Effect of pretreatment with pumpkin and flax seed enriched diets on anxiety in mice

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Abstract

Background: Biopsychosocial factors, including poor lifestyle, contribute to anxiety disorders. Thus modification of diet may be one option for management of anxiety.

Purpose: This study examined the association between intake of diets enriched with plants containing polyunsaturated fatty acids (PUFAs) viz. flaxseeds and pumpkin seeds, and the prevalence of anxiety in experimental animals.

Methods: Flaxseeds and pumpkin seeds enriched diets were prepared. Mice were fed these diets for one month, after which the anti-anxiety effect of the diets on the experimental animals was evaluated using the Elevated Plus Maze (EPM) model. The physicochemical parameters of standard and test diets were determined. Effect of n-hexane and methanol extracts of flaxseeds and pumpkin seeds were also examined using EPM in mice with the objective of confirming that the anxiolytic effect was due to fatty acids present in the seeds.

Results: Pretreatment with flaxseeds enriched diet (10% w/w) produced significant increase in time spent by mice in the open arms of EPM in comparison to standard diet. Furthermore, the n-hexane extract of flax seed (100 mg/kg; p.o.) showed marked anxiolytic-like effect, comparable to diazepam. Phytochemical evaluation of the n-hexane extract showed the presence of fatty acids.

Conclusion: These results suggest that dietary modification, by incorporation of flaxseeds, can reduce the incidence of anxiety in experimental animals and may be attributed to the PUFAs present in flax seeds.

Keywords: Dietary modification; Anti-anxiety; PUFAs; Flax seed; Pumpkin seed

Introduction

Anxiety is a complex psychological and physiological state characterized by cognitive, somatic, emotional and behavioral disturbances[1]. Various behavioral, genetic, psychoanalytic, psychodynamic, cognitive and biological theories have been proposed to explain the pathophysiology of mental disorders specially anxiety [2]. These include poor lifestyle habits such as consumption of inadequate or deficient diet [3-6]. It is reported that diet has major influence on mental health. The effect of dietary intake of omega-3 polyunsaturated fatty acids (PUFAs) for the management of anxiety disorders is being investigated. It is well documented that PUFAs affect the central nervous system by influencing cell membrane fluidity, altering the activity of receptors and proteins and neurotransmitter synthesis [6, 7]. Studies have shown the psychotropic effects of omega-3 PUFA [8, 9]. Evidence shows that deficient consumption of PUFAs could lead to the development of psychiatric disorders [10]. Thus incorporating PUFAs in diet may contribute to mental wellbeing.

In this context, plants which are a dietary source of PUFAs are receiving considerable attention and are being evaluated for various health benefits [11,12]. Among such plants are the seeds of *Linum usitatissimum* (flax seeds; family Linaceae) and *Cucurbita maxima* (pumpkin seeds; family Cucurbitaceae). These are reported to have numerous beneficial effects which were attributed to the high content of omega -3 fatty acids and omega -6 fatty acids, respectively [13-15]. However the potential of these plants for the management of anxiety has not been explored. Thus, the present study was designed to evaluate the effects of diet enriched with plants containing PUFAs on anxiety in experimental animals by employing the elevated plus maze (EPM) model.

Material and methods

Plant material

Seeds of *Linum usitatissimum* (flax) and *Cucurbita maxima* (pumpkin) were purchased from the local market of Barnala, Punjab in August 2011. The seeds were authenticated by Dr. H.B. Singh, Director, National Institute of Sciences Communication and Information Resources (NISCAIR), New Delhi (vide voucher specimen no- NISCAIR/RHMD/Consult/-2011-12/1967/267).

Preparation of diet

The seeds of flax and pumpkin were procured and dried. These were ground to fine powder. Diets were prepared by mixing 10% w/w seed powders of flax and pumpkin, separately, with standard chow. Starch solution (1% w/v) was added as a binder to the mixture. This mixture was kneaded into dough with a semi-solid consistency. The dough was then spread on a tray and cut into pieces of suitable size.

Preparation of extracts

Flax seeds and pumpkin seeds (250 g each), were ground to a coarse powder, separately, and subjected to successive exhaustive extraction, using Soxhlet apparatus, with n-hexane followed by methanol. The extracts were concentrated under vacuum and percentage yield was calculated. *Standardization of prepared diet*

Regular chow diet and prepared diets were standardized on the basis of physicochemical parameters viz. fat content, protein content, carbohydrate content, fatty acid content, total phenol content, total ash value, fibre content, acid value, spanofication value, iodine value and ester value [16-20]. Each parameter was evaluated in triplicate.

Animals

Swiss albino mice of either sex weighing 20-25 g were procured from the Animal House, Punjabi University, Patiala. Animals had access to water and food *ad libitum*. The experiments were conducted in a semi sound proof laboratory. All animal procedures have been approved by the Institutional Animal Ethics Committee (Reg No. 107/99/CPCSEA-2011-14).

Experimental protocol

Dietary treatment:

Animal were randomly divided into three groups of 6 mice each.

Group I. Control group. Mice fed with regular chow diet for 30 days before the evaluation of anxiety-like behavior on the EPM.

Group II and III. Test Groups. Mice fed with a chow diet enriched with flax and pumpkin seeds, respectively, for 30 days before the evaluation of anxiolytic activity on the EPM.

Extract treatment:

Experimental animals were divided into 14 groups of 6 mice each. These were given the following treatment:

Group I. Control group. Mice were administered vehicle (simple syrup + 0.2% CMC, p.o., 10 ml/kg).

Group II. Standard group. Mice were orally administered diazepam (2 mg/kg) 60 min prior to evaluation of anxiolytic efficacy.

Group III, IV, V, VI, VII, VIII. Flax seed extract treated groups. Mice were orally administered n-hexane and methanol extracts of flax seeds at a dose of 50, 100 and 200 mg/kg respectively, 60 min prior to evaluation of anxiety-like behavior.

Group IX, X, XI, XII, XIII, XIV. Pumpkin seed extract treated groups. Mice were orally administered n-hexane and methanol extracts of pumpkin seeds at a dose of 50, 100 and 200 mg/kg respectively, 60 min prior to evaluation of anxiety-like behavior.

These experiments were carried out in a semi sound-proof laboratory between 5am and 8am. The experimental animals were fasted overnight prior to the experiment.

Evaluation of physiological parameters of mice during study

Body weight change:

The body weight of mice on '0' day and '30th'day during the study period was determined to calculate body weight gain.

Average fed intake:

During the experimental period, feed intake of mice was measured daily during 30 days. The amount of diet ingested was calculated as the difference between the weight of feed that remained in the food bin and the amount placed one day before. These data were then used to calculate a daily average feed intake.

Food conversion efficiency:

Food conversion efficiency was calculated by dividing the feed intake with weight gained.

Apparent digestibility:

Apparent digestibility of macronutrients was assessed as the relative difference between daily intake and 24 h excretion of feces by following formula:

Digestibility = [(dietary intake – fecal excretion)/ dietary intake] X 100

Anxiolytic activity (EPM model):

The anxiolytic activity was evaluated using the EPM model [21]. The EPM consisted of two open arms (30 cm long and 5 cm wide) and two enclosed arms (30 cm long, 5 cm wide and 15 cm high) extended from a central platform (5 cm x 5 cm); arranged so that the two open arms are opposite to each other. The maze was elevated to a height of 40 cm from the floor in a dimly lit room. The experimental animals are placed individually at the central platform of the EPM with their head facing towards one of the enclosed arm. The behavior of the animals on the plus-maze apparatus was recorded for 5 min in terms of the average time spent by the mouse in open arms. An arm entry was defined when all four limbs of the mouse were on the arm [22].

Average time spent in open arms of the EPM = Total time spent in open arms/ number of entries

Data analysis

Results were expressed as mean ± SEM. The results were analysed using one way analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple range test. The significance level was taken at $P < 0.05$.

Results

Standardization of prepared diets

The physicochemical parameter of test and regular chow diet was found as mentioned in Table 1.

Effect of prepared diets and regular diet on anxiety in mice

Animals pretreated with both test diets have shown significant changes in anxiety-like behavior (Fig. 1) in comparison to regular diet treated animals. A diet enriched with flaxseed (10 % w/w) showed the maximum amelioration of anxiety-like behavior among animals as it increased the time spent by mice in the open arms of elevated plus maze.

Physiological parameters of mice during study

The effects of the test diets on physiological parameters of mice are shown in Table 2. Quantities of feed consumed by mice were the same in all groups, but body weight gain differed significantly between flaxseed and pumpkin seeds treated mice. The significant difference was observed in digestibility between flaxseed and pumpkin seed diets. The diet enriched with seeds of flax showed lower food conversion efficiency ratio.

Percentage yield and phytochemical screening of extracts

The percentage yield and results of phytochemical screening of prepared extracts is reported in table 3.

Effect of flax and pumpkin seed extracts on anxiety in mice

The relative anxiolytic activity at different doses (50, 100 and 200 mg/kg) of n-hexane and methanol extracts of both plant materials was evaluated using the EPM model. Results of administering extracts on anxiety-like behavior in mice are presented in Fig. 2 and 3. All the prepared extracts showed amelioration of anxiety-like behavior in mice in comparison to control group. However, the results were not dose dependent. N-hexane extract of flax seeds at a dose of 100mg/kg showed a more pronounced anxiolytic efficacy.

Discussion

Epidemiological research suggests that anxiety disorders have the highest prevalence rate among psychiatric disorders. Management of anxiety with conventional drugs is limited due to side effects. Dietary modifications with the use of PUFAs for management of anxiety is one avenue of research.

Omega-3 and omega-6 PUFAs are key components of brain membranes. They are provided in dietary form as mammals cannot synthesize them. The ratio of omega-6/omega-3 is an important factor for brain health [7]. An optimal ratio (1:1) is required for normal brain development and functions. However the ratio has shifted to approximately 30:1 as a consequence of unhealthy diet. This has been due to substitution of omega-3 PUFAs by omega-6 PUFAs and saturated fatty acids resulting in increased mental illness including anxiety [23-25]. Studies have shown that supplementation with omega 3-fatty acid in an early phase of rat brain development has resulted in decreased anxiety-like behavior [26]. Vinot et al. [27] showed efficacy of omega-3 fatty acids in lowering anxiety in non-human primates. Progressive decline in anxiety was reported in patients received 3 g of eicosapentaenoic acid and docosahexaenoic acid for 3 months as compared with patients receiving a placebo [28]. Administration of PUFAs (both omega-3 and omega-6) to students who had experienced significant anxiety associated with examinations reduced anxiety in comparison to placebo [29].

In this study, the effect of exposure to diet enriched with plants containing PUFAs on anxiety-like behavior in mice was examined. Two plant materials i.e. flax seeds and pumpkin seeds were selected as they are reported to contain PUFAs. In the second part of the experiment, it was investigated whether the observed anxiolytic effects with diet was due to PUFAs. Increase in average the time spent by animal in open arm of EPM was taken as an index of amelioration of anxiety [21].

The result showed that pretreatment with diets of flax and pumpkin seeds, separately, for 30 days significantly prevented the anxiety-like behavior in experimental animals when compared to mice fed with regular chow diet. Among both diets, mice fed with flax seeds diet showed reduced anxiety-like behavior in comparison to pumpkin seed diet fed mice. Also, higher amounts of fat and total free fatty acid content were found in diet enriched with flax seed (Table 1). The same diet has also shown significant lower food conversion efficiency in mice which resulted in less body weight gain (Table 2). This might be a positive effect as lower body weight gain represents lower health problems [30].

To confirm that PUFAs present in flax seed diet were responsible for antianxiety effect, different extracts (n-hexane and methanol) of flax and pumpkin seeds were prepared. In accordance with literature, carbohydrates and fatty acids were confirmed in n-hexane extracts by phytochemical screening [31]. The n-hexane extract of flax seed at a dose of 100 mg/kg was found to have statistically significant anxiolytic effect in comparison to control group. This was comparable to the effect of the standard drug –diazepam. Interestingly, treatment with both flax seed extracts at higher dose (200 mg/kg) resulted in decreased time spent by animal in open arm of EPM. Previously non-linear relationship of PUFAs or herbal extracts in treating mental disorders has been reported [32-34]. Similar results were obtained in our study indicating a biphasic dose-response effect of prepared extracts on anxiety-like behavior.

Short and long term changes in the composition of dietary macronutrients alter neurochemistry and behavior in animals [35]. The association between diet quality and anxiety is supported by the data in our study. Our study is in accordance with previous work that had shown higher content of omega-3 fatty acids in flaxseeds as compared to pumpkin seeds [13]. Thus, the marked anxiolytic effect of flaxseed diet may be attributed to the presence of fatty acids i.e. PUFAs (omega-3 fatty acids), in the plant. The anxiolytic effect was confirmed by the activity of nonpolar extract (n-hexane) as it contains only fatty acids.

Conclusion: Significant anxiolytic activity was observed with pretreatment with flax seed enriched diet as compared to the standard chow diet and the pumpkin seed enriched diet. This may be due to the presence of higher amount of fatty acids in flax seed. This study confirms the beneficial effects of dietary intervention with PUFAs containing plants in the management of anxiety.

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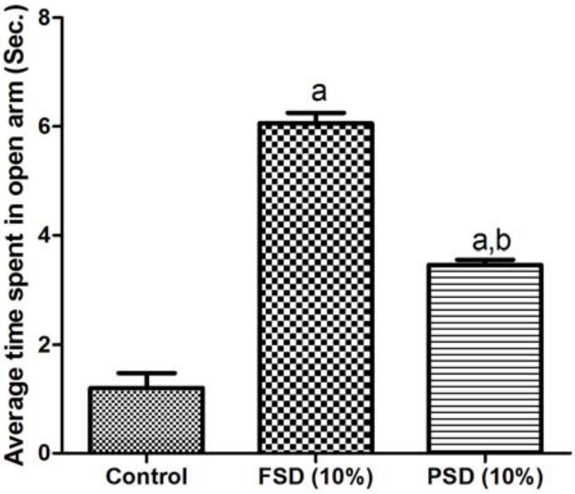

Fig. 1: Effect of diets enriched with flax and pumpkin seeds on time spent in open arms of EPM. Values are expressed as Mean ± SEM (n=6). The data was analyzed by one way ANOVA and *post hoc* Tukey's multiple range test. a= P<0.05 vs. control group (regular chow diet); b= P<0.05 vs. test diet with 10% flax seeds; FSD= Flax seed diet; PSD= Pumpkin seed diet.

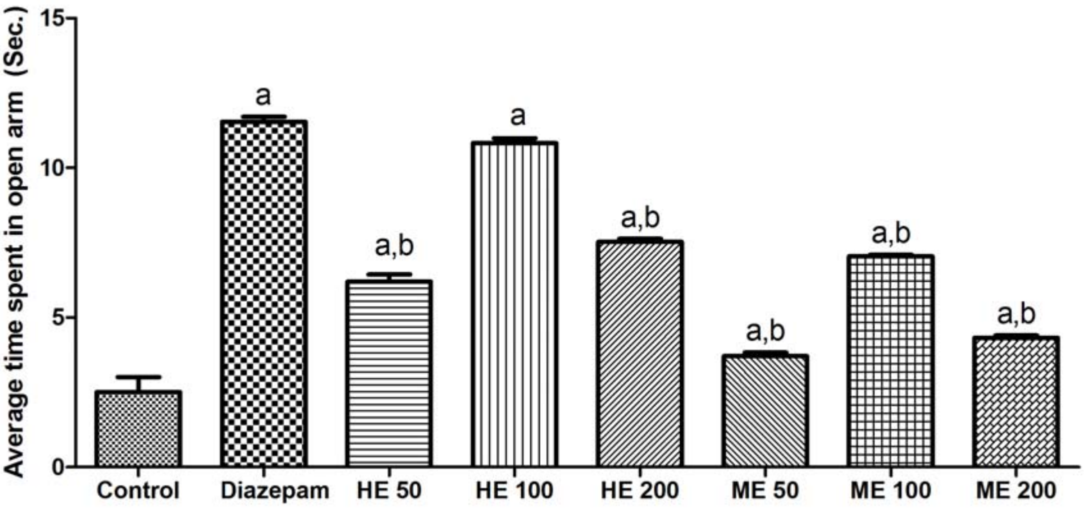

Fig. 2: Effect of n-hexane and methanol extracts of flax seed on time spent by mice in open arms. Values are expressed as Mean ± SEM (n=6). The data was analyzed by one way ANOVA and *post hoc* Tukey's multiple range test. a= P<0.05 vs. control group; b= P<0.05 vs. Diazepam (standard group); Diazepam = Diazepam 2 mg/kg; HE 50 = n-hexane extract (50 mg/kg); HE 100 = n-hexane extract (100 mg/kg); HE 200 = n-hexane extract (2000 mg/kg); ME 50 = Methanol extract (50 mg/kg); ME 100 = Methanol extract (100 mg/kg); ME 200 =Methanol extract (200 mg/kg)

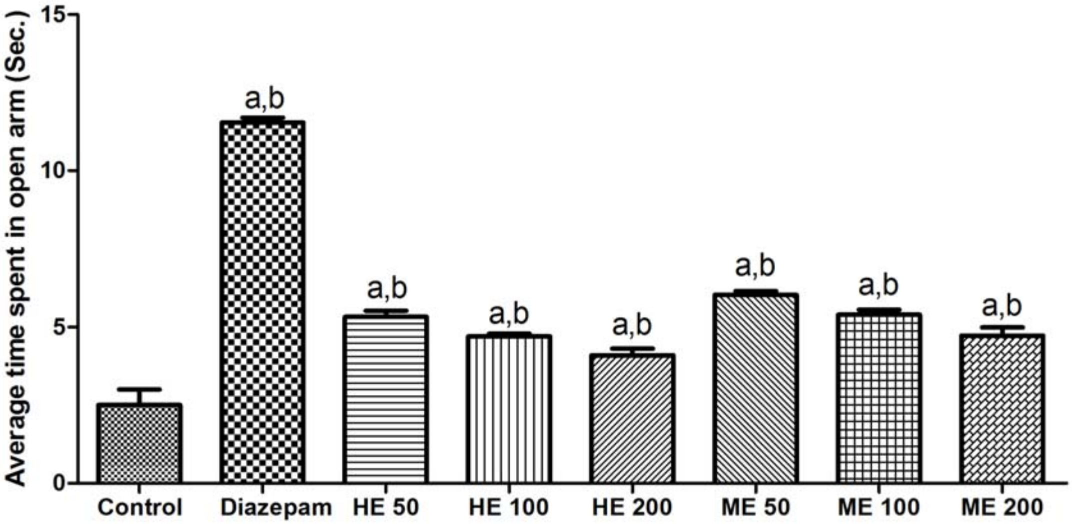

Fig. 3: Effect of n-hexane and methanol extracts of pumpkin seed on time spent by mice in open arms. Values are expressed as Mean ± SEM (n=6). The data was analyzed by one way ANOVA and *post hoc* Tukey's multiple range test. a= P<0.05 vs. control group; b= P<0.05 vs. Diazepam (standard group); Diazepam = Diazepam 2 mg/kg; HE 50 = n-hexane extract (50 mg/kg); HE 100 = n-hexane extract (100 mg/kg); HE 200 = n-hexane extract (2000 mg/kg); ME 50 = Methanol extract (50 mg/kg); ME 100 = Methanol extract (100 mg/kg); ME 200 =Methanol extract (200 mg/kg)

Table 1: Physicochemical parameters of test and regular chow diet

Physicochemical parameter	Diet containing Flaxseed seeds (10 % w/w)	Diet containing Pumpkin seeds (10 % w/w)	Regular chow diet
Carbohydrate content (mg glucose equivalent/100 mg)	29.87	28.94	34.96
Protein content (mg BSA equivalent/10 mg)	0.671	0.474	0.569
Fat content	5.4 %	2.60 %	2.50 %
Total phenol content (µg gallic acid equivalent/10 mg)	50.625	43.00	51.85
Total ash value	8.37	9.80	8.74
Crude fibre content	87.10 %	69.23 %	62.50 %
Total free fatty acid	7.80 %	4.20 %	1.30 %
Acid value (mg KOH/g)	8.98	7.52	4.7
Saponification value (mg KOH/g)	249.65	232.82	215.98
Ester value (mg KOH/g)	246.00	225.30	211.28
Iodine value (mg iodine/g)	125.52	120.43	116.42

Table 2: Effects of test diets on physiological parameters of mice

Physiological parameters	Flax seed diet	Pumpkin seed diet
Initial body weight (g)	25	20.05
Final body weight (g)	26.4	22.06
Body weight gain (g)	1.4	2.01
Average feed intake (g/day)	1.245	1.92
Food conversion efficiency	0.889	0.957
Average feaces weight (g/day)	0.77	0.32
Apparent digestibility (%)	28.15	66.45

Table 3: Yield and phytochemical screening of test extracts

Plant	Extract	% yield (w/w on dry weight basis)	Phytoconstituents present
Flaxseeds	n-hexane	37.00	Fixed oil, fatty acids
	Methanol	29.00	Amino acids, Carbohydrates, Phenolic compounds
Pumpkin seeds	n-hexane	37.10	Fixed oil, fatty acids
	Methanol	31.70	Amino acids, Carbohydrates, Phenolic compounds, Steroids