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Pharmacological validation of the anxiolytic, muscle relaxant and sedative like activities of *Capsicum annuum* in animal model

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Abstract

The current study deals with anxiolytic, muscle relaxant and sedative like activities of *Capsicum annuum* in animal models. The crude extract was found safe in acute behavior and toxicity studies. However, the *n*-hexane fraction caused severe acute toxicity. Pretreatment of crude extract and *n*-hexane fraction elicited marked dose-dependent antianxiety-like effects in elevated plus-maze and dark and light models. The results showed significant ($p < 0.05$) antianxiety-like effects of crude extract while highly significant ($p < 0.001$) for *n*-hexane fraction. Similarly, an effect on muscle coordination was tested in traction model, where both crude extract and *n*-hexane fraction evoked marked dose-dependent activity. The effect of thiopental-induced sleep test of crude extract and *n*-hexane fraction was highly significant ($p < 0.001$). All together, the results revealed potent anxiolytic, muscle relaxant and sedative like activities of *C. annuum* in animal models and the pharmacologically active constituents are mostly non-polar in nature.

Introduction

Global statistics revealed that approximately 450 million people throughout the world suffer from the mental illness or behavioral disorders, which account for 12.3% of the global burden of disease. It is supposed to be reached 15% by the year 2020. Amongst the many mental illnesses and behavioral disorders, anxiety and depression are the two most prevailing psychiatric disorders (He et al., 2015). Anxiety, a state of uncontrollable fear, is denoted by a hyperactive sympathetic nervous system, psychomotor tension, perturbation and vigilance syndromes (Mansouri et al., 2014). Worldwide, anxiety disorders constitute the most common forms of psycho-pathology (Hellian-Ibarrola et al., 2006) nearly inflicting almost one-eighth of the total population globally (Komaki et al., 2014).

Benzodiazepines are the most common class of drugs prescribed in anxiety and they continue to be prescribed for the ailment as such (Lader and Morton, 1991). Although as a class benzodiazepines are quite effective, acting rapidly and exhibiting good tolerability. But along with these advantages, there are certain issues that are clinically important to consider, such as, may cause dependence, disruption can lead to rebound anxiety and also memory impairment (Chouinard, 2004). This has compelled researchers to evaluate new compounds specially plant-based drugs having less undesirable effects (Tiwari et al., 2014). *Capsicum annuum* commonly known as pepper belongs to Solanaceae family. Worldwide, it is one of the most highly consumed season-free vegetables (Kim et al., 2014). Epidemiological facts indicated the useful effects of food/dietary anti-oxidant compounds in the



prevention of a number of disease states, which includes cancer, neurodegenerative disorders and cardiovascular disease (Hollman, 1999; Harborne and Williams, 2000; Ferrari and Torres, 2003).

C. annuum is reported to be an excellent source of polyphenols, particularly flavonoids such as quercetin, myricetin, luteolin, and capsaicin (Lee and Villalon, 1995; Miesan and Mohamed, 2001; Chen and Hwa, 2013), caffeic, coumaric and 3,4-dimethoxy cinnamic acid glucosides (Mattila and Hellstrom, 2007). In addition to that, in pepper leaves some other flavonoids were also identified, apigenin and its analogue apigenin 7-pioprofuransyl [1→2] glucopyranoside (Choi et al., 2007). Health promoting phytochemical constituents like quercetin, myricetin, apigenin, luteolin, are commonly exist in *C. annuum*. These show antidepressant and anxiolytic activities in the previous studies (Kumar and Sharma, 2006; Aguirre-Hernandez et al., 2010; Ishisaka et al., 2011; Impellizzeri et al., 2013). *C. annuum* is also one of the constituents in a well-known poly-herbal product Xtend[®] which is widely used in the Egypt for the antidiabetic, antianxiety and antidepressant effects (El-Ashmawy et al., 2014).

Based on its traditional uses and its potential phytochemical composition, the aims of the present study were to investigate the anxiolytic like effects of the methanolic extract and the *n*-hexane fraction of *C. annuum* in elevated plus-maze (EPM) and light and dark (LD) model behavioral test for anxiolytic drug. Additionally, the possible sedative and muscle relaxant like effects of the *C. annuum* extracts were also evaluated *in vivo*.

Materials and Methods

Drugs and chemicals

Diazepam (valium injection 10 mg/2 mL manufactured by Martin Dow), thiopental sodium, Tween 80, methanol and *n*-hexane were purchased from the local chemical market.

Animals

Healthy (male) albino mice having a weight of 25-35 g were purchased from the National Institute of Health Islamabad, Pakistan. Prior to the experiments, all mice were kept in cages having control temperature 22 ±1°C with light and dark cycle of 12 hours each. Food pellets and water were supplied regularly. Mice were grouped so that each group contained 6 animals. All experimental works were performed between 9:00 a.m. and 2:00 p.m. Pharmaceutical Ethics Committee of the Pharmacy Department at Peshawar University approved the study.

Collection of the plant material

Mature fruits of *C. annuum* (8 kg) were collected from

the District Swabi. The plant was identified as *C. annuum* in the Department of Botany, University of Islamia, Peshawar, KPK, Pakistan. For future reference, a specimen having voucher number 102 was kept in their herbarium.

After cleaning the fruits, it was dried for 15 days and then crushed into powder, having a weight of 1,800 g.

Preparation of extract

After harvesting, the fruit of *C. annuum* (8 kg) was dried in the sun. The maceration process was used. The fruit powder was macerated for 14 days with methanol at 25°C and with occasional shaking the methanolic extracts after filtration were concentrated in vacuum, resulting in a crude extract. Half of the crude extract (900 g) was dissolved in distilled water and further fractionated with *n*-hexane. The resultant fraction was filtered and dried yielding about 63 g (7%) of crude extract.

Acute toxicity test

For any possible toxicity, *C. annuum* was passed from the acute toxicity test and confirm its safety. Mice were divided into groups and treated with different doses of crude extract (500, 1000 and 1500 mg/kg) and *n*-hexane fraction of *C. annuum*, while the control group received saline and Tween 80 (10 mL/kg). For any gross effects, all experimental groups were observed during the first four hours of the experiment. The mortality was observed if any after 24 hours (Khan et al., 2010).

Elevated plus maze (EPM) model

The EPM test was used for the evaluation of anxiety like effect in the animal model (Pellow and File, 1986; Nogueira and Silvia, 1996). Briefly, The EPM model was designed with two open arms (30 cm x 5 cm) and two closed arms (30 cm and x 5 cm x 15 cm). These arms were arranged in such a way that the closed arms were located directly opposite to each other. The maze consists of the central square (5 cm x 5 cm) due to which the model seems like a plus sign. The EPM was 50 cm above the floor. The floor and the walls of all arms were painted black and wooden. The mice were divided into different groups. Each group consisted of 6 animals. The normal saline and 2.5% Tween 80 (10 mL/kg) were administered to all animals in the control group and diazepam (1 mg/kg) to the standard group. Three groups of animals were treated with *C. annuum* crude extract at a dose of (2.5, 5 and 10 mg/kg) and *n*-hexane fraction of *C. annuum* at a dose of 2.5, 5 or 10 mg/kg were administered to animals in the next three groups. During experiment, the mouse was placed one by one on the center and face of the mouse towards the open arm. For the analysis of experimental work, the number of entries and time spent on the apparatus were recorded over a period of 5 min (Gupta et al., 2013).

Light and dark (LD) model

This LD model was elaborated (Crawley and Goodwin,

1980). The LD apparatus consisted of a box with compartments having dimensions (25 cm x 33 cm x 24 cm). The compartments were connected each other by an opening (7 cm x 7 cm). Through this opening, the mouse was allowed to move from one compartment to the other. One of the compartments had 100 W bulb which emit visible light placed in at a height of 30 cm while the other compartment remained dark. Mice were divided into different groups. Each group consisted of 6 animals. The normal saline and 2.5% Tween 80 (10 mL/kg) were administered to all the animals in the control group and diazepam (1 mg/kg) to the standard group. Three groups of animals were treated with *C. annuum* crude extract at a dose of 2.5, 5 or 10 mg/kg. The *n*-hexane fraction of *C. annuum* at a dose of 2.5, 5 or 10 mg/kg was administered to animals in the next three groups. The transition of mice between the light and dark compartments and the time spent by them in it was recorded for a period of 5 min immediately just after the mouse stepped in the dark compartment (Gupta et al., 2013).

Muscle relaxant activity (Traction test)

In this procedure model, the traction test was designed as two supports placed. In these supports, a rubber-coated metal wire was crimped at a height of 60 cm. Mice were divided into different groups. Each group consists of 6 animals. These groups were treated with diazepam (1 mg/kg), normal saline and 2.5 % Tween 80 (10 mL/kg) and *C. annuum* crude (50, 100 and 200 mg/kg) and *n*-hexane fraction (5, 10 and 15 mg/kg). After 30 min of administration of the test and standard doses, the mice were exposed to the traction test. Normally mice grasp the wire with one hind foot within 5 sec. Failure to grasp the wire shows muscle relaxant activity of the test compound (Muhammad et al., 2013, Rauf et al., 2013).

Thiopental-induced sleep test in mice

The animals were divided into groups ($n=6$). The control group was treated with normal saline and 2.5% Tween 80 (10 mL/kg). The standard group was treated with diazepam (1 mg/kg). The next three groups were treated with the crude extract of *C. annuum* at a dose of 50, 100 or 200 mg/kg i.p. and remaining three groups were treated with *n*-hexane fraction of *C. annuum* at a dose of 2.5, 5 or 10 mg/kg. Thirty minutes after the administration of test doses, thiopental sodium 30 mg/kg (i.p.) was injected intraperitoneally. The onset of sleep and the duration of sleep were observed in each animal. The duration of time between the loss and regaining of righting reflex were considered sleeping time (Dandiya, 1959, Rauf et al., 2016).

Statistical analysis

The results obtained from all the experiments were expressed as the mean \pm S.E.M. All these results were analyzed with the help of One-way Analysis of Variance (ANOVA).

Results

Effect of acute toxicity test

The effect of acute toxicity test of the crude extract and *n*-hexane fraction of *C. annuum* is present in Table I. Following intraperitoneal administration of *C. annuum* crude extract at the following doses 500, 1000 and 1500 mg/kg, there showed no toxicity and also no significant changes in behavior were demonstrated. The groups tested with *n*-hexane fraction of *C. annuum* caused bleeding from the mouth of the mouse and thus lead to death within 5 min, of three animals at a dose of 15 mg/kg and all the mice died on 20 mg/kg.

	Control	Crude extract (mg/kg)				<i>n</i> -hexane fraction (mg/kg)		
Observation	10	500	1000	1500	10	15	20	
Alertness	+	+	+	+	+	+	-	
Awareness	+	+	+	+	+	+	-	
Touch response	+	+	+	+	+	+	-	
Sound response	+	+	+	+	+	+	-	
Temperature	-	-	-	-	-	-	-	
Food intake	+	+	+	+	+	+	-	
Sedation	-	-	+	+	+	+	+	
Drowsiness	-	-	-	+	-	+	+	
Diarrhea	-	-	-	-	-	-	-	
Survived	All	All	All	All	All	3	6	
Death	-	-	-	-	20%	50%	100%	
LD ₅₀		-				15 mg/kg		

Effect in EPM model

The effect of elevated plus maze model test of the crude extract and *n*-hexane fraction of *C. annuum* is present in Figure 1 and Figure 2 respectively. The administration of diazepam (1 mg/kg) significantly increased the entries into the open arms and also time spent in there. The pre-treatment of the crude extract (50,100,200 mg/kg) produced a significant ($p < 0.05$) reduction in the time spent in the closed arm. However, the *n*-hexane fraction was comparatively potent and evoked highly significant ($p < 0.001$) effect at the 2.5, 5, and 10 mg/kg treated mice by increasing in the number entries to open arms and also time spent in the open arms. Time spent in the closed arms were also markedly decreased.

Effect in LD model

The effect of light and dark test of the crude extract (Figure 3) and *n*-hexane fraction (Figure 4) of *C. annuum* is present in Figures. The crude extract of *C. annuum* at the dose of 100 and 200 mg/kg produced a significant ($p < 0.01$) increase in the time spent on the illuminated box of the LD apparatus compared to the control group. At these doses, it also produced a significant ($p < 0.001$) increase in the transitions of mice to the light side. Those groups tested with the *n*-hexane fraction of *C. annuum* (5 mg/kg) showed a significant increase in the time spent on illuminated side as well as the number of

transitions were also increased extremely significantly.

Effect of traction test

The effect of traction test of the crude extract and *n*-hexane fraction of *C. annuum* is present in Table II. The crude extract of *C. annuum* at 50 and 100 mg/kg did not show any significant muscle relaxant effect (0% failure). However, the crude extract after the administration of 200 mg/kg caused a significant increase in the reestablishment time at that dose 37% mice fail to made reestablishment within 5 sec. The groups tested with the *n*-hexane fraction at dose 2.5 mg/kg showed the normal reestablishment time while at 5 and 10 mg/kg the reestablishment time increased in resulting in 37% and 87% failure respectively of mice.

Effect in thiopental-induced sleep in mice

The effect of thiopental-induced sleep test of the crude extract and *n*-hexane fraction of *C. annuum* is presented in Figure 5. The group treated with the crude extract at 200 mg/kg showed significant ($p < 0.01$) decrease in the onset time and significantly ($p < 0.05$) increased the duration of sleep. The group tested with *n*-hexane fraction at a dose of 10 mg/kg elicited significantly ($p < 0.001$) decrease in the onset of action and significantly increased the duration of sleep.

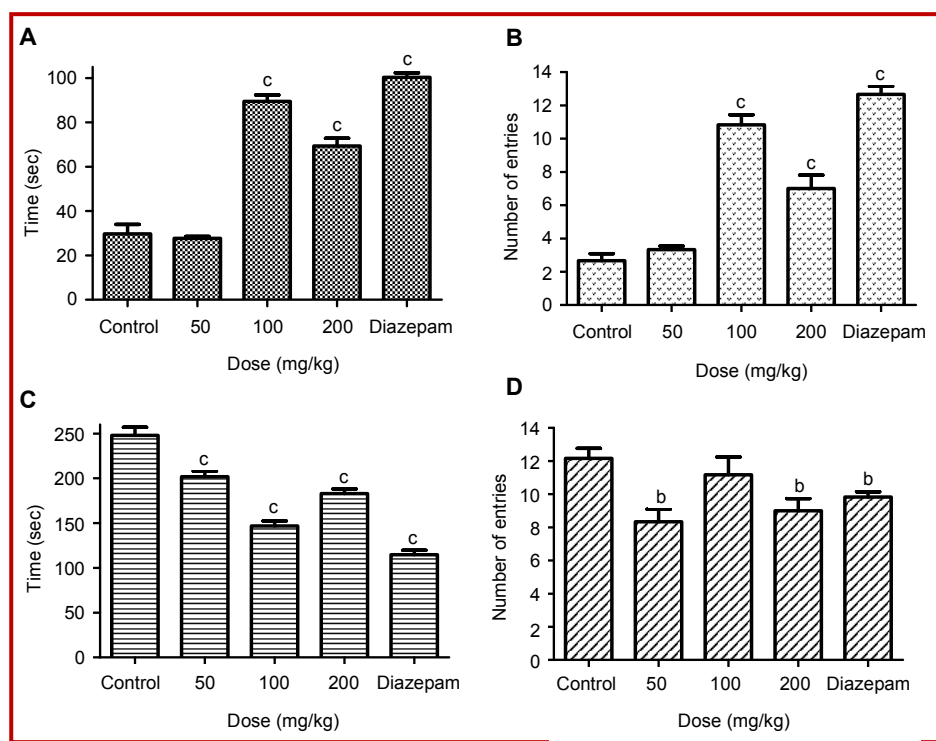


Figure 1: Effect of crude extract of *C. annuum* at test doses in elevated plus-maze model. Time spent in open arm (A), Number of entries to open arm (B), Time spent in close arm (C), Number of entries to closed arm (D). Control (Saline and tween 80 2.5%), 50,100,200 mg/kg (crude extract of *C. annuum*), and Diazepam 1 mg. All values are expressed as mean \pm SEM, $n = 6$, ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$

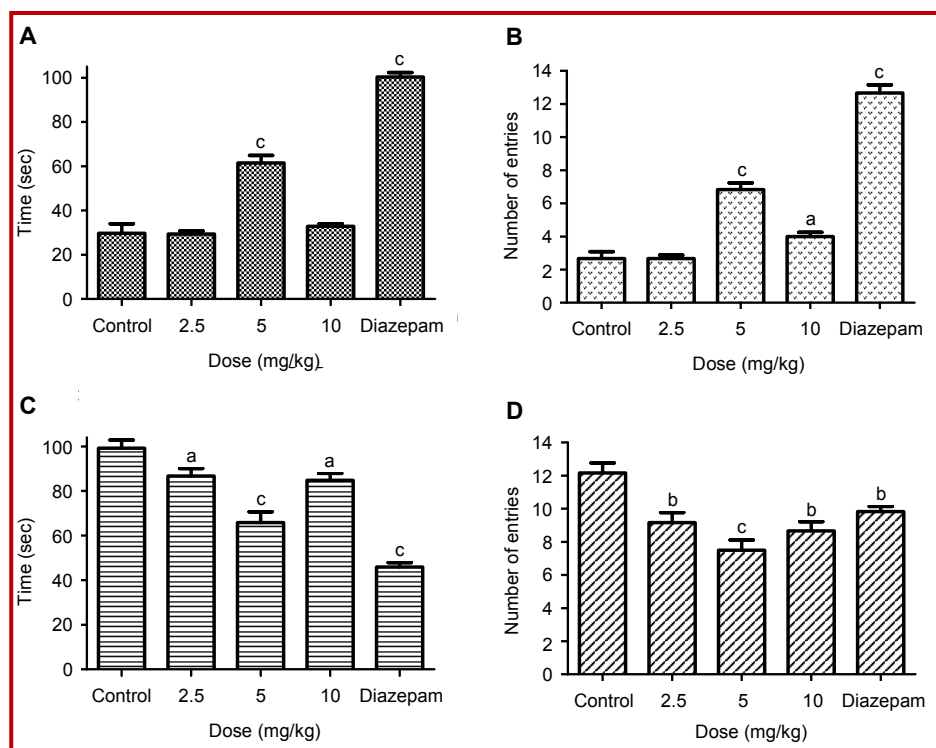


Figure 2: Effect of *n*-hexane fraction of *C. annuum*, at test doses in elevated plus maze model. Time spent in open arm (A), Number of entries to open arm (B), Time spent in close arm (C), Number of entries to closed arm (D). Control (Saline and Tween 80 2.5%), 2.5,5,10 mg/kg (*n*-hexane fraction of *C. annuum*), and diazepam 1 mg. All values are expressed as mean \pm SEM; n = 6; *p<0.05, ^bp<0.01, ^cp<0.001

Table II			
Effect of crude and hexane fraction of <i>C. annuum</i> in traction test			
Traction test			
SL. No.	Treatment	Dose	% Failure
1	Normal saline	10 mL/kg	0.0%
2	Diazepam	1 mg/kg	75 ^c
		2 mg/kg	100 ^c
3	Crude extract	50 mg/kg	0.0
		100 mg/kg	0.0
		200 mg/kg	37.5 ^b
4	<i>n</i> -Hexane fraction	2.5 mg/kg	0.0
		5 mg/kg	37.5 ^b
		10 mg/kg	87.5 ^c

Discussion

The current study revealed marked anti-anxiety (elevated plus maze and light-dark models), muscle relaxant (traction test) and sedative (thiopental-induced sleep in mice) like effects of crude extract and *n*-hexane fraction of *C. annuum*. However, the *n*-hexane fractions elicited actions that were highly pronounced than crude extract including its toxicity.

In acute toxicity assessment of the crude extract and *n*-hexane fraction of *C. annuum*, the acute toxicological effect was observed for *n*-hexane fraction only. Previous studies showed that hexane fraction of *Capsicum* fruit at 200 mg/kg cause death of all the mice (Johnson, 2007). Among the capsinoids found in the pepper capsaicin and its analogue dihydrocapsaicin are the most abundant one, these two almost comprises 80 to 90% of the total flavonoids (Wesolowska et al., 2011).

The capsaicin is very potent and toxic in nature. It was already reported that in acute oral toxicity study the capsaicin LD₅₀ value is 118 mg/kg for oral route. Those animals which were died, their examination showed that hemorrhage of the gastric fundus occurred in them. In comparison to oral toxicity the intravenous, subcutaneous and intraperitoneal, LD₅₀ values were lower (Johnson, 2007). The large variation in the acute toxicity of capsaicin was found which depends on the route of administration. LD₅₀ varies from 0.56 mg/kg to 60–75 mg/kg (in ethanol) and 19 mg (122–294) mg/kg (in dimethyl sulfoxide), following intragastric intubation. Respiratory paralysis was the possible cause of death in all the mice.

The EPM is a widely used paradigm for the assessment of antianxiety like effects of test articles. Indeed, it is considered as a versatile and specifically designed method for the compounds that mediate the antianxiety

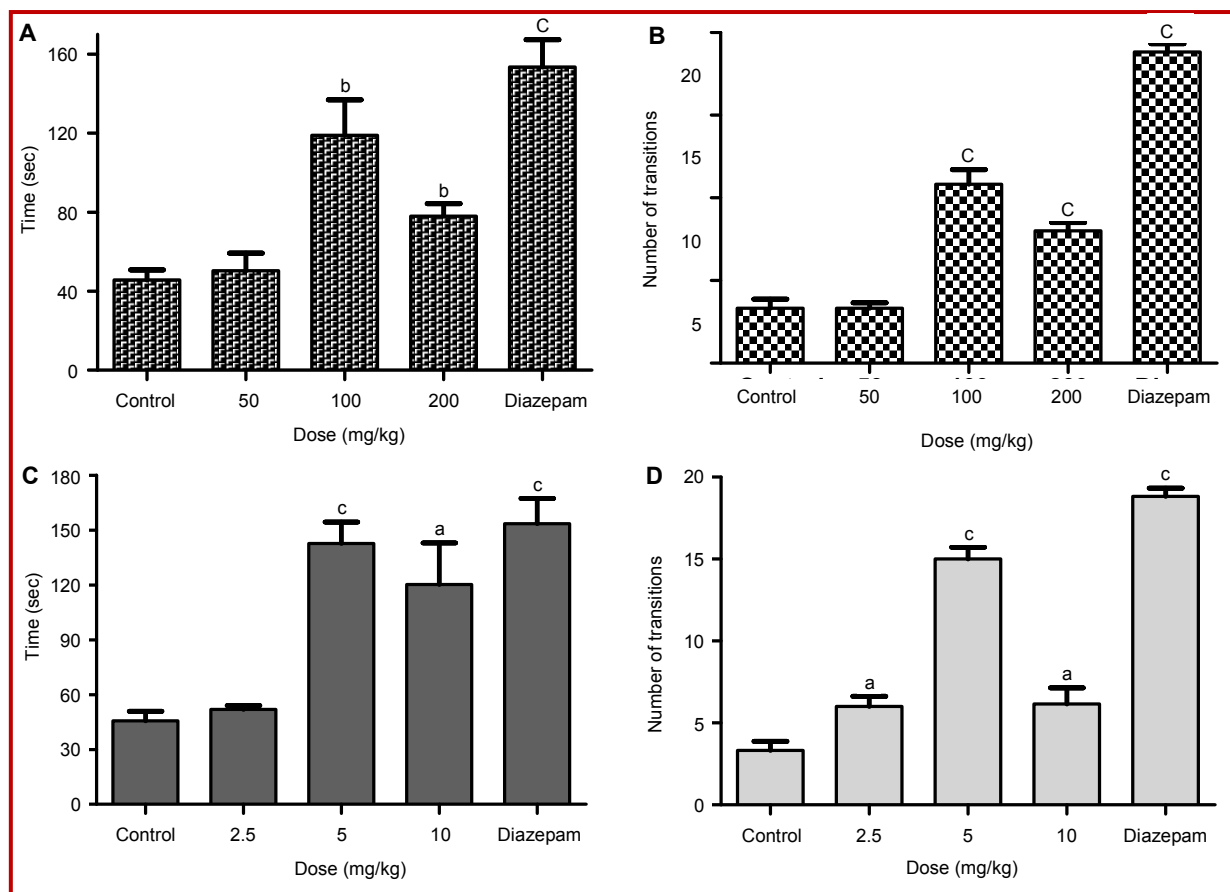


Figure 3: Effect of crude extract and n-hexane fraction of *C. annuum* at test doses in Light and dark model. Time spent on illuminated side (A), No of Transitions (B) for crude extract and Time spent on illuminated side (C), No of Transitions (D) for n-hexane fraction. Control (Saline and tween 80 2.5%), 50,100,200 mg/kg (crude extract of *C. annuum*), 2.5, 5,10 mg/kg (n-hexane fraction of *C. annuum*) and Diazepam 1 mg. All values are expressed as mean \pm SEM, n=6, *: P<0.05, **: P<0.01,***: P<0.001

like through benzodiazepine receptor (Silva and Brandao, 2000; Walf and Frye, 2007). In this study, it was observed that the crude extract and hexane fraction produced anxiolytic like effect at the dose of 100 and 5 mg/kg respectively in EPM. It significantly increased the time spent in open arms as well as the entry to the open arms. At these doses, it significantly decreased the time spent in the close arms. In the light and dark model, the hexane fraction at 5 mg/kg elicited almost the same effect as the standard diazepam. It significantly increased the time spent in the illuminated side of the model and also enhanced the number of transitions to the illuminated side.

Traction test was used to determine the muscle relaxant activity of the extract and in comparison, with the standard diazepam. The n-hexane fraction at a high dose of 10 mg/kg increase the reestablishment time significantly. The mice failed to put the hind paw on the wire within 5 sec which reflects the strong muscle relaxant property the crude extract at a high dose of 200 mg/kg also displayed a significant muscle relaxant activity.

The centrally acting effect of the extract was indicated because the latency time was reduced and the duration of sleep was prolonged. The hexane fraction at 10 mg/kg shows almost the same effect as diazepam. The crude extract at a high dose of 200 mg/kg showed significant sedative and hypnotic activity while at a lower dose of 50 and 100 mg/kg, the results were almost same as for the control.

Diazepam evoked the anxiolytic, muscle relaxant and sedative like effects by impairing the action of GABA_A (Yemitan and Salahdeen, 2005; Rauf et al., 2013). Binding of diazepam to the gamma sub-unit of the GABA_A receptor results in the modification of their structure due to which the activity of GABA_A receptor increases. Which increases the frequency of channel opening as the frequency increase it ultimately results in an increased chloride ion conductance and inhibition of the action potential (Ali et al., 2008; Mahendran et al., 2014). This may explain the underlying mechanism of action for our tested extracts, because the overall expression was similar to diazepam. Anxiolytic, sedative and muscle relaxant like effects of diazepam are in

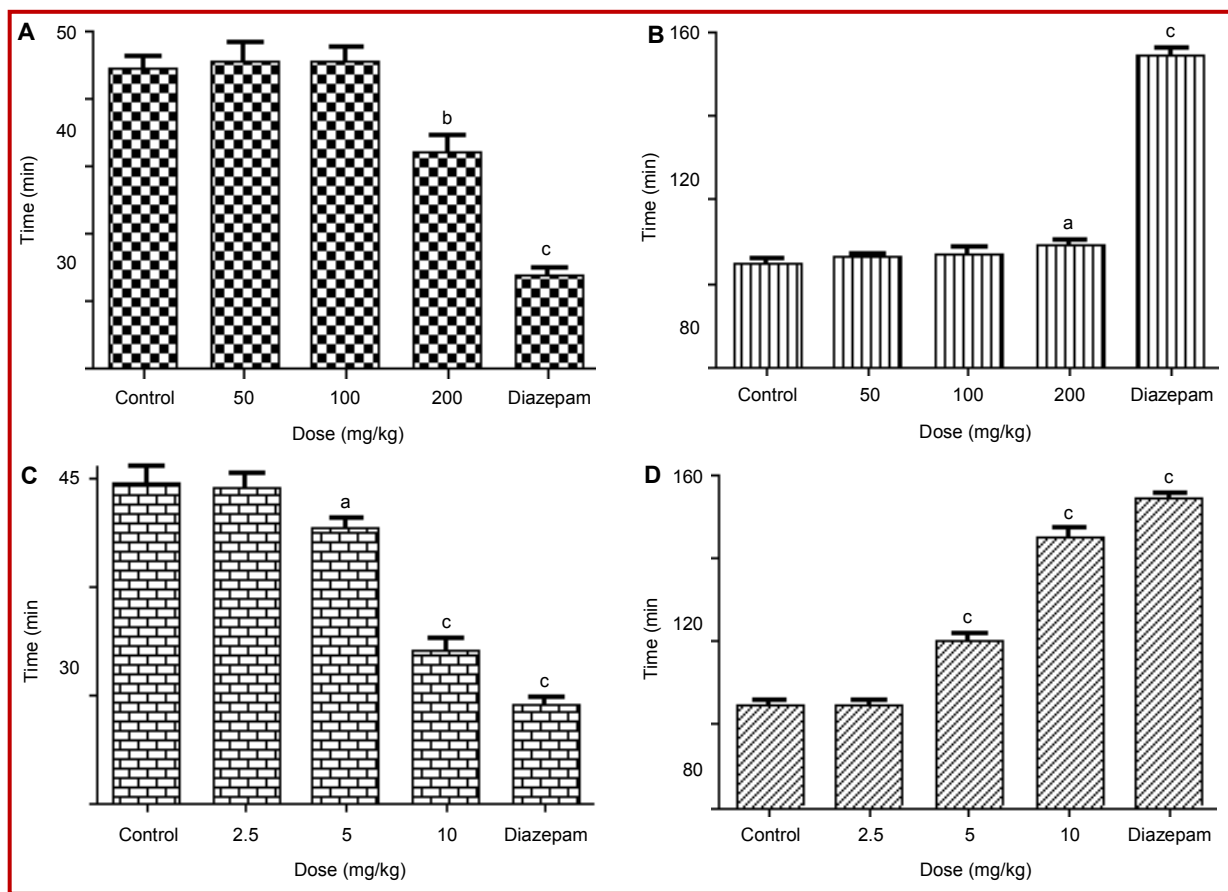


Figure 4: Effect of crude extract and *n*-hexane fraction of *C. annuum* at test doses in thiopental induced sleep in mice. onset time of sleep (A), duration of sleep (B) for crude extract and onset time of sleep (C), duration of sleep (D) for *n*-hexane fraction. Control (Saline and tween 80 2.5%), 50,100,200 mg/kg (crude extract of *C. annuum*), 2.5,5,10 mg/kg (*n*-hexane fraction of *C. annuum*), and Diazepam 1 mg. All values are expressed as mean \pm SEM, $n=6$, ^a $p<0.05$, ^b $p<0.01$, ^c $p<0.001$

ascending order of dose. Our extract showed similar behavior, so *C. annuum* extract might be worked through GABA which is similar to diazepam.

C. annuum is reported to be an excellent source of polyphenols, particularly flavonoids such as quercetin, myricetin, luteolin, and capsaicin (Lee and Villalon, 1995; Mian and Mohamed, 2001; Chen and Hwa, 2013), caffeic, coumaric and 3,4-dimethoxy cinnamic acid glucosides (Mattila and Hellstrom, 2007). In addition to that in pepper leaves some other flavonoides were also identified, apigenin and its analogue apigenin 7-piofuranosyl [1 \rightarrow 2] glucopyrano-side (Choi et al., 2007) and the previous data on these constituents suggest their anxiolytic and antidepressant activity (Lister, 1987, Crupi et al., 2013, Impellizzeri et al., 2013). So, the anxiolytics and sedative like effects may be due to these compounds. Hexane fraction is so potent because the HPLC analysis of it shows that this fraction contains most abundantly the three major compounds quercetin, luteolin and kamperol which have been already reported for its anxiolytic like effect.

Conclusion

The crude methanolic extract (100 mg/kg) and *n*-hexane fraction (5,10 mg/kg) of *C. annuum* possess strong muscle relaxant and anxiolytic like activity while the *n*-hexane fraction at a dose 10 mg/kg possesses strong sedative like activity. Therefore, this *C. annuum* may possibly be considered for the treatment of anxiety and related neuropsychiatric disorders by conducting further pharmacological studies and mechanism of sedative and anxiolytic like action.

Ethical Issue

The study was approved by the ethical committee of Department of Pharmacy, University of Peshawar.

Conflict of Interest

The authors declare that there is no conflict of interest.

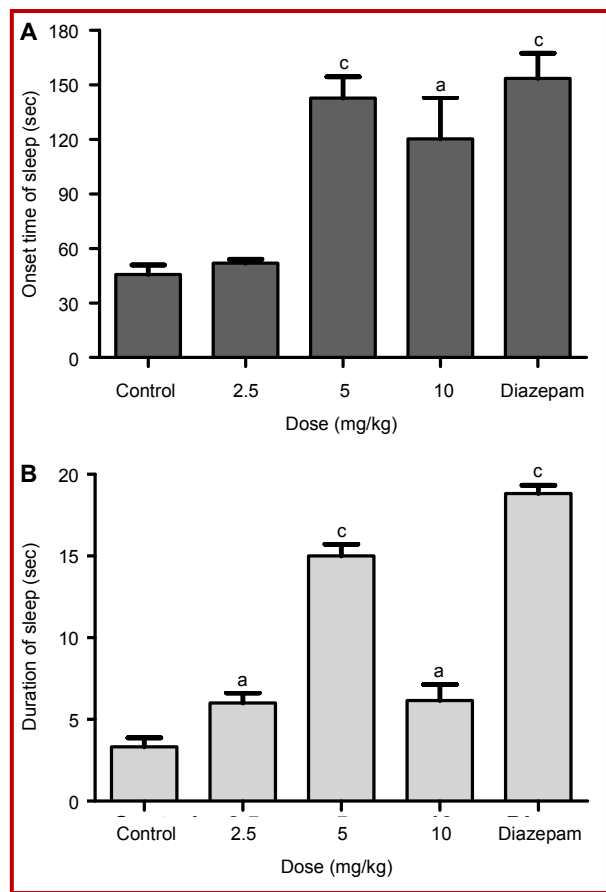


Figure 5: Effect of *n*-hexane fraction of *C. annuum* at test doses in thiopental induced sleep in mice. Onset time of sleep (A), duration of sleep (B). Control (Saline and Tween 80 2.5%), 2.5, 5, 10 mg/kg (*n*-hexane fraction of *C. annuum*), and diazepam 1 mg. All values are expressed as mean \pm SEM; n = 6; *p<0.05, **p<0.01, ***p<0.001

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