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Original Article

Anxiolytic-like effect of hydroalcoholic extract of ripe pistachio hulls in adult female Wistar rats and its possible mechanisms

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Abstract

The present study was designed to study the preventive effect of hydroalcoholic extract of ripe pistachio hulls (RPH) in the elevated plus maze model of anxiety. One hundred twenty female wistar rats in their estrous cycle were divided into 15 groups of 8 each and received various concentrations of hydroalcoholic extract of RPH except the control groups. Elevated plus maze was used to measure the level of anxiety. Percentage of time spent in the open arms (%OAT), percentage of the number of entries into the open arms (%OAE), locomotor activity, and time spent in the closed arms (CAT), and the number of entries in to the closed arms (CAE) were measured and compared. Dose-response experiments showed that only 10 mg/kg dose of RPH extract significantly increased %OAT ($P < 0.001$) and %OAE ($P < 0.05$) compared to the control group, indicating anti-anxiety effects of the extract. Also, pentylenetetrazol and an estrogen receptor antagonist (ERA) tamoxifen could block anti-anxiety effects of the extract ($P < 0.001$). It was also noticed that tamoxifen was able to significantly reduce locomotor activity. As the RPH extract showed a preventive effect in experimental model of anxiety, it might be concomitantly administered with other anxiolytic medications.

Keywords: Anxiety; Ripe pistachio hulls; PTZ; Estrogen; Plus maze

INTRODUCTION

Anxiety and fear are crucial adaptive components of the overall behavioral and autonomic stress response to dangerous situations which threaten to perturb homeostasis. Anxious states are controlled by a highly complex system of both inhibitory and facilitatory mechanisms which are reciprocally regulated in a highly dynamic fashion and display considerable redundancy (1).

The main inhibitory neurotransmitter system in the brain, the gamma-aminobutyric acid (GABA) system, is the target for many clinically used drugs to treat anxiety and epilepsy via two classes of GABA receptors including $GABA_A$ and $GABA_B$. $GABA_A$ receptors are ligand-gated ion channels, whereas $GABA_B$ receptors are G protein-coupled receptors (2).

Binding of GABA to its receptors causes a conformational change in the receptor leading to opening of chloride channels and influx of chloride ion into the cells, thus reducing excitability. In addition to GABA, other molecules such as benzodiazepines used to treat anxiety can also influence this receptor (3).

Pentylenetetrazol (PTZ), a prototypical anxiogenic drug which produces a reliable discriminative stimulus, has been extensively utilized in animal models of anxiety (4). While benzodiazepines block the effects of PTZ, $GABA_A$ antagonist drugs potentiate its effects (5). These findings emphasized the anxiogenic nature of the PTZ discriminative stimulus, and indicate a $GABA_A$-related mechanism.

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The biological effects of estrogen on mammalian target tissues are important for numerous physiological and pathological processes such as anxiety and depression (6,7). The effects of estrogen are induced via its two known receptors, estrogen receptor α (ERα) and β (ERβ).

Antidepressant action of estradiol is mediated through ERβ which is antagonized by the selective estrogen receptor modulator, tamoxifen (7). Furthermore, tamoxifen-treated animals showed increases the anxiety. These anxiogenic properties of tamoxifen attenuated with estrogen therapy. These findings show that tamoxifen administration increased anxiety levels (8).

There are several species of pistachio plant like *P. lentiscus, P. weinmonifolia, P. terebinthus, P. chines, and P. vera* that grow in different parts of Mediterranean region. In traditional medicine, it has been used for conditions such as gastrointestinal disorders, bloody diarrhea, headache, and influenza (9). Different compounds including procyanidin, neoflavone, penta- and tetra-cyclic triterpens, α-β pinene, oleanonic acid, lanostane, and flavonoids have been found in pistachio (10). Flavonoids are phytoestrogens in plants like pistachio especially in its hull (11) had been the focus of intense research for their biological activities.

It is believed that these components play a significant role in reducing the risks of age and life style-related diseases such as cancer, diabetes, and cardiovascular disease, declining neurodegenerative diseases like Parkinson and Alzheimer and improving cognitive function. Additionally, flavonoids have demonstrated anxiolytic, sedative and anticonvulsant activities (12,13).

There is some evidence that estrogen contributes in anxiety; and phytoestrogens might be effective in this situation. Considering the therapeutic effect of phytoestrogens, it can be concluded that its components are associated with anti-anxiety effects. The present study was designed to study the preventive effect of hydroalcoholic extract of ripe pistachio hulls (RPH) containing phytoestrogens in the elevated plus maze model of anxiety.

MATERIALS AND METHODS

Chemicals and preparation of ripe pistachio hulls hydroalcoholic extract

Pentylenetetrazol and diazepam were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Caspian Company (Rasht, Iran), respectively. Tamoxifen was donated by Iran Hormone Company (Tehran, Iran). Both chemicals were dissolved in saline. The ripe pistachio (*Pistacia vera* L.) nuts of Ohadi cultivar were obtained from 13 year-old trees grown in Damghan region and Semnan of Iran in 2013. The plants were authenticated by associate professor Davood Bakhshi, a pomologist in the Faculty of Agriculture, University of Guilan, Rasht, Iran. The fleshy fruit wall of fresh and ripe Pistachios was removed and dried in an oven. Fifty grams of fine powder of RPH was mixed with 70% ethanol and placed on a shaker incubator with gentle shaking for 72 h. Afterwards, the liquid phase of the container was placed on funnel and then dried. Dried extract was weighed and resuspended in normal saline. Animals received an appropriate amount of extract according to their weight and protocol.

Animals

In this experimental study, one hundred twenty female wistar rats weighting 180 ± 20 g were divided in to 15 groups of 8 each. Rats were housed with a 12-h light/dark cycle in a temperature-controlled (22 ± 2 °C) animal facility. Food and water were freely available with the exception of the brief test periods. All animal experiments were carried out in accordance with the national institute of health guide for the care and use of laboratory animals' publication No. 85-23 revised in 1985. All protocols were also approved by Ethical Committee of Guilan University of Medical Sciences. Animals in their estrous cycle which characterized by microscopic observation of vaginal secretion collected every morning during a month (14). Animals received a single dose of 0.1, 1, 10, 50, 100, 250, 500 mg/kg of RPH hydroalcoholic extract, 10 mL/kg saline (negative control group), 0.6 mg/kg diazepam (positive control group), 20 mg/kg PTZ + 10 mg/kg

hydroalcoholic extract of RPH, 20 mg/kg PTZ + 10 mL/kg saline, 10 mg/kg hydroalcoholic extract of RPH + 10 mL/kg saline, 15 mg/kg tamoxifen + 10 mg/kg hydroalcoholic extract of RPH just 5 min before starting the experiment. Animals received an appropriate amount of extract (as outlined in the following section) according to their weight and protocol. All drugs were administered intraperitoneally (i.p.) in a volume of 0.1 mL/10 g body weight.

Elevated plus maze model of anxiety

The apparatus (Borj Sanat Company, Tehran, Iran) used for the elevated plus maze test is in the configuration of a cross symbol and comprises two open arms (5 × 10 cm) across from each other and perpendicular to two closed arms (39.5 × 10 × 49.5 cm) with a center platform (10 × 10 cm) elevated to a height of 60 cm above the floor. Each rat received one trial in our test. Rats were allowed to move freely about the maze for 5 min. Rats had access to all arms and allowed to move freely between them. The number of entries into the open arms and the time spent in the open arms were used as indices of open space-induced anxiety in rat. Total number of entries into the open arms plus the number of entries into the close arms is index of locomotor activity. After each trial, all arms and the center area were cleaned with super hypochlorous water (15).

Statistical analysis

All data represent mean ± SEM values and were analyzed using the software SPSS 16 (SPSS Inc., Chicago, USA). One-way ANOVA followed by Tukey's test for multiple comparisons were used for statistical evaluation. $P < 0.05$ was the critical criterion for statistical significance.

RESULTS

Acute toxicity

To evaluate acute toxicity of RPH extract, rats were carefully observed for any signs of toxic effects during the first 6 h after the treatment and subsequent 24 h. Extract of RPH (0.1-500 mg/kg) did not produce any noticeable effects on animals' behaviors. Furthermore, no case of lethality was found.

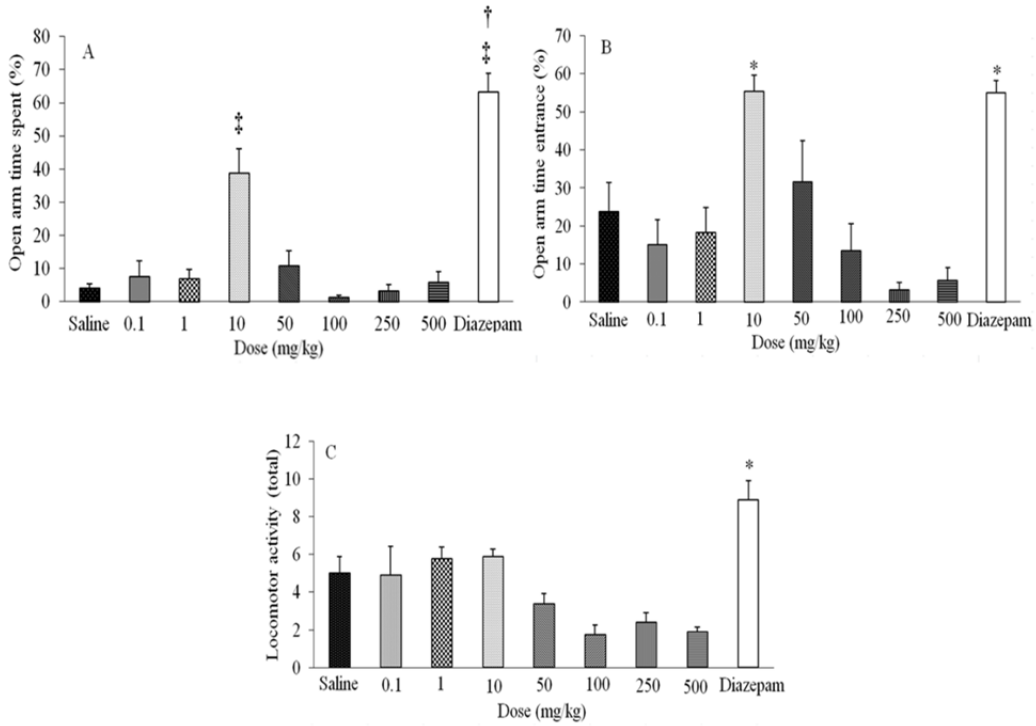

Fig. 1. The effect of different doses of hydroalcoholic extract of RPH on (A) percentage of time spent in the open arms (%OAT), (B) percentage of the number of entries into the open arms (%OAE), and (C) locomotor activity (total). *$P < 0.05$ and ‡$P < 0.001$ compared with saline receiving group (Mean ± SEM, n = 8). †$P < 0.01$ compared with dose 10 mg/kg of the extract receiving group (Mean + SEM, n = 8).

Effect of various RPH extract concentrations on fundamental behavior parameters

Among the various concentrations of RPH, only 10 mg/kg dose of the extract significantly increased the percentage of the time spent in open arms (%OAT) (38.75 ± 7.29 vs 3.91 ± 1.54, $P < 0.001$) as compared to the control group. Diazepam also showed significant increase in the %OAT compared to the saline ($P < 0.001$) and 10 mg/kg dose of the extract (63.21 ± 5.76 vs 3.91 ± 1.54, $P < 0.01$) (Fig. 1A).

Moreover, only 10 mg/kg of the extract (55.31 ± 4.32) and diazepam (54.90 ± 3.20) significantly increased the percentage of the number of entries into the open arms (%OAE) ($P < 0.05$) as compared to the control group (Fig. 1B). Since 10 mg/kg dose of the extract had significant effect on %OAT and %OAE, we used it as the effective dose for further subsequent studies. Although, 1 and 10 mg/kg doses of the extract showed an insignificant increase on locomotor activity, diazepam increased this parameter significantly as compared to the control group ($P < 0.05$) (Fig. 1C).

Effect of PTZ, tamoxifen and the RPH extract on fundamental behavior parameters

There was a significant increase in %OAT and %OAE in extract-receiving group compared to control, PTZ, and tamoxifen-treated groups ($P < 0.001$) (Fig. 2A and 2B). Tamoxifen decreased locomotor activity as compared to the control group. On the other hand, the extract significantly increased locomotor activity as compared to tamoxifen-receiving group ($P < 0.01$) while PTZ decreased non-significantly the locomotion as compared to the control group (Fig. 2C).

Effect of co-administration of RPH extract with PTZ and tamoxifen on fundamental behavior parameters

Percentage of OAT was significantly decreased in groups receiving PTZ or tamoxifen along with the extract compared to the extract receiving group ($P < 0.001$) (Fig. 3A). Percent OAE was significantly decreased in group receiving the extract with PTZ as compared to the group treated with the extract ($P < 0.001$). On the other hand, %OAE was decreased in tamoxifen plus extract-treated group compared to the extract receiving group, but was not significant (Fig. 3B). Locomotor activity (total) was decreased in tamoxifen or PTZ plus extract receiving groups as compared to the group treated only with the extract (Fig. 3C).

The RPH extract effect on closed arms time and number of entries into the closed arms

In the time spent in closed arms (CAT), the different doses of RPH did not show any statistically significant alteration. However, diazepam significantly decreased this parameter ($P < 0.001$) as compared to that of the control group (Fig. 4A). No significant changes were observed on the number of entries into the closed arms (CAE) for various concentrations of RPH extract compared to the control groups (Fig. 4B).

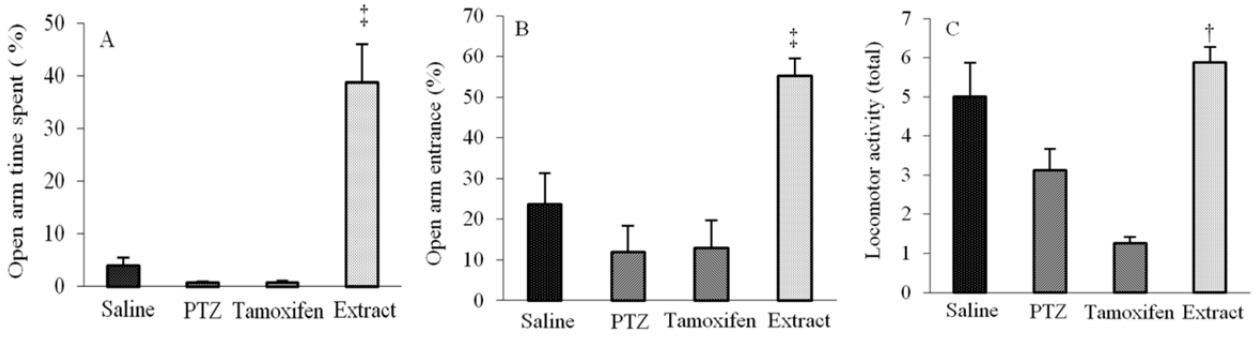

Fig. 2. The effect of RPH extract (10 mg/kg), saline, PTZ, and tamoxifen on (A) percentage of time spent in the open arms (%OAT), (B) percentage of the number of entries into the open arms (%OAE), and (C) locomotor activity (total). ‡$P < 0.001$ compared with saline, PTZ, and tamoxifen receiving groups (Mean ± SEM, n = 8) (A and B). †$P < 0.01$ compared with tamoxifen receiving groups (Mean + SEM, n = 8) (C).

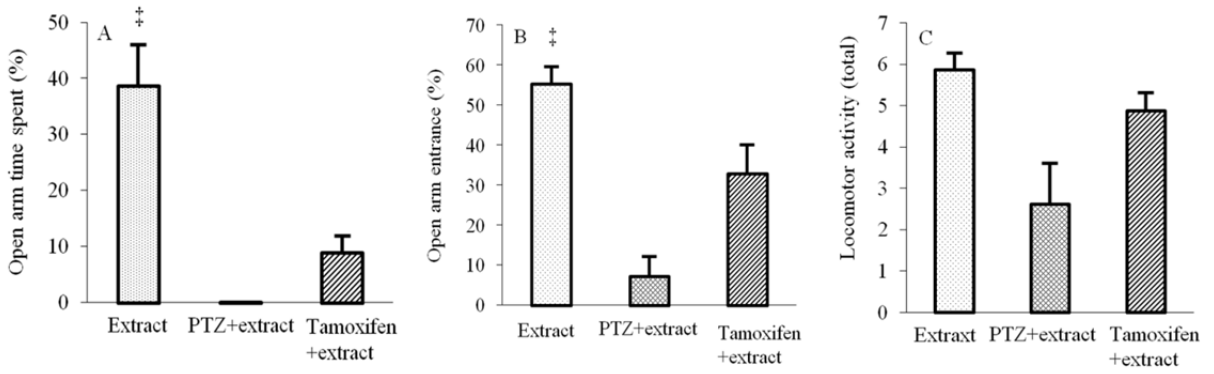

Fig. 3. The effect of RPH extract (10 mg/kg), PTZ + extract, and tamoxifen + extract on (A) percentage of time spent in the open arms (%OAT), (B) percentage of the number of entries into the open arms (%OAE), and (C) locomotor activity (total). ‡$P < 0.001$ RPH extract compared with PTZ + RPH extract and tamoxifen + RPH extract receiving groups (Mean ± SEM, n = 8) (A) and compared with PTZ + RPH extract receiving groups (Mean ± SEM, n = 8) (B).

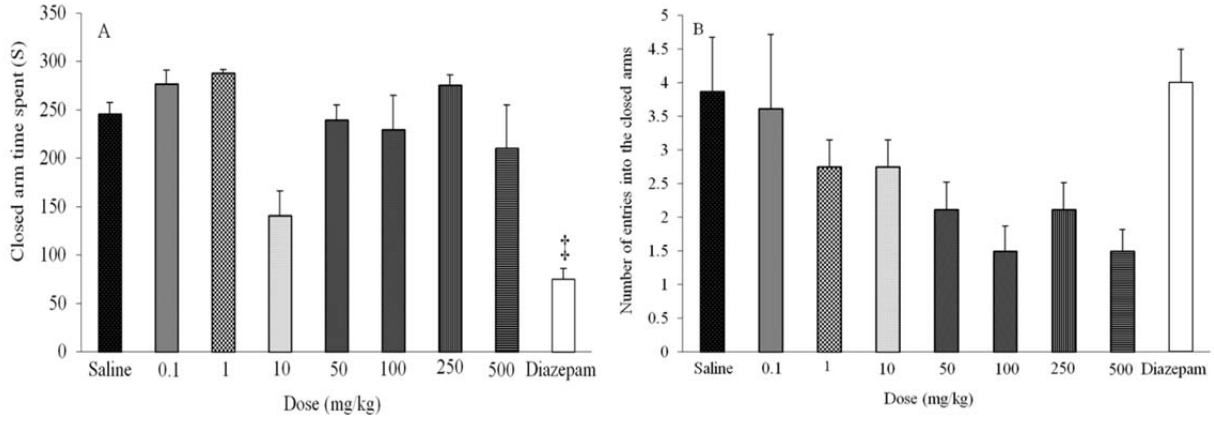

Fig. 4. The effect of different doses of hydroalcoholic extract of RPH on (A) time spent in the closed arms (CAT), and (B) the number of entries in to the closed arms (CAE). ‡$P < 0.001$ compared with saline receiving group (Mean ± SEM, n = 8).

DISCUSSION

Our results showed that the defined concentration of hydroalcoholic extract of RPH made a significant increase in %OAT and %OAE compared to saline group. Phytochemical studies have shown that RPH contain antraquinone, tannin, and flavonoids which exert estrogen-like activities (10). Elevated levels of estradiol achieved during rodent proestrus or after exogenous hormone injection to ovariectomized females, demonstrate anxiolytic actions in the elevated plus maze (16). Therefore, one might postulate that RPH extract has anxiolytic action based on the physiological actions of phytoestrogens (17).

There is some evidence that estrogens have both anxiolytic and anxiogenic activity (18). In order to avoid sexual cycle variety in our study, all animals were synchronized in estrous cycle based on their vaginal secretion and its cellular contents (14). The two types of estrogen receptor α (ERα) and β (ER_{β}) independently regulate different behavioral function synergically or antagonistically (19). ER_{β} selective agonist treatment decreased anxiety-like behaviors in elevated plus maze and open field tests. Moreover, anxiogenic behaviors including the time spent grooming were decreased by ER_{β} agonist and increased by ERα selective agonist (18,20). According to cumulative data, time spent in open arms significantly increases in dietary-rich

phytoestrogen compared to dietary lacking phytoestrogen in male and female intact rats (21). Additionally, increased anxiety and synaptic plasticity in estrogen receptor β-deficient mice has been reported (22). In fact, these molecules have the ability to selectively bind estrogen receptor ($\alpha < \beta$) (17). Although, in this study 10 mg/kg dose of RPH extract significantly increased %OAT and %OAE, other concentrations of extracts exhibited conflicting effects. It is pivotal to keep in mind that phytoestrogens compete for binding on ERs, since their effect depend on the level of endogenous estradiol. In a state of high levels of endogenous estrogens, phytoestrogens should be blocked the ERs, whereas, in a state with low levels of endogenous estrogens such as ovariectomized animal the estrogen activity of these components may be revealed (17). Taken together, other concentrations above 10 mg/kg dose of RPH extract revealed no decreasing effect on anxiety indices. Based on our observation, co-treatment of tamoxifen with extract significantly reduced the incremental effect of RPH extract on %OAT. Tamoxifen blocks estrogen receptor in the brain and results in increased anxiety levels as shown by some other researchers (8,23).

In an attempt to model human pathological anxiety in rodents, a wide range of behavioral testing patterns have been developed. The elevated plus maze test is one of the most widely used tests for measuring anxiety-like behavior (15). In this model, the sensitivity to anxiety may differ between males and females depending on numerous hormonal and neuronal factors involved in pathophysiology of anxiety (24). It has been reported that anxiolytic agents increase locomotor activity on the open arms (25,26). In the present study, tamoxifen (15 mg/kg, i.p) significantly decreased locomotor activity compared to control group. Whereas, co-treatment of RPH extract with tamoxifen diminished extract effect on locomotor activity.

Furthermore, our study showed that PTZ (20 mg/kg, i.p) reduced %OAT and %OAE compared to saline control group. PTZ is a central nervous system convulsant that is thought to act at the picrotoxine site of $GABA_A$ receptor and inhibits GABA activated channels (27). In animal studies, anxiety-like properties of PTZ have been demonstrated in a variety of anxiety models, including the elevated plus maze method (28). In this study, we showed that blockade of $GABA_A$ receptor by PTZ significantly decreased the anxiolytic effect of RPH extract. It could be assumed that to the some extent the extract effect may be mediated via $GABA_A$ receptor. In agreement with our study, Pourabbas, ***et al.*** showed that co-administration of PTZ with fennel extract decreased its anxiolytic effect (29). Besides, reducing effect of *Hypericum perforatum* extract containing flavonoid on fear behavior has been suppressed by co-administration with PTZ (30). It seems that PTZ with binding to benzodiazepine site of $GABA_A$ receptor prevents the anxiolytic effect of extract containing flavonoids. Although, we were unable to determine mRNA expression of GABA receptor in this study, krezel, ***et al.*** suggested a potential correlation between reduced inhibitory GABAergic neuro-transmission and increased anxiety in ER_β mutant rodents (31).

CONCLUSION

The present study showed that the hydroalcoholic extract of RPH has a preventive effect in experimental model of anxiety. Anxiolytic effect of RPH extract was antagonized by tamoxifen through blockade of estrogen receptor. Moreover, PTZ decreased anti-anxiety effect of RPH extract. Taken together, it might be concluded that GABA and estrogen receptors have imperative role in antianxiety effect of hydroalcoholic extract of fleshy fruit wall of pistachio in adult female wistar rats. Although, the hydroalcoholic extract of RPH was effective in this experimental model, it should be considered the pharmacological studies of all fragments of RPH extract. Moreover, interaction of RPH ingredients with selective GABA and estrogenic agonist and antagonist would be useful for further studies.

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