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Research Article

ANTI-ANXIETY EFFECT ON ETHANOLIC EXTRACTS OF *AGAVE AMERICANA* LINN

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ABSTRACT

The aim of present study is to demonstrate the anti anxiety effects of ethanolic extract of *Agave Americana* (L) leaves in rat and mice. Diazepam 2mg/kg (p.o) was used as positive control. The animal treated orally (p.o) with dose of 200 and 400 mg/kg of ethanolic extract. It showed significant action in the elevated plus maze, time spent and number of entries in open, close arm. The hole board test showed a significant increase in the time spent in head dip latency, head dip count, rearing, 1st head dip latency, and decrease locomotion. In light dark model treatment with this extract showed increase in time spent in light compartment, entries in light, dark compartment and number of crossing. In social interaction the ethanolic extract significantly increase social interaction time of low light, unfamiliar test condition. These results indicate an anxiolytic action from *Agave Americana* (L) leaves extract on mice and rat, probably due to the action of flavonoid present in the *Agave Americana* (L) leaves.

Keywords: *Agave Americana*, Anxiolytic, Elevated plus maze, Hole board, Light dark, Social Interaction, per oral.

INTRODUCTION

Human anxiety is defined as a feeling of apprehension, uncertainty or tension stemming from anticipation of an imagined / unreal threat[1]. Anxiety related disorders such as generalized anxiety, panic, obsessive-compulsion, phobias or post traumatic stress disorders are common and major cause of disability[2] and 1/8th of the total population worldwide affected with anxiety and became a very important area of research interest in psychopharmacology[3]. Anxiety is also an important component of many other psychiatric or medical conditions. Effective treatments such as anxiolytic drug therapy or cognitive behavioral therapy exist but, many patients remain untreated, experience adverse effects of benzodiazepines, or do not benefit from full symptom control. It has been estimated that 43% of anxiety sufferers use some form of complementary therapy. The most popular treatments include herbal medicines. Similarly, anxiety disorders are amongst the most common reason for people to try with herbal medicines[4].

The *Agave americana* Linn Family-Agavaceae, is traditional medicinal plant has been used in ayurvedic medicine for fish poison, it is given internally as a febrifuge in malaria and various other fevers; externally it is applied to wounds as an antiseptic and tonic[5]. The leaves and root is used as a cure for toothache[6]. It is reported, that possess active constituents flavonoids like Dihydroxy-6, 5'-dimethoxy-3', and 4' methylenedioxyflavanone has been isolated from *Agave americana*[7]. Recent study on anxiety claims that the flavonoids, alkaloids and terpenoids are responsible for anxiolytic (anti anxiety) and sedative activity[8, 9, 10]. In view of this, the primary aim of the present study was to investigate the possible antianxiety activity of *Agave Americana* leaves of ethanolic extract in laboratory animal.

MATERIALS AND METHODS

Plant Material

The young leaves of the plant *Agave Americana* Linn were collected during November 2010 from Gulbarga district, Karnataka. It was identified and authenticated by Mr. Y.N.Seetharam, Botanist from Gulbarga University from Gulbarga district, Karnataka, India where a voucher specimen with the number (HGUG S797) was deposited.

Preparation of extracts [11, 12, 13]

The plants collected were carefully protected and leaves were separated. The leaves were carefully washed with tap water and left to dry for 20 days in the shade at room temperature. Then they were stored in well sealed cellophane bags, so as to prevent from the environmental effects. 335 g of the harvested fresh leaves of *Agave Americana* Linn was completely extracted in 1L of 99.9% ethanol for 08-12 h hours using Soxhlet extraction procedure. The extract was filtered and the filtrate evaporated to dryness under reduced pressure using a rotatory evaporator into an aromatic green-to brown sticky residue over a water-bath, giving a yield of 9.24% (w/w). This was then stored in air-proof container, which was kept in the refrigerator at a temperature of 4ºC for about a week before the experiment begun. From this stock, fresh solution of the extract dissolved in distilled water was prepared.

Drug and Chemical

The drug Diazepam purchased from Reliance Formulation Private Limited, Chaekosons Chemic, Ahmadabad, Gujarat and Ethanol-Ghangshu Yangyuan chemical; China.

Preliminary phytochemical screening[14, 15]

The preliminary phytochemical screening revealed the presence of alkaloids, glycosides, carbohydrates, amino acid, flavonoids, tannins and saponins. (Table - 1)

Table1:Preliminary phytochemical group tests for the ethanol extract of leaves of *Agave Americana* Linn.

Phytoconstituents	Ethanol extract of leaves of *Agave americana* Linn
Alkaloids	+
Glycosides	+
Carbohydrates	+
Amino Acids	-
Flavonoids	+
Tannins	+
Saponins	+

-Absence +Presence

Experimental animals

Albino male wistar rats and albino mice weighing between 150 to 250g and 20 to 30 gm respectively were procured form registered breeders (Mahavir Enterprise, Hyderabad).The animals were housed under standard conditions of temperature 20 ± 25 ^{0}C and relative humidity 30- 70% with a 12:12 light-dark cycle. The animals were fed with standard pellet diet and water ad libitum. Approval of CPCSEA for the Institutional Animal Ethics Committee (IAEC) no. 346/CPCSEA of Luqman College of Pharmacy, Gulbarga was taken for conducting anxiolytic activity.

Acute toxicity studies[16]

Acute oral toxicity study for the formulation was carried out using OECD guideline 425 (Adopted 3rd October 2008). The test procedure minimizes the number of animals required to estimate the oral acute toxicity of a chemical and in addition estimation of LD50, confidence intervals. The test also allows the observation of signs of toxicity and can also be used to identify chemicals that are likely to have low toxicity.

Elevated Plus-Maze Test in mice[17, 18, 19, 20]

The maze consisted of two open (28 cm x 5 cm) and two closed (28 cm x 5 cm x 14 cm) arms, extending from the central platform (5 cm x 5 cm) and elevated up to the height of 25 cm above the floor. The entire maze was made of clear Plexiglass. Mice of either sex weighing between 20 to 30gm were individually placed on the centre of the maze facing an open arm, and the number of entries and the time spend in closed and open arm were recorded during a 5 min observation period. Arm entries were defined as entry of all four paws in to the arm. Standard drug (Diazepam) was administered 45 min prior to testing and extracts were administered p.o 45 min prior to testing. The following parameters were calculated for each animal; a) open arm time, b) close arm time, c) open arm entry, d) closed arm entry

Hole board test in rats[21]

The hole-board apparatus was an open-field arena with 16 equally spaced holes of 3 cm diameter in the floor. Rats were placed singly in the centre of the hole-board, and during a 5-min trial. Albino rats of either sex weighing between 150-250 gm were dividing into four groups of six animals each. Standard drug (Diazepam) was administered 30 min prior to testing and extracts were administered p.o 45 min prior to testing. Following measures were recorded: a) Head dip latency, b) Number of head dips in the holes, c) Total locomotors activity (numbers of squares crossed), d) Number of rearing, e) Latency to the first head dips

Light-dark model transition test in mice[22, 23, 24, 25]

The apparatus for light/dark transition test consist of two compartments: one light area (27 L x 27 W x 27 H cm), illuminated by 100 W desk lamp was painted white, and the other dark area (18 L x 27 W x 27 H cm) was painted black. The two compartments were separated by a partition with a tunnel (7.5 x 7.5 cm) to allow passage from one compartment to the other. Animal was placed in the centre of the light area with its back to the opening. Albino mice of either sex weighing between 18-25 gm were dividing into four groups of six animals each. Standard drug (Diazepam) was administered 30 min prior to testing and extracts were administered p.o 45 min prior to testing. The following parameters were recorded during 5 min: a) Total time spend in the illuminated part of the cage, b) Time spend in the dark part of the cage, c) Number crossing between the light and dark area, d) Total number of crossing

Social interaction test[26]

A total of 24 male albino rats (Rattus norvegicus), 150-250 gm divide in to four groups of six animals (three pairs) each. Standard drug (Diazepam) was administered 30 min prior to testing and extracts were administered p.o 45 min prior to testing. They were housed singly for 5 days before the experimental test, and were allowed food and water ad libitum. During this period they were weighed and handled daily and the position of the cages in the rack was changed so that all rats received equal experience of the different levels of illumination. The rats were randomly assigned to 'low light and unfamiliar' test conditions. The test box was 65 x 65 cm with walls 47 cm high. Pairs of rats were placed in this box for 10 min and their behaviour observed on a television monitor in an adjacent room. The following behaviors were scored: sniffing, nipping, grooming, following, mounting, kicking, boxing, wrestling, and jumping on, crawling and under or over the partner.

Statistical analysis

The values were expressed as (n=6) mean ± SEM. The results were subjected to statistical analysis by using ANOVA followed by Dennett's- t - test to calculate the significance difference if any among the groups. P<0.05 was considered as significant.

RESULT

Effect of ethanolic extract on elevated plus maze test

The effects of ethanolic extract (Eth1 and Eth2 - 200mg/kg and 400mg/kg) and diazepam were summarized in Table 2 and Figure. 1to4. Diazepam has increased the percentage of time spent (P < 0.001, Figure 1) in open arms and entries in open(P < 0.01, Figure. 3) and close arm (P < 0.01, Figure. 4) significantly, whereas in closed arm time spent decreased significantly (P < 0.001, Figure. 2) as compare to control group. It was seen that the ethanolic extract (200mg/kg) has significantly increased percentage of time spent in open arm (P < 0.01, Figure. 1) whereas in time spent in close arm (P < 0.01, Figure. 2) decreased as compare to control. The studies with that of ethanolic extract (400mg/kg) shows significant results as compare to control group. Eth 2 has significantly increased the percentage time spent (P < 0.001, Figure. 1) in open arm and entries in open (P < 0.01, Figure. 3) and close arm (P < 0.01, Figure. 4) whereas in closed arm it has decreased time spent (P < 0.001, Figure. 2) as compare to control group.

Table 2 : Effects of ethanolic extract on elevated plus maze test in mice.

Treatment	% open arm time	% close arm time	% open arm entry	% close arm entry
Control	88.33 ± 1.40	185.7 ± 2.78	4.50 ± 0.99	6 ± 0.93
Diazepam (2 mg/kg)	176.2 ± 5.28***	107 ± 2.69***	9.66 ± 1.43**	10.83 ± 0.79**
Ethanolic (200 mg/kg)	109.8 ± 4.16**	159.5 ± 1.50***	5.66 ± 0.33ns	5.50 ± 0.61ns
Ethanolic (400 mg/kg)	172.2 ± 3.07***	119.8 ± 2.30***	9.33 ± 0.61**	9.50 ± 0.56**

All values are mean ± SEM, (n =6), one way ANOVA, followed by Dunnet's test.
*** P< 0.05, **P< 0.01, *** P < 0.001 when compared to vehicle treated group**.

Effects of ethanolic extract on elevated plus maze test after acute treatment with 200 mg/kg and 400 mg/kg extract in mice.

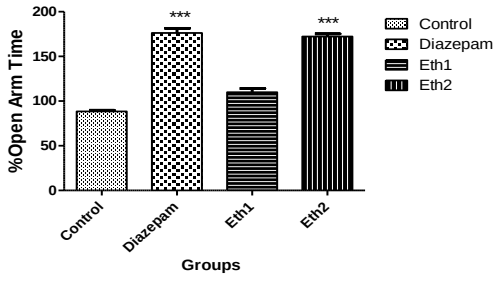

Figure 1:Percentage open arm time in 5 min EPM.

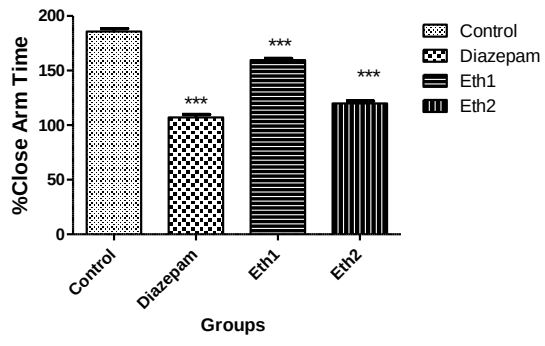

Figure 2: Percentage close arm time in 5 min EPM.

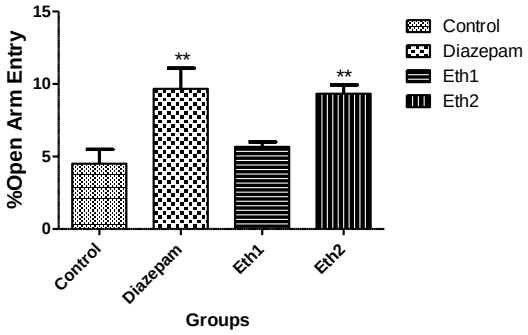

Figure 3:Percentage open arm entry in 5 min EPM.

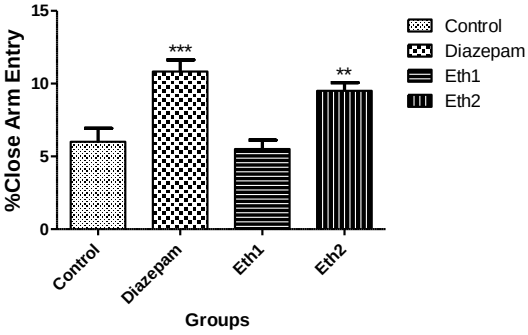

Figure 4:Percentage close arm entry in 5 min EPM.

Effect of ethanolic extract on Hole board test

The effects of ethanolic extract (200mg/kg), ethanolic extract (400mg/kg) and diazepam were summarized in Table- 3. Diazepam revealed that, significantly increase head dip latency ($P < 0.01$, Figure 5), head dip count ($P < 0.001$, Figure. 6), rearing ($P < 0.001$, Figure 8), 1st head dip latency ($P < 0.05$, Figure 9) and decrease locomotion ($P < 0.001$, Figure 7). Ethanolic extract (200mg/kg) decrease locomotion ($P < 0.05$, Figure 7), whereas ethanolic extract (400mg/kg) showed increased head dip latency ($P < 0.01$, Figure 5), head dip count ($P < 0.01$, Figure 6), rearing ($P < 0.01$, Figure 8) and 1st head dip latency ($P < 0.05$, Figure 9) significantly. The locomotion ($P < 0.01$, Figure 7) showed significant result as compare to control group.

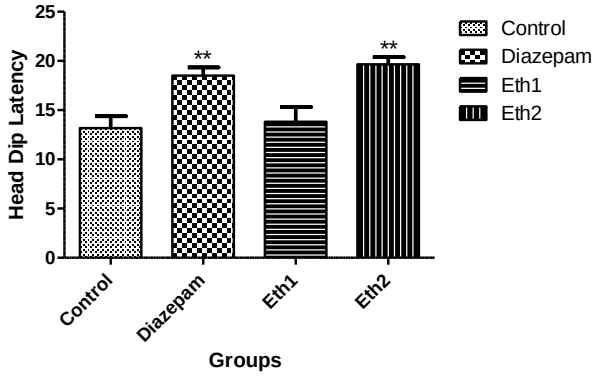

Figure 5:Head dip latency (s) in 5 min Hole Board test.

Table 3: Effect of ethanolic extract on hole board test in rat.

Treatment	Head dip latency	Head dip count	Locomotion	Rearing	1stHead Dip Latency
Control	13.17 ± 1.22	17.17 ± 1.24	53.50 ± 1.64	4.66±0.49	3.66 ± 0.61
Diazepam 2 mg/kg	18.50 ± 0.84**	23.17 ± 0.79***	44 ± 1.46***	8.5±0.42***	6.66 ± 0.66*
Ethanolic 200 mg/kg	13.83 ± 1.49ns	18.50 ± 0.34ns	47 ± 1.88*	6.16±0.65ns	4.33±0.80ns
Ethanolic 400 mg/kg	19.67 ± 0.71**	21.67 ± 1.20**	45.33±1.05**	7.50±0.34**	6.16 ± 0.65*

All Values are mean ± SEM, (n =6), one way ANOVA, followed by Dunnet's test.
*** P< 0.05, **P< 0.01, *** P< 0.001 when compared to vehicle treated group.**

Effects of ethanolic extract on hole board test after acute treatment with 200 mg/kg and 400 mg/kg extract in rat.

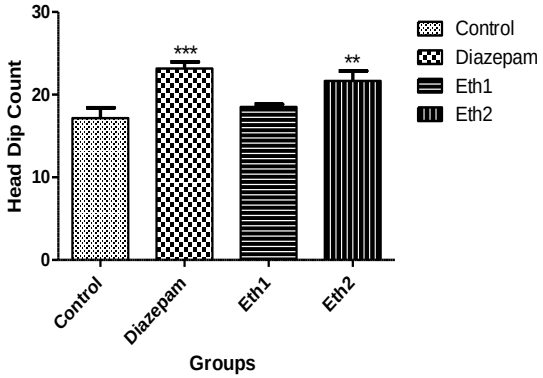

Figure 6:Head dip count in 5 min Hole Board test.

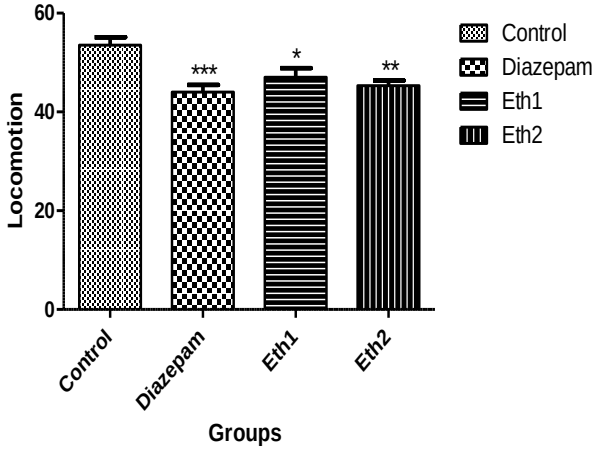

Figure 7:Locomotion in 5 min Hole Board test.

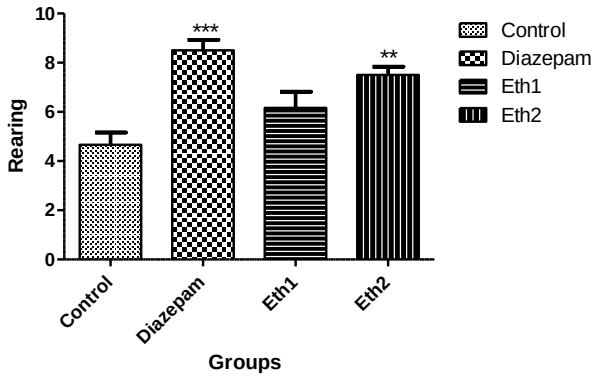

Figure 8:Rearing in 5 min Hole Board test.

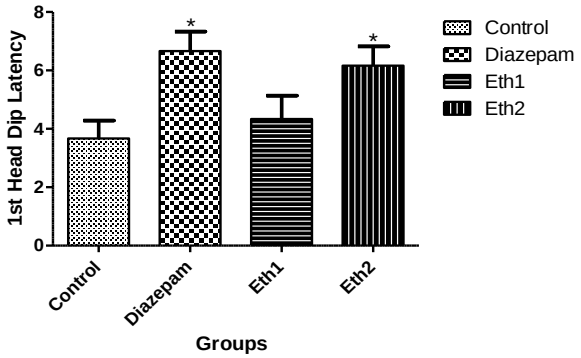

Figure 9 :1st Head dip latency (s) in 5 min Hole Board test.

Effect of ethanolic extract on light/dark transition test

Result of the light/dark transition test shown in table 4 and figure 10 to 14. Diazepam treatment group showed significantly increased time spent in light area (P < 0.001, Figure 10), entries in light, dark compartment (P < 0.01, Figure 12 and 13), and number of tunnel crossing (P < 0.01, Figure 14) whereas decrease time spent in dark area (P < 0.001, Figure 11). The ethanolic extract (200mg/kg) was showed significantly increase the time spent (P < 0.001, Figure 10) in light area and decrease the time spent (P < 0.05, Figure11) in dark area. whereas ethanolic extract (400mg/kg) has showed significantly increase the time spent in light area (P < 0.001, Figure 10), entries in light (P < 0.01, Figure 12) and dark (P < 0.05, Figure 13) compartment, and number of tunnel crossing (P < 0.01, Figure 14) whereas decrease time spent in dark area (P < 0.001, Figure 11).

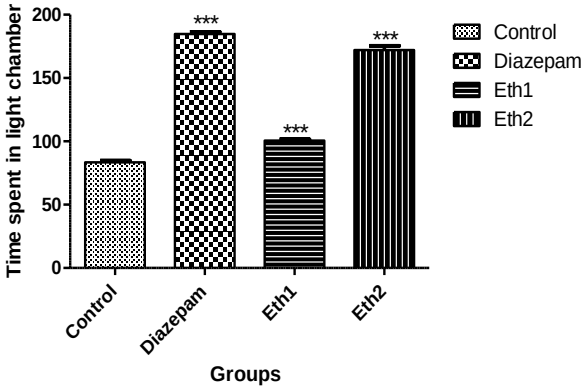

Figure 10:Time spent in light chamber (sec) in 5 min Light/Dark test.

Table 4 : Effect of ethanolic extract on light/dark transition test in mice.

Treatment	Time in light area	Time in dark area	Entry in light area	Entry in dark area	Tunnel crossing
Control	83.33 ± 1.33	194.7 ± 1.54	7.50 ± 0.56	8.66 ± 0.76	16.17 ± 1.13
Diazepam 2 mg/kg	184.7±1.66***	109.3±3.53***	10.83±0.79**	11.50±0.61**	22.33±1.14**
Ethanolic 200 mg/kg	100.5±1.05***	185.7 ± 0.84*	9.33 ± 0.42ns	9.50 ± 0.34ns	18.83±0.74ns
Ethanolic 400 mg/kg	172 ± 3.37***	111.2±2.79***	10.50±0.50**	11 ± 0.63*	21.50±1.02**

All Values are mean ± SEM, (n =6), one way ANOVA, followed by Dunnet's test.
*** P< 0.05, **P< 0.01, when compared to vehicle treated group.**

Effects of ethanolic extract on Light/dark transition test after acute treatment with 200 mg/kg and 400 mg/kg extract in mice.

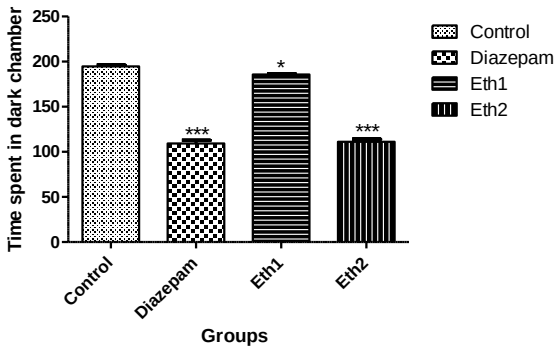

Figure 11 :Time spent in dark chamber (sec) in 5 min Light/Dark test.

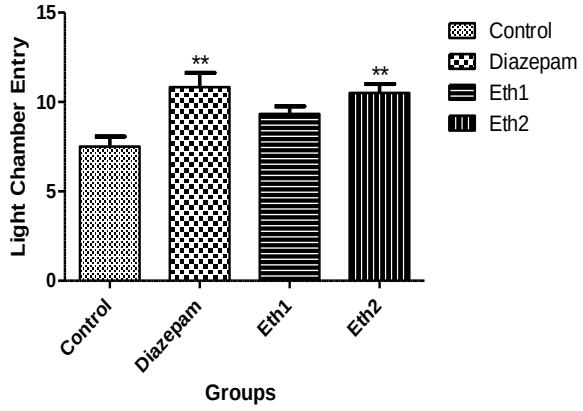

Figure 12: Light chamber entry (sec) in 5 min Light/Dark test.

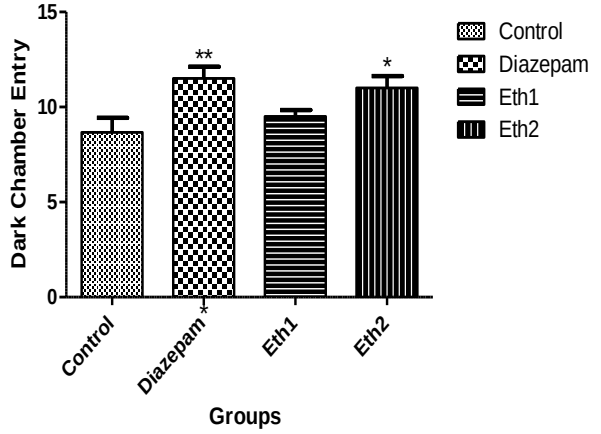

Figure 13:Dark chamber entry (sec) in 5 min Light/Dark test.

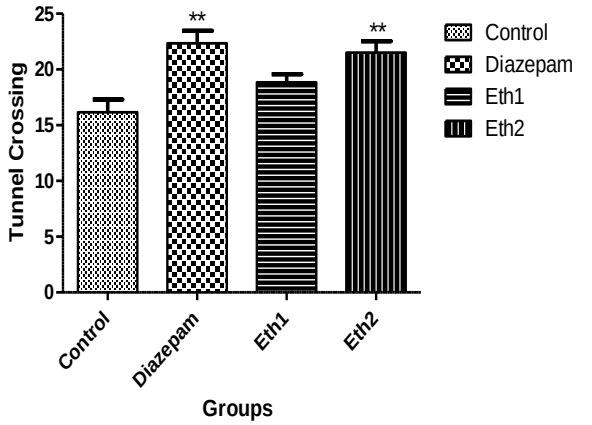

Figure 14: Tunnel crossing in 5 min Light/Dark test.

Effect of ethanolic extract on social interaction test

The effects of ethanolic extract (200mg/kg), ethanolic extract (400mg/kg) and diazepam on social interaction test were shown in Table 5 and Figure 15 and 16. Diazepam has increased in social interaction time (sec) significantly (P < 0.001, Figure 15) and decreased locomotor activity (P< 0.05, Figure 16). The ethanolic extract (200mg/kg) has shown significant result of S.I time (P< 0.05, Figure 15) but not in locomotor activity as compare to control group. The ethanolic extract (400mg/kg) has shown increased social interaction time significantly (P < 0.001, Figure 15) and decreased locomotor (P < 0.05, Figure 16) activity as compare to control group.

Table 5 :Effects of ethanolic extract on social interaction test in male rats.

Treatment	Social interaction time (sec)	Locomotion
Control	131 ± 5.85	151.3 ± 1.85
Diazepam (2mg/kg)	190.7 ± 2.60***	130 ± 5.77*
Ethanolic (200mg/kg)	159 ± 3.78*	143.3 ± 6.66ns
Ethanolic (400mg/kg)	183.3 ± 8.81***	128.3 ± 4.41*

All values are mean ± SEM, (n = 3), one way ANOVA, followed by Dunnet's test.
*** P< 0.05, **P< 0.01, When compared to vehicle treated group.**

Effects of ethanolic extract on social interaction test after acute treatment with 200 mg/kg and 400 mg/kg extract in male rats.

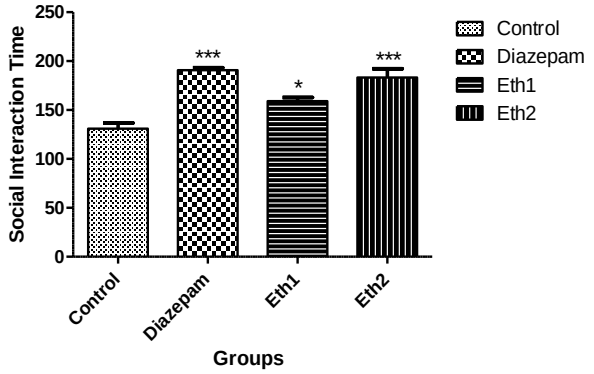

Figure 15 :Social interaction time (sec) in 10 min test.

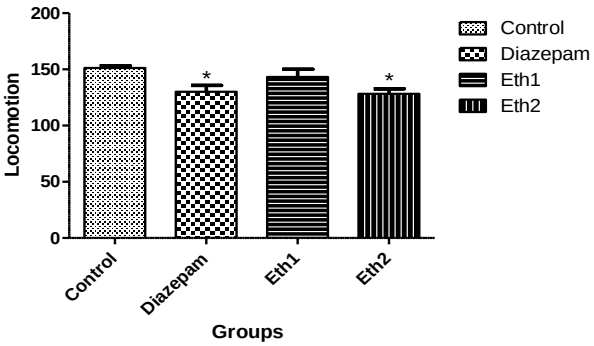

Figure 16 :Locomotion in 10 min S.I test.

DISCUSSION

The decrease in aversion to the open arm is the result of an anxiolytic effect, expressed by the increased time spent and entries in to the open arm are sensitive to agents thought to act via the GABAA receptor complex, justifying the use of diazepam as a positive control in this study[27], even when the compound being screened does not act via benzodiazepine receptors. Diazepam increases the entries and time spent in open arm confirms its anxiolytic effects. The ethanolic extract at 200mg/kg has significantly increase time spent in open arm and decrease time spent in close arm as compare to control. The ethanolic extract at 400 mg/kg had increased the percentage time spent in open arm and entry in to open and close arm with decreased percentage time spent in closed arm.

In hole board test the head dipping behavior was sensitive to changes in the emotional state of the animal, and suggested that the expression of the anxiolytic state in animals may be reflected by an increase in head dipping behavior[28]. Ethanolic extract at 400mg/kg had significantly increased head dip latency; head dip count, rearing, 1st head dip latency and decrease locomotion. Diazepam a putative anxiolytic agent compared to control group.

Drugs induced increase in behavior in the white part of a two compartment box, in which a large white compartment is illuminated and a small black compartment is darkened, is suggested as an index of anxiolytic activity[25]. The ethanolic extract at 200mg/kg increased the time spent in light area and decreased the time spent in dark area whereas ethanolic extract at 400mg/kg had significantly increased the time spent in light area, entry in light, dark chamber, tunnel crossing and decreased the time spent in dark area similar to standard drug, suggesting that anxiolytic activity of leaves extract as compare to control group.

The social interaction test was reported by File and Hyde[26] as the first animal test of anxiety, which used a natural form of behavior as the dependent measure. In this test low light and unfamiliar condition is used that generate moderate level of anxiety. Ethanolic extract at 400mg/kg that had not only significantly increased the time of social interaction in low light and unfamiliar condition but also decrease the locomotion, that suggest of anxiolytic effect does not support with benzodiazepines.

The results obtained from these experimental models clearly confirmed that the anxiolytic activity of ethanolic extracts of *Agave Americana* leaves. The ethanolic extract (400mg/kg) had significant anxiolytic activity comparable to standard drug diazepam (2 mg/kg; p.o) clearly demonstrate a dose dependant anxiolytic effect in all experimental models of anxiety. The phytoconstituents like flavonoids were reported for their anxiolytic effect and these constituents was present in ethanolic extracts of *Agave Americana* leaves, so this active principle might be responsible for anxiolytic effect. The mechanism of anxiolytic activity of *Agave Americana* leaves extracts is unclear hence further studies are needed to identify the anxiolytic mechanism(s) and the phytoconstituents responsible for the observe central effects of the ethanolic extracts of *Agave americana* leaves.

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