldwide and test the anti-anxiety activity of leaf extracts as leaf extracts can be made available at commercial scale. So, the present study was designed to develop an economic herbal based drug substitute for existing anti-anxiety drug modules using different varieties of different extracts of *Citrus paradisi* using the EPM, an exteroceptive behavior animal model.

Materials and Methods

Plant material

The leaves of *Citrus paradisi* of four different varieties i.e *duncan, foster, marshseedless* and *star ruby* were procured from a identified and cultivated source: Punjab Agricultural University, Regional Centre, Abohar (Punjab, India) in the month of March-April, 2013.

Preparation of extracts

Leaves of different varieties were dried in shade and powdered. The powdered leaves (100 g) were subjected to successive Soxhlet extraction by solvents in increasing order of polarity viz. petroleum ether (60-

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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 and then evaporating to dryness on the water-bath.

Test animals

The experimental animals [Swiss albino mice (20-30 g) of either sex] were procured from the approved animal House, Akal College of Pharmacy and Technical Education, Mastuana Sahib, Sangrur. (IAEC No. ATRC/05/13). The animals were given standard laboratory feed and water ad libitum. The experiments were performed between 6.00 am to 11.00 am. The experiments were conducted in a semi-sound proof laboratory. All the experimental procedures and protocols used in the study were duly reviewed and approved by the Institutional Animal Ethics Committee.

Anti-anxiety activity

Animals were divided into six (I-VI) groups for each variety of source plant.

For each variety

1. Group I was a negative control and administered only vehicle (consisting of simple syrup IP and carboxy methyl cellulose (5%).
2. Group II was a positive control and was given standard drug, diazepam (2 mg/kg, orally), suspended in the vehicle.
3. Group III-VI were treated as test groups and were given petroleum ether (60-80°C), chloroform, methanol and water extracts of different varieties from the leaves of *Citrus paradisi* at different doses viz. 50, 100, 200 and 400 mg/kg body weight respectively. Mice were treated orally with all the test solutions, standard drug and control for 5 days once daily at a stipulated time and last dose was given on the 5th day, 45 minutes prior to study.

Elevated plus maze model (EPM)

The elevated plus-maze model is well established animal model for testing anxiolytic drugs. The elevated plus-maze apparatus consist of two open arms (16×5 cm for mice and 50×10 cm for rats), two closed arms (16×5×12 cm for mice and 50×10×40 cm for rats), and an open roof with the entire maze elevated (25 cm for mice and 50 cm for rats) from the floor. The animals were placed individually in the centre of the maze, head facing towards open arms [11,12].

Statistical analysis

The anxiolytic activities of the extracts, diazepam and control were analyzed by one-way analysis of variance (ANOVA). The test groups were compared with standard/control by Dunnett Multiple Range Test. Difference were considered significant at $p<0.05$.

Results and Discussion

Acute toxicity study

Acute oral toxicity studies revealed the non-toxic nature of different extracts of different varieties of *Citrus 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

The results obtained from the EPM model, indicate that methanolic extract of all the varieties showed significant ($p<0.05$) anti anxiety activity as compared to control and was almost at par with standard drug diazepam. At 50 mg/kg of body weight there was an increase in the time spent in open arms when the mice were administered leaf extracts from all the varieties. The average time spent in open arms further increased from 7.987 ± 0.613 (sec) in control to 21.750 ± 0.243 (sec) in methanolic extract at a dose of 100 mg/kg of *Citrus paradisi var. foster*, from 8.642 ± 0.351 (sec) in control to 23.143 ± 0.520 (sec) in *Citrus paradisi var. duncan*., from 10.023 ± 0.850 (sec) in control to 25.050 ± 0.369 (sec) *Citrus paradisi var. marshseedless* and from 5.837 ± 0.585 (sec) in control to 20.948 ± 0.651 (sec) in *Citrus paradisi var. staruby*. At a higher dose of 200 mg/kg the leaf extracts demonstrated

Group	Treatment	Average time spent in open arms (in sec)					
		Extracts				Control	
		Petroleum ether (Mean ± SEM)	Chloroform (Mean ± SEM)	Methanol (Mean ± SEM)	Aqueous (Mean ± SEM)	Negative (Mean ± SEM)	Positive (Mean ± SEM)
I	Vehicle	-	-	-	-	7.987 ± 0.613	-
II	Diazepam	-	-	-	-	-	24.567 ± 0.683*
III	50 mg/kg	13.258 ± 0.269*	16.742 ± 0.331*	18.382 ± 0.321*	13.422 ± 0.820*	-	-
IV	100 mg/kg	13.516 ± 0.490*	17.940 ± 0.330*	21.750 ± 0.243*	12.122 ± 0.759*	-	-
V	200 mg/kg	11.425 ± 0.298	14.345 ± 0.691*	18.218 ± 0.226*	14.685 ± 0.354*	-	-
VI	400 mg/kg	10.887 ± 0.217	11.910 ± 0.437	13.988 ± 1.056*	11.197 ± 0.997	-	-

Values are Mean ± SEM (n=6); One way ANOVA and Dunnett multiple range test. *p<0.05 compared to control

Table 1: Anti-anxiety activity of various extracts of leaves of *Citrus paradisi var. foster* using EPM.

Group	Treatment	Average time spent in open arms (in sec)					
		Extracts				Control	
		Petroleum ether (Mean ± SEM)	Chloroform (Mean ± SEM)	Methanol (Mean ± SEM)	Aqueous (Mean ± SEM)	Negative (Mean ± SEM)	Positive (Mean ± SEM)
I	Vehicle	-	-	-	-	8.642 ± 0.351	-
II	Diazepam	-	-	-	-	-	25.502 ± 0.684*
III	50 mg/kg	11.987 ± 0.568	16.583 ± 0.463*	18.560 ± 0.549*	14.060 ± 1.065*	-	-
IV	100 mg/kg	15.113 ± 0.714*	15.137 ± 0.649*	23.143 ± 0.520*	15.437 ± 0.812*	-	-
V	200 mg/kg	13.818 ± 0.412*	15.078 ± 0.681*	18.900 ± 0.655*	13.837 ± 0.546*	-	-
VI	400 mg/kg	10.895 ± 0.434	10.747 ± 0.622	13.47 ± 0.951*	10.753 ± 0.828	-	-

Values are Mean ± SEM (n=6); One way ANOVA and Dunnett multiple range test. *p<0.05 compared to control

Table 2: Anti-anxiety activity of various extracts of leaves of *Citrus paradisi var. duncan* using EPM.

Group	Treatment	Average time spent in open arms (in sec)					
		Extracts				Control	
		Petroleum ether (Mean ± SEM)	Chloroform (Mean ± SEM)	Methanol (Mean ± SEM)	Aqueous (Mean ± SEM)	Negative (Mean ± SEM)	Positive (Mean ± SEM)
I	Vehicle	-	-	-	-	10.023 ± 0.850	-
II	Diazepam	-	-	-	-	-	26.133 ± 0.692*
III	50 mg/kg	13.165 ± 0.568*	16.887 ± 0.561*	19.150 ± 0.571*	15.322 ± 0.700*	-	-
IV	100 mg/kg	16.690 ± 0.461*	18.328 ± 0.392*	25.050 ± 0.369*	18.695 ± 0.695*	-	-
V	200 mg/kg	13.728 ± 0.541*	15.772 ± 0.227*	18.155 ± 0.326*	14.920 ± 0.396*	-	-
VI	400 mg/kg	11.003 ± 0.414	11.378 ± 0.379	13.298 ± 0.522*	12.735 ± 0.677	-	-

Values are Mean ± SEM (n=6); One way ANOVA and Dunnett multiple range test. *p<0.05 compared to control

Table 3: Anti-anxiety activity of various extracts of leaves of *Citrus paradisi var. marshseedless* using EPM.

Group	Treatment	Average time spent in open arms (in sec)					
		Extracts				Control	
		Petroleum ether (Mean ± SEM)	Chloroform (Mean ± SEM)	Methanol (Mean ± SEM)	Aqueous (Mean ± SEM)	Negative (Mean ± SEM)	Positive (Mean ± SEM)
I	Vehicle	-	-	-	-	5.837 ± 0.585	-
II	Diazepam	-	-	-	-	-	22.158 ± 0.963*
III	50 mg/kg	11.218 ± 0.648*	16.430 ± 0.891*	18.527 ± 0.729*	15.207 ± 0.481*	-	-
IV	100 mg/kg	15.840 ± 0.743*	18.292 ± 0.695*	20.948 ± 0.651*	14.598 ± 0.774*	-	-
V	200 mg/kg	13.030 ± 0.839*	12.848 ± 0.885*	15.857 ± 0.525*	15.633 ± 0.400*	-	-
VI	400 mg/kg	7.953 ± 0.400	10.995 ± 0.717*	11.492 ± 0.628*	8.758 ± 0.941	-	-

Values are Mean ± SEM (n=6); One way ANOVA and Dunnett multiple range test. *p<0.05 compared to control

Table 4: Anti-anxiety activity of various extracts of leaves of *Citrus paradisi var. starruby* using EPM.

a sedative effect in all the varieties which further accentuated at a dose of 400 mg/kg of body weight. Results obtained have been presented in Tables 1-4.

The fear due to height induces anxiety in the animals when placed on the EPM. The ultimate manifestation of anxiety and fear in the animals is exhibited by decrease in the motor activity and preference to remain at safer places. Anxiolytic agents are expected to increase the motor activity, which is measured by the time spent by the animal in the open arms [13]. The methanol extract of four varieties of *Citrus paradisi* (100 mg/kg), markedly increased the percentage of average time spent by the animals in the open arms. The anxiolytic effect of the plant extract was more prominent at 100 mg/kg and doses higher or lower than this did not show a consistent anxiolytic effects. The lack of significant anxiolytic effects at doses higher than 100 mg/kg could be due to strong sedative properties of the plant extracts. Lower doses (less than 100 mg/kg) of the plant extract did not show any significant anxiolytic effects. The anxiolytic effects of methanolic extract of four varieties of *Citrus paradisi* may be related to their flavonoid content. The extracts from the plant shows the presence of flavonoids and the flavonoids exert anti-anxiety activity through GABA receptors. In the CNS several flavones bind to the benzodiazepine site on the GABA A receptor resulting in sedation, anxiolytic or anti-convulsive effects. Flavonoids of several classes are inhibitors of monoamine oxidase A or B, thereby working as anti-depressants or to improve the conditions of Parkinson's patients. Flavonoids with anxiolytic activity have been described in many plant species used in folk medicine such as *Passiflora coerulea* [14]. This effect has been attributed to the affinity of flavonoids for the central benzodiazepine receptors [15-17]. In another study a sedative effect on the central nervous system has been shown for quercetrin and isoquercetin glycosides in mice [18-20]. However, further studies are being carried out to identify the phytoconstituent responsible for the observed anxiolytic effect of methanol extract at dose 100 mg/kg. Natural herbs/herbal mixtures that act synergistically promise to provide an effective remedy for anxiety.

Conclusion

There is a paradigm shift towards use of herbal remedies or herbal based formulations. The role of medicinal plants in disease prevention and treatment has always been remarkable. This study was conducted with an aim towards develop of safe anti-anxiety economic drug of easy availability. In normal course aromatic oils from plant are being used however authors selected four varieties of plant *Citrus paradisi* available worldwide and tested the anti-anxiety activity of leaf extracts as leaf extracts can be made available at commercial scale. All the selected varieties have demonstrated a potential diazepam like effect that strongly justify the use of *Citrus paradisi* leaf extracts for treatment of anxiety in human trials. Further studies are in progress to find out active component involved in the anti-anxiety effect. Synthetic drugs and medications possess enormous side effects whereas toxicity studies of our proposed extract have proven to be safe, so these herbs with a wide therapeutic applicability promise to alleviate anxiety with very few adverse effects.

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