POSSIBLE ANXIOLYTIC PROFILE OF AQUEOUS FRUIT EXTRACTS OF A MEDICINAL PLANT SEA BUCKTHORN (HIPPOPHAE RHAMNOIDES L. SPP. TURKESTANICA) IN EXPERIMENTAL MODELS

FARHAT BATOOL1, ASAD HUSSAIN SHAH2, SYED DILNAWAZ AHMED2, ZAFAR SAEID SAIFY3 AND DARAKHSHAN JABEEN HALEEM1

1Neurochemistry and Biochemical Neuropharmacology Research Laboratory, Department of Biochemistry, University of Karachi, Karachi-75270. Pakistan, 2Department of Plant Breeding and Molecular Genetics, Faculty of Agriculture, Rawalakot, Azad Jammu and Kashmir Pakistan, 3International Centre for Chemical and Biological Sciences, H.E.J. Research Institute of Chemistry, University of Karachi.

Abstract

The present study was designed to examine possible anxiolytic profile of aqueous fruit extracts of a medicinal plant Sea buckthorn (Hipppophae rhamnoides L. spp. Turkestanica) in experimental animal models. Sea buckthorn (SBT) is a very potent medicinal and multipurpose plant which has gained global significance due to its biochemical and nutritional utility in folk medicine. Diazepam is a benzodiazepine with CNS depressant properties and a sedative-hypnotic drug traditionally used to treat anxiety. Animal models for anxiety-related behavior are based on the assumption that anxiety in animals is comparable to anxiety in humans. To unravel neurobiological mechanisms underlying normal anxiety as well as its pathological variations, animal models are indispensable tools. In this investigation rats were treated with the aqueous fruit extracts of Sea buckthorn (SBT-FE) (20 and 40 mg/kg P.O.) and diazepam at doses of 3.0mg/kg I.P. 1 hr before introducing the groups of animals to various experimental models of anxiety. Anti anxiety activity was evaluated using elevated plus maze (EPM), light-dark model (LDM) and open field test (OFT). Results revealed that in elevated plus maze, treatment with aqueous extracts of SBT-FE increased the time spent in open arm and total locomotion time in aversive environment. In light-dark model treatment with these extracts showed significant (p<0.01) increases in time spent in lit-box and in open field test treatment with SBT-FE exhibited significant increases in the exploratory activity and latency time as compared to controls. The results indicate that aqueous SBT-FE is an effective anxiolytic agent and could be useful in primary medical care.

Introduction

Medicinal and nutritional applications of the supernatural plant Sea Buckthorn (Hipppophae rhamnoides L. spp. Turkestanica) are well-known in Asia, Europe and North America (Beveridge et al., 2002; Tiitinen et al., 2005; Shah et al., 2007; Batool et al., 2009) since last decade. Fruit extracts from Sea buckthorn (Hipppophae rhamnoides L. spp. Turkestanica) seeds and berries have been customarily used in the treatment of different clinical and psychotic disorders and have large implication in modern medicinal psychotherapy (Batool et al., 2009; Li & Schroeder, 1996; Zeb, 2004). Sea buckthorn berry enriches nutritional components and contains total soluble sugar (totally fructose and glucose), organic acid and various vitamins, for example carotene, Vitamin C, Vitamin E, Vitamin B1, Vitamin B2 etc. Fruit extract contains many kinds of microelements people needed as a food supplement for better health and mental performance (Figs. 1 & 2).

1Corresponding author E-mail: batool@uok.edu.pk; batool_fb@yahoo.com Mobile: 03333097217
Fig. 1. Sea buckthorn (*Hippophae rhamnoides* L. spp. *Turkestanica*) fresh fruit berries collected from Northern areas (Hussainabad, Sakardu) of Pakistan used for the fruit extract preparation and P.O. (per os) administration in the experiment.

Fig. 2. Aqueous fruit extract of Sea buckthorn (*Hippophae rhamnoides* L. spp. *Turkestanica*) berries (SBT-FE) in pure (100%) form used for P.O. administration in different dilutions (20mg/kg & 40mg/kg) during experiment.
The treatment of anxiety is also one of the leading problems in medicine today. Anxiety is a normal reaction but when it is severe and disabling it becomes pathological. Anxiety is an almost ubiquitous component of mental illnesses (Handley & McBlane, 1993). It is present in its purest form in the so-called anxiety disorders, but also found in depression, schizophrenia and personality disorders (Clarke & File, 1982). Experimental evidence based primarily on drug therapy suggests that anxiolytic effects of benzodiazepines (BZs), the conventional anxiolytics, are manifested by the stimulation of BZ-GABA (Gamma amino butyric acid) receptor complex and a concomitant decrease in serotonergic neuronal activity (Batool et al., 1995; Haleem & Batool, 1996; Batool & Haleem, 1997; Batool & Haleem, 1999). Despite a trend of reduced prescribing the BZs remain the most widely used psychotropic drugs and this is due to their considerable effectiveness as anxiolytic, hypnotic and anticonvulsant (Feely et al., 1982). The use of components from sea buckthorn fresh fruit extract or other medicinal plants is a protected method for fighting against anxiety or anxiety-related symptoms in various brain-related personality disorders (Bernath & Foldesi, 1992; Guliyev et al., 2004). Investigations on modern medicinal applications of Sea buckthorn were instigated in Russia during the 1950s (Ahmad & Kamal, 2003). The fruit, leaves, bark and seeds of sea buckthorn contain over 190 nutrients. Its active ingredients include carotene, flavonoids, phytosterols, serotonin, and eight essential amino acids (Sabir et al., 2005). Herbal nutrients of the plant have been shown to help heal many maladies, improve general health and specific pathological conditions (Shah et al., 2007, Batool et al., 2009). Different preparations of sea buckthorn in the form of oils and pulp (fresh fruit extract) are available in the market and recommended for various clinical and psychotic conditions (Yang & Kallio, 2003). Despite several usages of Sea buckthorn plant, the most important and popular part are berries, from which the juice is extracted for research. Animal models shape the backbone of preclinical research on the neurobiology of psychiatric disorders, and are employed as screening tools in the search for novel therapeutic agents. On the basis of these considerations, it was the purpose of this study to characterize the possible anxiolytic-like activity of aqueous fruit extracts prepared from the berries of Sea buckthorn in experimental models of anxiety.

Materials and Methods

Plant material and preparation of fruit extracts: Sea buckthorn (Hippophae rhamnoides L. spp. Turkestanica) (SBT) fresh fruit berries were collected from Northern areas (Hussainabad, Sakardu) of Pakistan in the first week of October when the plant grows wildly under natural conditions (Fig. 1). These berries were kept in plastic pots and transported to University of Agriculture, Rawalakot, Azad Kashmir. The fresh fruit were then cleaned and pounded to pieces with a squeezer. The extract was filtered and the filtrate was stored at -20°C in a refrigerator. The fruit pulp was extracted manually by extracting seeds from 50 berries of Sea buckthorn. The calculation was done after weighing 100 berries and 100 sample seeds. The crude extract was then diluted in sterile double-distilled water to make aqueous fruit extracts of sea buckthorn (SBT-FE) in different dilutions (20mg/kg and 40mg/kg) during the experiment (Fig. 1). These fruit extracts were administered orally per os (P.O.) to groups of rats and diazepam injected at doses of 3.0mg/kg body weight at an equivalent volume of 1.0 mL/kg body weight in another group of rats respectively in a balanced design. Control animals received an equal volume of saline (0.9% NaCl) as vehicle. The route of administration for diazepam and saline used was intraperitoneal (I.P.) under controlled conditions.
Animals and treatment schedule: Male Albino-Wistar rats with an average weight of 180 ± 20 g on arrival were purchased from H.E.J. Research Institute of Chemistry and group-housed (two rats per cage) in an environmentally controlled room (ambient temperature 21 ± 1°C and relative humidity 55 ± 5%) in a 12:12 h light/dark cycle (lights on at 7:00 A.M.). A 5-day adaptation period was allowed before animals were used in experiments. After this period and 24 h before the behavioral tests, the animals were individually housed in an environmentally controlled test room in transparent Perspex cages (dimensions 26×26×26 cm, w×l×h). Food (standard rat diet) and tap water were continuously available to animals during experiment. The rats used for the treatment were all experimentally naive animals. All experiments were conducted in a balanced design in proper conditions (like room temperature, room lights and isolation). All solutions were freshly prepared on test days. All experimental protocols were approved and performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Sciences, US National Research Council, 1996) and the Institutional Ethical Committee’s guidelines for animal research.

Behavioral analysis

Assessment of anxiolytic activity

Elevated plus-maze model of anxiety: In the present study, the plus-maze apparatus, consisting of two open arms (OA) (16 x 5 cm) and two closed arms (CA) (16 x 5 x 12 cm) having an open roof, was elevated (60 cm) from the floor was used to observe anxiolytic behavioral parameters in rats. Rats were treated with vehicle (0.9% NaCl; Saline), diazepam (3.0 mg/kg, I.P.) and aqueous extracts of SBT-FE (20mg/kg P.O.) and (40mg/kg P.O.). One hour after the oral administration of SBT-FE or drug treatment, each rat was placed at the center of the elevated plus maze with its head facing the open arm. During the experiment following behavioral parameters were recorded for 5 min.: 1) Number of entries in open arms, 2) Total time spent in open arms in %. During the entire experiment, rats were allowed to socialize. During the experiment every precaution was taken to ensure that no external stimuli, other than the height of the plus-maze could invoke maze anxiety.

Light-dark model of anxiety: Light-dark box is a rectangular box of 46 x 27 x 30 cm (l x b x h), which is divided in to 2 equal compartments one of the box used for the dark compartment (painted black) and the other served as light compartment (or Lit-Box) centrally compartmentalized by a small open doorway at the center of the partition. Aqueous fruit extracts (SBT-FE) of plant were administered through per oral route (P.O.) while drug or vehicle was injected via I.P. One hour after the oral administration of SBT-FE or drug treatment the rats were placed individually in the Lit-Box and observed for a period of 5 min. Behavioral parameters observed for 5 min., during the experiment were number of entries in Lit-Box, and time spent in Lit-Box in %. All the parameters were recorded in a balanced design.

Open field model of anxiety: The open field apparatus used in the present investigation consisted of a square area 76x76 cm with walls 42 cm high. The floor was divided by lines into 25 equal squares. To determine the activity, a rat was placed in the centre square of the open field and latency to leave the centre square and numbers of squares crossed with all four paws were scored for 5 min as described earlier (Batool et al., 2009). One hour after the oral administration of SBT-FE or drug treatment, each animal were placed at the centre of the apparatus and the following behavioral parameters were
scored in the next 5 min. All the behavioral parameters were monitored in a balanced design in the following order:
Latency time: Time taken by animal to leave center square in which it was placed.
Ambulation: Numbers of squares crossed by the four paws of animal.
Total ambulation time in Open field arena in %.

**Experimental protocol:** Twenty four animals were randomly divided into four equal groups (six animals in each group) and treated as: (1): Saline (0.9% NaCl) injected, (2): Diazepam (3.0mg/kg; I.P.) injected, (3): aqueous SBT-FE (20mg/kg; P.O.) and (4): aqueous SBT-FE (40mg/kg; P.O.). One hour after the oral administration of SBT-FE at different dilutions (20mg/kg and 40mg/kg; P.O.) and acute injections of diazepam (3.0mg/kg body weights; I.P.) to different groups of rats, Control and test animals were individually subjected to elevated plus maze (EPM), Light-dark model (LDM) and open field test (OFT) models of anxiety for testing the potent anxiolytic activity of medicinal plant Sea with different time intervals and scoring. Each experiment was conducted in a separate room in balanced design with proper schedule, time factor and isolation.

**Statistical analysis:** In this investigation, the results are presented as a means ± S.D. Data on exploratory activity and total ambulation time in elevated plus maze (EPM), light-dark model (LDM) and open field test (OFT) were analyzed by one analysis of variance (1-ANOVA). *Post-hoc* comparisons, done with the Newman-Keuls test, when values p <0.05 were considered statistically significant.

**Results**

Table 1 shows the anxiolytic activity of aqueous SBT-FE in elevated plus maze model (EPM). Treatment with acute injections of diazepam (3.0mg/kg) and P.O. administration of different dilutions of aqueous SBT-FE (20mg/kg & 40mg/kg) in rats showed significant increases in number of entries in OA (p<0.01) and time spent in OA (p<0.01). The results revealed that the increases observed in group of rats administered with different dilutions of SBT-FE (20mg/kg & 40mg/kg) were well equivalent to the increases observed by the administration of diazepam when compared with controls.

Table 2 shows the anxiolytic activity of aqueous SBT-FE in light-dark model (LDM). Treatment with acute injections of diazepam (3.0mg/kg) and P.O. administration of different dilutions of aqueous SBT-FE (20mg/kg & 40mg/kg) in rats showed significant increases in number of entries in Lit-box (p<0.01) and time spent in Lit-box (p<0.01). The results revealed that the increases observed in group of rats administered with different dilutions of SBT-FE (20mg/kg & 40mg/kg) were well equivalent to the increases observed by the administration of diazepam when compared with controls.

Table 3 shows the anxiolytic activity of aqueous SBT-FE in open field test (OFT). Treatment with acute injections of diazepam (3.0mg/kg) and P.O. administration of different dilutions of aqueous SBT-FE (20mg/kg & 40mg/kg) in rats showed significant increases in latency period (p<0.01) in open field arena. Treatment with similar pattern also showed significant increases in numbers of squares crossed (p<0.001) by the animals when compared with saline injected group of animals. The results revealed that the increases observed in group of rats administered with different dilutions of SBT-FE (20mg/kg & 40mg/kg) were well equivalent to the increases observed by the administration of diazepam when compared with controls.
Table 1. Anxiolytic activity of aqueous fruit extracts of Sea buckthorn (*Hippophae rhamnoides* L. spp. *Turkestanica*) and DZP in Elevated Plus Maze Model.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of entries in OA</th>
<th>Time spent in OA (sec/5min.)</th>
<th>Time in OA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (0.9% NaCl)</td>
<td>25.5 ± 3.2</td>
<td>65.0 ± 2.7</td>
<td>21.6%</td>
</tr>
<tr>
<td>Diazepam (3.0mg/kg)</td>
<td>71.0 ± 5.8**</td>
<td>225.0 ± 10.5***</td>
<td>75%</td>
</tr>
<tr>
<td>SBT-FE (20mg/kg)</td>
<td>65.5 ± 6.5**</td>
<td>195.0 ± 11.6***</td>
<td>55%</td>
</tr>
<tr>
<td>SBT-FE (40mg/kg)</td>
<td>51.5 ± 1.7**</td>
<td>205.0 ± 7.8***</td>
<td>80%</td>
</tr>
</tbody>
</table>

Values expressed are means ± S.D. (n = 6). Significant differences by Neuman-Keuls test: p<0.05*, <0.01** and <0.001*** as compared to saline injected group following 1-ANOVA.


<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of entries in lit-box</th>
<th>Time spent in lit-box (sec/5min.)</th>
<th>Time in lit-box (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (0.9% NaCl)</td>
<td>10.5 ± 1.5</td>
<td>28.0 ± 0.4</td>
<td>10%</td>
</tr>
<tr>
<td>Diazepam (3.0mg/kg)</td>
<td>35.0 ± 2.7**</td>
<td>85.0 ± 2.5**</td>
<td>28%</td>
</tr>
<tr>
<td>SBT-FE (20mg/kg)</td>
<td>48.5 ± 3.3**</td>
<td>175.0 ± 6.5**</td>
<td>58%</td>
</tr>
<tr>
<td>SBT-FE (40mg/kg)</td>
<td>40.7 ± 5.5**</td>
<td>92.0 ± 8.4**</td>
<td>30%</td>
</tr>
</tbody>
</table>

Values expressed are means ± S.D. (n = 6). Significant differences by Neuman-Keuls test: p<0.05*, <0.01** and <0.001*** as compared to saline injected group following 1-ANOVA.


<table>
<thead>
<tr>
<th>Treatment</th>
<th>Latency time (sec)</th>
<th>Numbers of squares crossed by rat (counts/5min.)</th>
<th>Total ambulation time in open field arena (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (0.9% NaCl)</td>
<td>7.0 ± 0.5</td>
<td>175.0 ± 21.5</td>
<td>20%</td>
</tr>
<tr>
<td>Diazepam (3.0mg/kg)</td>
<td>45.0 ± 2.5**</td>
<td>115.0 ± 22.4***</td>
<td>65%</td>
</tr>
<tr>
<td>SBT-FE (20mg/kg)</td>
<td>64.5 ± 5.8**</td>
<td>95.0 ± 10.7***</td>
<td>70%</td>
</tr>
<tr>
<td>SBT-FE (40mg/kg)</td>
<td>51.0 ± 3.0**</td>
<td>110.0 ± 15.6***</td>
<td>80%</td>
</tr>
</tbody>
</table>

Values expressed are means ± S.D. (n = 6). Significant differences by Neuman-Keuls test: p<0.05*, <0.01** and <0.001*** as compared to saline injected group following 1-ANOVA.
Discussion

In recent years, research on medicinal plants has attracted a lot of attention globally. Large body of evidence has accumulated to demonstrate promising role of a medicinal plant Sea Buckthorn (*Hippophae rhamnoides* L. spp. *Turkestanica*) fruit extract as a dietary nutrient is well established in folk medical discipline. Diazepam, a standard anxiolytic used clinically, is also employed in behavioral pharmacology as a reference compound for inducing anxiolytic-like effects, even when the compound being screened dose not act via benzodiazepine receptors. The anxiolytic effects of drugs such as benzodiazepines are accompanied by decreased locomotor activity and sedation. The aqueous fruit extract SBT-FE did not inhibited locomotor activity in this experiment. However, effects of SBT-FE at doses of 40mg/kg on locomotion were comparable to the effects of diazepam (3.0mg/kg) and thus have a better profile for an anxiolytic agent (Table. 3). There is a considerable interest in the development of new anxiolytics that do not induce sedative effects and do not inhibit locomotion. Our most recent research has shown that oral supplementation of HRL-FE is effective in modifying haloperidol-induced behavioral deficits. In addition, HRL-FE reversed brain TRP and 5-HT decreases induced by repeated haloperidol treatment (Batool *et al*., 2009). It is suggested from our previous data that SBT-FE has the ability to normalize the depression in spontaneous and/ or locomotor activity in rats. The present behavioral data are in accord with the reported study on SBT-FE when supplemented orally in laboratory animals and tested in experimental models of anxiety in the present investigation. In the field of anxiety research, animal models are used as screening tools in the search for compounds with therapeutic potential and as simulations for research on mechanisms underlying emotional behavior (Lister, 1990; Wolfman *et al*., 1994).

Behavioral effects of diazepam generally thought to be a reflection of its anxiolytic properties (Batool *et al*., 2001). There is a wide range of animal models showing changes in activity in a familiar and novel environment (File *et al*., 1991). Depression of spontaneous locomotor activity following the administration of diazepam has been shown in reported animal studies (Elliot & White, 2001). Chronic administration of diazepam also decreases locomotion (Baldwin & Rudge, 1995). Our results show that both single administration of diazepam at doses of 3.0mg/kg and P.O. administration of aqueous SBT-FE (20mg/kg & 40mg/kg) increased spontaneous locomotor activity in novel and aversive environments (Batool & Haleem, 1995; Batool & Haleem, 1997). The elevated plus maze (EPM) is a rat model of anxiety that is used as a screening test for putative anxiolytic compounds such as diazepam and as a general research tool in neurobiological anxiety research (Lader, 1991; Hogg, 1996). The conventional indices of anxiety in this test, percent of open arm entries and percent time spent in the open arm, are exquisitely sensitive to agents thought to act via the GABA<sub>A</sub> receptor complex (i.e., benzodiazepines, barbiturates, ethanol, and neurosteroids). For this reason, we chose this paradigm to investigate the anxiolytic potential of SBT-FE. The model is based on animal's aversion of open spaces. This aversion leads to the behavior termed thigmotaxis, which involves avoidance of open areas by confining movements to enclosed spaces or to the edges of a bounded space (Treit *et al*., 1993). In EPM this translates into a restriction of movement to the enclosed arms. Anxiety reduction in the plus-maze is indicated by an increase in the proportion of time spent in the open arms (time in open arms/total time in open arms), and an increase in the proportion of entries into the open arms (entries into open arms/total entries into open arms). Studies from our laboratory have confirmed that
full and partial benzodiazepine receptor agonists produce behavioral changes in the maze consistent with anxiety reduction (Haleem & Batool, 1996). In the present study, increases in the proportion of number of entries and time spent in OA are the best index of reduced anxiety levels in rats following the P.O. administration of aqueous SBT-FE (Table. 1).

The light-dark model is a rat model of anxiety and useful to predict anxiolytic-like or anxiogenic-like activity in rats (Chaouloff et al., 1997). Transitions have been reported to be an index of activity-exploration because of habituation over time and the time spent in each compartment to be a reflection of aversion. Classic anxiolytics (benzodiazepines; diazepam) as well as the newer anxiolytic-like compounds (e.g., serotonergic drugs; buspirone) can be detected using this paradigm (Barrett, 1991). In the present study, diazepam at 3.0mg/kg showed significant effect on all parameters monitored during the experiment. An increase in locomotion in terms of number of entries in Lit-box and time spent in Lit-box are the indicators of anxiolytic activity of aqueous fruit extracts of SBT-FE used in the study (Table. 2).

The standard open field test is commonly used to assess locomotor, exploratory and anxiety-like behavior in laboratory animals. This test is particularly useful in evaluating the effects of anxiolytic and anxiogenic drugs, locomotor responses to drug and as well as behavioral responses to novelty (Choleris et al., 2001). The open field test task approaches the conflict between the innate fear that rats have of the central area of a novel or brightly lit open field versus their desire to explore new environments. When anxious, the natural tendency of rats is to prefer staying closed to the walls (thigmotaxis). In this context, anxiety-related behavior is measured by the degree to which the rat avoids the center of the pen field test (Haleem et al., 2002). Results obtained from all doses of aqueous SBT-FE (20mg/kg & 40mg/kg) and acute injections of diazepam at doses of 3.0mg/kg showed equipotent increases in latency time and increases in number of squares crossed by the animal when compared with saline injected group of rats which showed that decreases in fear of animal, indicates the preferential anxiolytic activity of the aqueous extracts of SBT-FE and support the findings that sea buckthorn is a medicinal plant with potent anxiolytic activity and could be useful in primary medical care. It has been reported that the effect of most of the anxiolytic agents is to enhance the response to GABA, by facilitating the opening of GABA-activated chloride channels. GABA_A receptors were involved in anxiety and their direct activation would have anxiolytic effect. This is in accord with the pharmacological effects of benzodiazepines and highlights the relevance of putative anxiolytic-like effects of SBT-FE. Although further major active components and precise anxiolytic mechanisms are need to be identified on the basis of these suggestions.

**Conclusion**

The present study advocates the proposal that P.O. administration of SBT-FE in rats possibly exhibits anxiolytic-like effects in different experimental models and results are in accord with the traditional use of Sea buckthorn in history of herb and medicine. Keeping in view the medicinal, biochemical and nutritional characteristics of sea buckthorn fruit extract in combination with modern medical drug therapy, neurobiological mechanism of anxiety and its eradication can be better understood and SBT-FE may be used as a therapeutic nutritive supplement in psychotic patients. In the same way, identification of compound(s) responsible for biological activity could be used as prototype(s) to design new substances with anxiolytic activity. Hence more queries will be addressed in future protocols with novel paradigms.
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