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Evaluation of Anxiolytic Activity of *Hygrophila auriculata* Seeds on Experimental Animals

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Abstract
To study the anxiolytic activity of ethanolic and aqueous extracts of *Hygrophila auriculata* seeds in experimental animals. *Hygrophila auriculata* seeds powder extracted in two different solvents, one is ethanol and other is aqueous. standardization of both extracts and identification of phytochemical analysis test. The extracts were orally administered at the dose of 300-600 mg/kg of body weight. Elevated plus Maze, Light-Dark model, Open Field test for studying the anxiolytic behaviour and the Rotarod was used to check the motor co-ordination and Photoactometer used for locomotor activity. Both extracts showed significantly increased the percentage of time spent and number of entries in open arm in Elevated plus maze model. In Light-Dark model, the extracts produced significant increase in time spent in Light area. In Open Field test, the extract showed significant increase in number of squares crossed, all of which are demonstrations of exploratory behaviour. In Rotarod model showed significant decrease in the motor co-ordination score with fall of time of the mice from the rotating rod which indicates its anxiolytic-like effect. In locomotor activity evaluated with photoactometer in lower cut off number point out from extracts treated groups as per diazepam treated standard group. Both extracts showed prominent anxiolytic activity, but the best result obtained with 600 mg/kg dose of ethanolic extract of *Hygrophila auriculata* in models. The present research suggests that *Hygrophila auriculata* may be a therapeutic candidate for the treatment of Anxiety disorder.

Keywords
Anxiety, *Hygrophila auricula*, elevated plus maze, light-dark model, locomotor activity, antioxidant.

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INTRODUCTION
Anxiety is an unpleasant state of tension, apprehension, or uneasiness a fear that seems to arise from a sometimes-unknown source. Disorders involving anxiety are the most common metal disturbances. The physical symptoms of server anxiety are similar to those of fear (such as tachycardia, sweating, trembling, and palpitations) and involve sympathetic action. Episodes of mild anxiety are common life experiences and do not warrant treatment.[1]

Hygrophila auriculata belonging to the family Acanthaceae called Talimkhana is described in ayurvedic literature as Ikshura, Ikshugandha, and Kokilasha "having eyes like Kokila or the Indian cuckoo," common in moist places - on the banks of tanks, ditches, and paddy fields. It is believed to be indigenous to India from the Himalayas to Srilanka, Myanmar, Malaysia, and Nepal. The plant contains various groups of phytoconstituents, phytosterols, fatty acids, minerals, polyphenols, proanthocyanins, mucilage, alkaloids, enzymes, amino acids, carbohydrates, hydrocarbons, flavonoids, terpenoids, vitamins, and glycosides. The parts of this plant are widely used in traditional medicine for the treatment of various disorders, which include

anasarca, diseases of the urinogenital tract, dropsy from chronic Bright's disease, hyperdipsia, vesical calculi, flatulence, diarrhoea, dysentery, leukorrhea, gonorrhoea, asthma, blood diseases, gastric diseases, inflammation, cancer, rheumatism, painful micturition, menorrhagia. It is also scientifically proved to have a variety of pharmacologic functions.[2]
Hygrophila auriculata seeds contain fatty acids in myristic acid, palmitic acid, steric acid, oleic acid, linoleic acid. Myristic acid produced anxiolytic-like effects only when the concentration corresponded to the one identified in human amniotic fluid (30 μg/mL) but did not alter locomotor activity. It concludes that of the eight fatty acids contained in the fatty acid mixture, only myristic acid produces anxiolytic-like effects when administered individually at a similar concentration detected in human amniotic fluid.[3]
Myristic acid produced anxiolytic-like effects only when the concentration corresponded to the one identified in human amniotic fluid (30 μg/mL) but did not alter locomotor activity. It concludes that of the eight fatty acids contained in the fatty acid mixture, only myristic acid produces anxiolytic-like effects when administered individually at a similar concentration detected in human amniotic fluid.[4]
Oleic acid produced a significant increase in norepinephrine, dopamine, and serotonin levels in a dose dependent manner. The fatty acid mixture significantly increased the frequency of entries into, and time spent on the open arms of the elevated plus maze and decreased burying behaviour in the defensive burying test, without producing significant changes in spontaneous locomotor activity. The fatty acids that are contained in maternal fluid may reduce anxiety activity.[5]
When the production of oxygen-derived metabolites prevails over the brain defence systems, however, oxidative damage to nucleic acids, proteins, and neuronal membrane lipids, which are rich in highly polyunsaturated fatty acids, can occur. oxidative stress can alter neurotransmission, neuronal function and overall brain activity. Oxidative damage in the brain causes nervous system impairment and also has been implicated in depression, anxiety disorders and high anxiety levels.[6]

MATERIALS AND METHODS

Animals

36 either sex swiss albino mice each weighing 20 to 25 grams, obtained from animal house, shree naranjibhai lalbhai patel collage of pharmacy, Umrakh was utilized in this study. animals were allowed standard diet and tap water.

Standard drug

Diazepam is mixed with distil water to obtain solution of concentration 0.05 mg/ml and administered by orally at the dose of 1 mg/kg.[7]

Test drug

Hygrophila auriculata seed was procured from Neoteric India, Coimbatore, Tamil Nadu.
Preparation of Ethanolic Extract: 100 g of seed powder was dissolved in 500 ml absolute ethanol with periodic stirring for 72 hr. Kept at incubator at 37°C to avoid any modification of active component. Crude extract was filtered and evaporated at 37°C. [8]
Preparation of Aqueous Extract: 50 g of the seed powder was mixed with 500 ml of water and put on the orbital shaker for 72 hours. This was done due to the formation of a gelatinous mass when smaller quantities of water were mixed with seed powder. The extracts were preserved at 4ºC till further use.[9]

Phytochemical analysis of *Hygrophila auriculata* seeds extract:

TLC of extract:[10] Mobile phase – 90:10 (Hexane: Ethyl ether)

Fig.No.:1 TLC of EEHA

Fig.No.:2 TLC of AEHA

Table 1: Phytochemical analysis of *Hygrophila auriculata* seeds extract:

Phytochemical Constituents	Ethanolic extract	Aqueous extract
Carbohydrates	+	+
Steroids	+	-
Alkaloids	+	-
Glycoside	+	+
Saponins	+	+
Flavonoids	-	+
Tannins	-	+
Fats & Oil	+	+
Amino acid	-	-
Resin	-	-
Phenols	-	-

Table 2: Identification of phytoconstituents in Extract:

Rf value	EEHA	AEHA
Myristic acid (0.50)	+	+
Palmitic acid (0.34)	+	+
Stearic acid (0.30)	+	+
Oleic acid (0.41)	+	+
Linoleic acid (0.55)	+	-

Pharmacological evaluation for Anxiolytic activity:

Experimental Design:

The animals were divided into six groups consisting of six animals per each group:

- Group I – Normal group.
- Group II – Diazepam 1 mg/kg orally.
- Group III –EEHA 300 mg/kg orally.
- Group IV –EEHA 600 mg/kg orally.
- Group V–AEHA 300 mg/kg orally.
- Group VI –AEHA 600 mg/kg orally.

1.Elevated plus-maze model:[11]

The plus – maze consists of two open arms and two closed arms elevated to a height of Extract and diazepam was administered to groups. 30 minutes post treatment, each mouse was placed in turn in the centre of the maze facing one of the closed arms. The cumulative times spent by each mouse in the open and closed arms of the maze were recorded for 5 to 7 min.

2.Light-Dark model:[12]

Transfer the mouse from its home cage to the testing room. It is recommended to wait 30 minutes before the experiment to habituate to the new environment. Put the mouse in the light compartment of the cage. Place the cage inside the system in a way that the dark compartment locates at the back. Let the mouse move freely between the two compartments for 5 minutes.

3.Open Filed Test:[13]

The apparatus size 50 cm in length, 50 cm in width, and 25 cm in height. The plain floor of the box was divided into 8 cm by 8 cm, with 16 squares on it. 16 squares were defined as the center and the others adjacent to the walls as the periphery. Groups of preselected animals was treated with extracts and diazepam (1 mg/kg). 30 minutes after the treatment, the animals were placed in center of open field and observed the number of squares were crossed in 5 min.

4.Rotarod model:[14]

The animals were preselected in a training session 24 h before the test based on their ability to remain on the bar (at 12 rpm). Groups of preselected animals was treated with vehicle, diazepam (1 mg/kg). 30 minutes after the treatment, the animals were placed with all four paws onto the bar, and the fall down latency was evaluated.

5.Photoactometer:[15]

This test measures the exploration and the voluntary locomotion within an enclosed area. The objective value for the spontaneous motor activity was obtained using a photo actometer. The animal was placed individually into a 30 cm × 30 cm black metal chamber with a screen floor and a light-tight lid. Six beams of red light were focused 2 cm above the floor into photocells on the opposite side. Each beam

interruption was registered as an event on the external counter. The light beam breaks were counted for 5 min.

Statistical analysis:

was carried out using Graphpad Prism Version 8. The results are expressed as mean ± SEM (Standard Error of Mean) for anxiolytic models' parameters & antioxidant parameters with one-way Analysis of Variance (ANOVA) by Dunnet's multiple comparison test. $p<0.05$, $p<0.01$ was considered as statistically significant, respectively.

RESULTS:

1. Elevated plus Maze model:

Table 3: Effect of ethanolic & aqueous extract of *Hygrophila auriculata* in Elevated plus maze model in 5 min.

Groups	Dose mg/kg	No. of entries in open arm	No. of entries in enclosed arm	% Time spent Open arm	% Time spent Close arm
Normal	**-**	2.667±0.3333	18.67±1.764	12.67±0.8819	69.22±2.248
Diazepam	**1**	9.333±0.8819**	13.67±0.8819**	50.90±1.308**	40.30±1.966**
EEHA	**300**	4.333±0.3333*	9.33±0.8819*	28.51±4.042**	59.33±1.020*
EEHA	**600**	9±0.5774**	12±1.155**	40.20±2.139**	40.56±2.162**
AEHA	**300**	3±0.5774*	16±0.5774**	18.30±1.328*	68.45±1.747*
AEHA	**600**	7.333±0.3333**	12.67±0.8819**	31.87±2.101**	50±5.357**

Values are mean ± SEM; Statistical analysis by One-way ANOVA followed by Dunnett's Multiple Comparison test. ∗P < 0.05; ∗∗P < 0.01

2.Light-Dark model:

Table 4: Effect of ethanolic & aqueous extract of *Hygrophila auriculata* in Light-Dark model in 5 min.

Group	Dose mg/kg	No. of Transition	% Time in Light	% Time in Dark
Normal	**-**	7.333±0.3333	25.33±1.333	66.33±2.33
Diazepam	**1**	14.33±0.3333**	65.33±3.93**	34.67±1.764**
EEHA	**300**	10.67±0.8819**	42.67±2.96**	46±3.512**
EEHA	**600**	13±0.5774**	50.67±1.202**	42.67±2.902**
AEHA	**300**	10±0.5774*	39.67±1.856*	52.33±4.256*
AEHA	**600**	10.67±0.3333**	45±3.786**	47.33±2.404**

Values are mean ± SEM; Statistical analysis by One-way ANOVA followed by Dunnett's Multiple Comparison test. ∗P < 0.05; ∗∗P < 0.01

3. Open filed test:

Table 5: Effect of ethanolic & aqueous extract of *Hygrophila auriculata* in Open filed test in 5 min.

Group	Dose mg/kg	No. of Square crossed (5 min.)
Normal	**-**	182.0±4.726
Diazepam	**1**	329.3±5.812**
EEHA	**300**	265.0±2.887**
EEHA	**600**	294.3±2.963**
AEHA	**300**	231.0±5.568*
AEHA	**600**	249.0±4.726**

Values are mean ± SEM; Statistical analysis by One-way ANOVA followed by Dunnett's Multiple Comparison test. ∗P < 0.05; ∗∗P < 0.01

4.Rotarod model:

Table 6: Effect of ethanolic & aqueous extract of *Hygrophila auriculata* in Rotarod model.

Group	Dose mg/kg	Fall of time (sec)
Normal	**-**	164.3±6.438
Diazepam	**1**	62.33±5.364**
EEHA	**300**	113.3±11.1**
EEHA	**600**	94.67±10.74**
AEHA	**300**	128.3±2.028*
AEHA	**600**	105.3±4.41**

Values are mean ± SEM; Statistical analysis by One-way ANOVA followed by Dunnett's Multiple Comparison test. ∗P < 0.05; ∗∗P < 0.01

5.Photoactometer:

Table 7: Effect of ethanolic & aqueous extract of *Hygrophila auriculata* in Photoactometer in 5 min.

Group	Dose mg/kg	Cut off No. (5 min.)
Normal	**-**	616.7±13.02
Diazepam	**1**	300±6.429**
EEHA	**300**	534.7±12.72**
EEHA	**600**	493±6.658**
AEHA	**300**	560±13.09**
AEHA	**600**	528.3±6.009**

Values are mean ± SEM; Statistical analysis by One-way ANOVA followed by Dunnett's Multiple Comparison test. ∗P < 0.05; ∗∗P < 0.01

5 Biochemical Parameters:

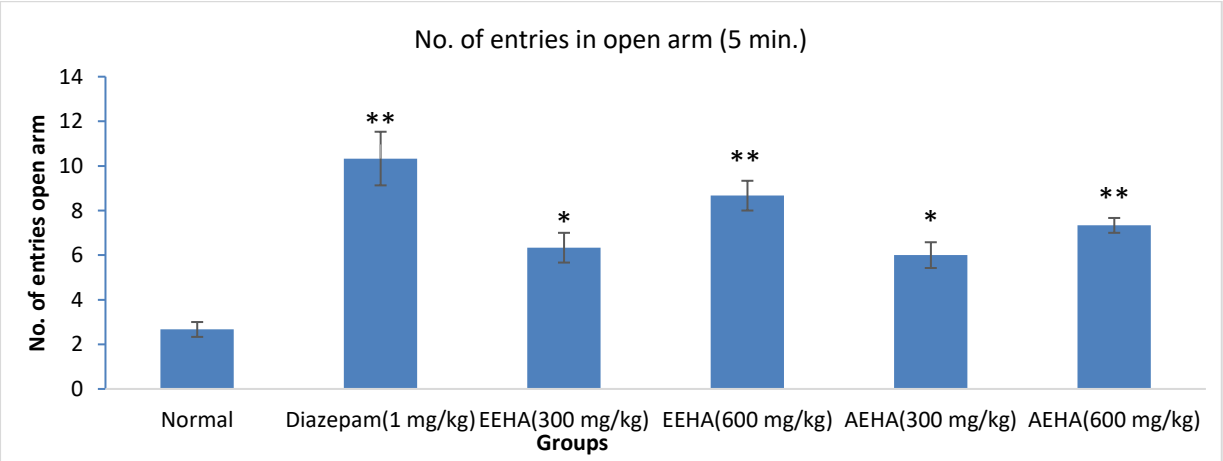
1. Antioxidant Properties: Brain tissue homogenate for the estimation of Superoxide dismutase (SOD), Catalase (CAT), Reduced glutathione (GSH) measured.

Table 7: SOD, CAT, GSH Levels.

Group	Dose mg/kg	SOD (U/mg protein)	CAT (U/mg Protein)	GSH (ug GSH/mg protein)
Normal	**-**	4.767±0.2333	1.300±0.1155	79.33±2.963
Diazepam	**1**	2.273±0.08193**	0.2967±0.03283**	43.67±0.8819**
EEHA	**300**	3.667±0.2404**	0.7267±0.02906**	61.00±2.082**
EEHA	**600**	2.733±0.122**	0.4500±0.03215**	53.33±1.667**
AEHA	**300**	4±0.1155*	0.8867±0.02028**	68.67±1.202**
AEHA	**600**	3.133±0.1856**	0.6233±0.04333**	60.33±1.856**

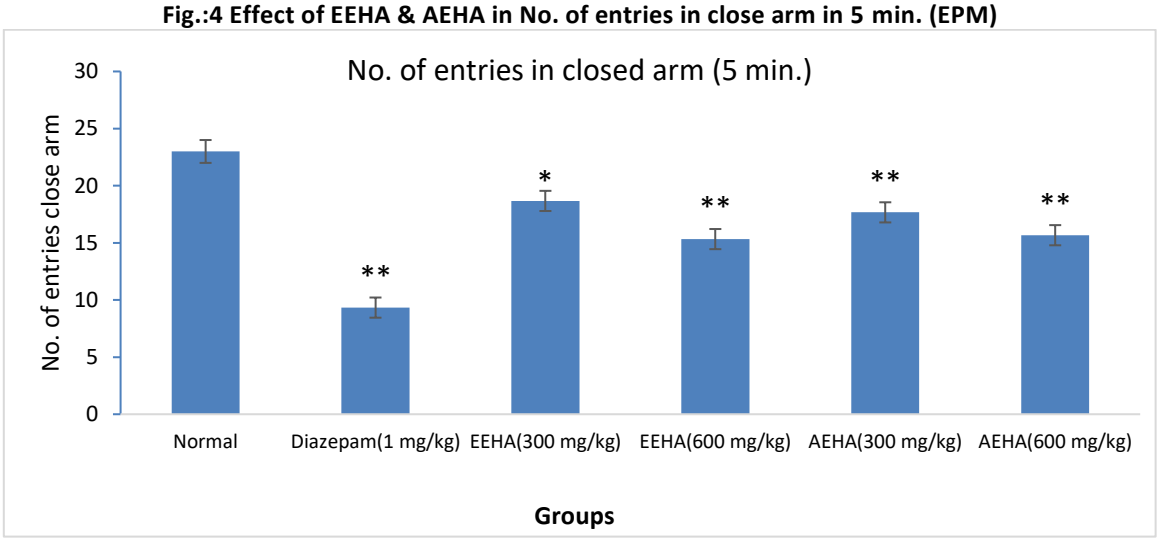
Values are mean ± SEM; Statistical analysis by One-way ANOVA followed by Dunnett's Multiple Comparison test. ∗P < 0.05; ∗∗P < 0.01

Fig.:3 Effect of EEHA & AEHA in No. of entries in open arm in 5 min. (EPM)



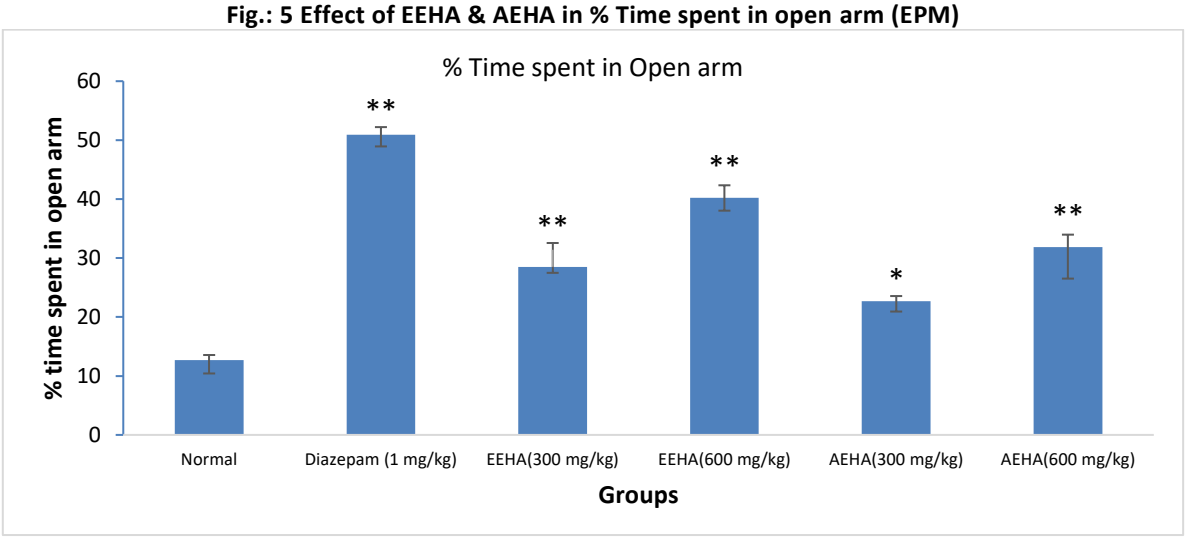
The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.:4 Effect of EEHA & AEHA in No. of entries in close arm in 5 min. (EPM)



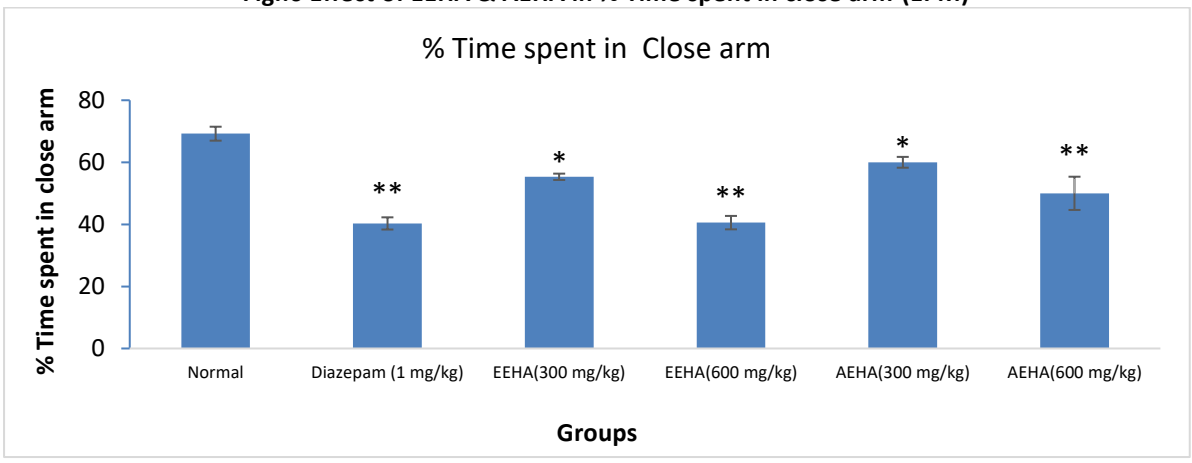
The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.: 5 Effect of EEHA & AEHA in % Time spent in open arm (EPM)



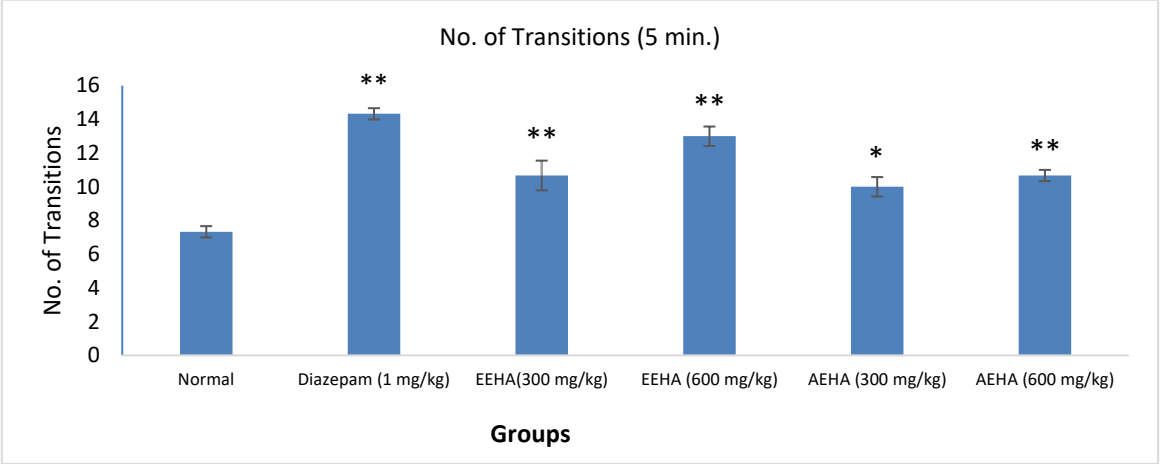
The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.:6 Effect of EEHA & AEHA in % Time spent in close arm (EPM)



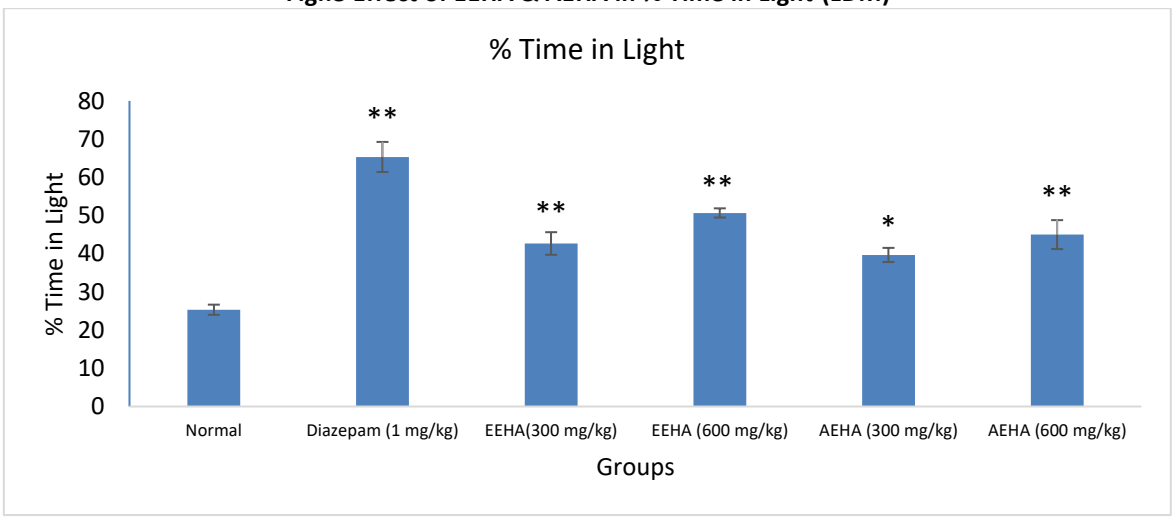
The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.:7 Effect of EEHA & AEHA in No. of Transitions (LDM)



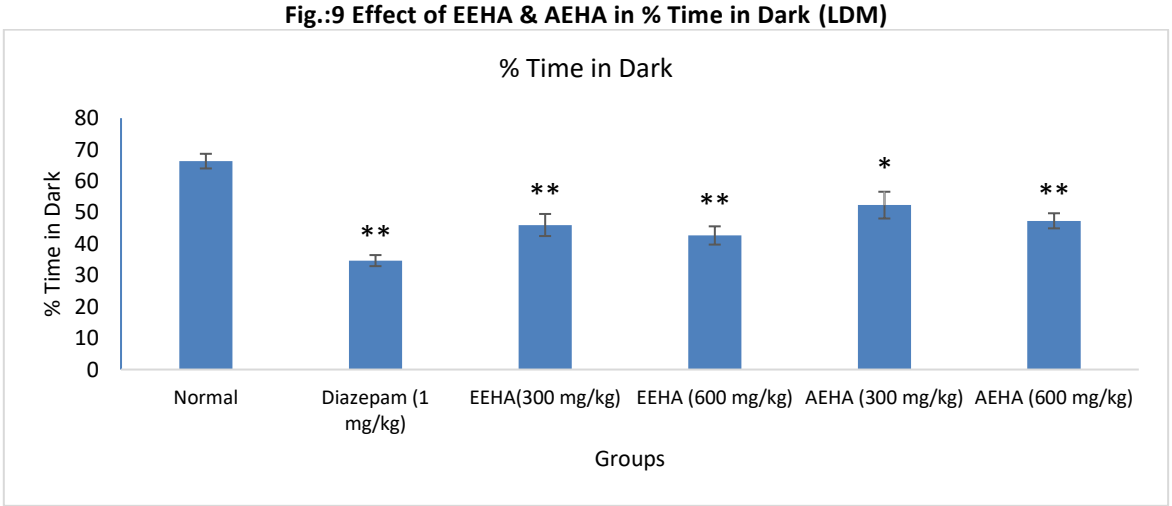
The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.:8 Effect of EEHA & AEHA in % Time in Light (LDM)



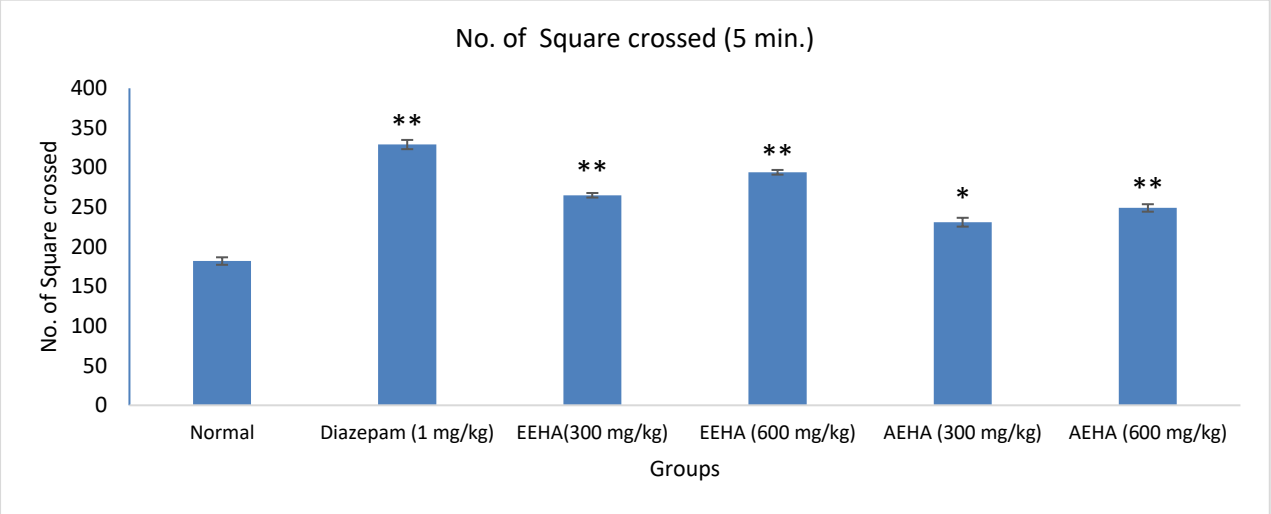
The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.:9 Effect of EEHA & AEHA in % Time in Dark (LDM)



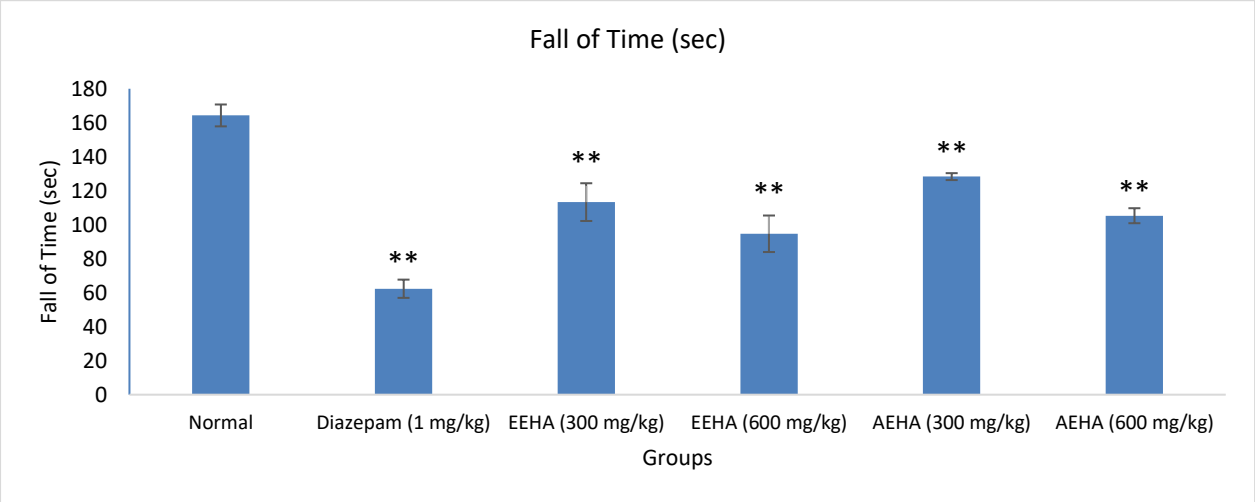
The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.:10 Effect of EEHA & AEHA in No. of Square crossed (OFT)



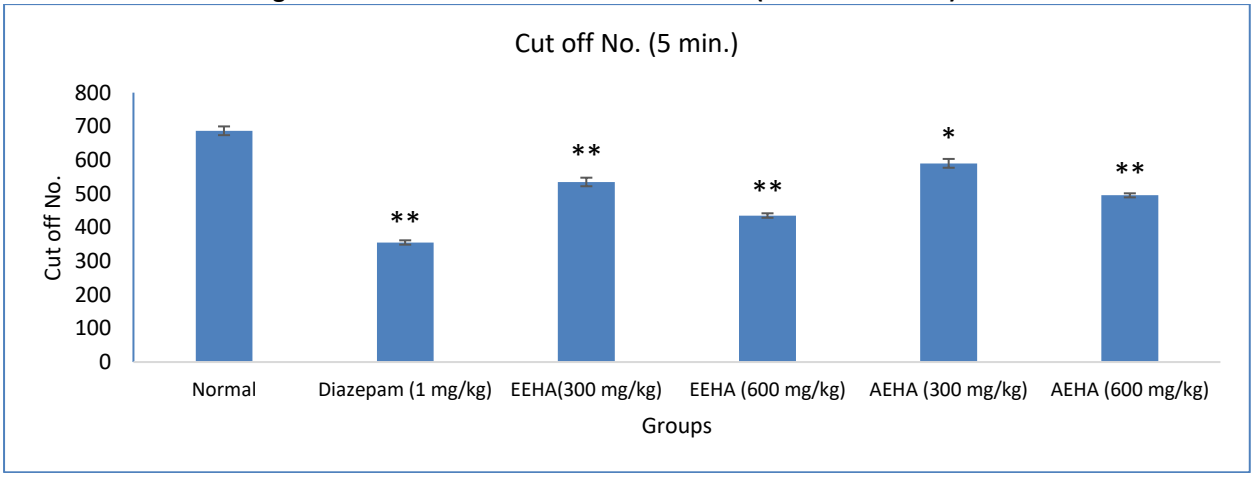
The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.:11 Effect of EEHA & AEHA in Fall of Time (sec)(Rotarod)



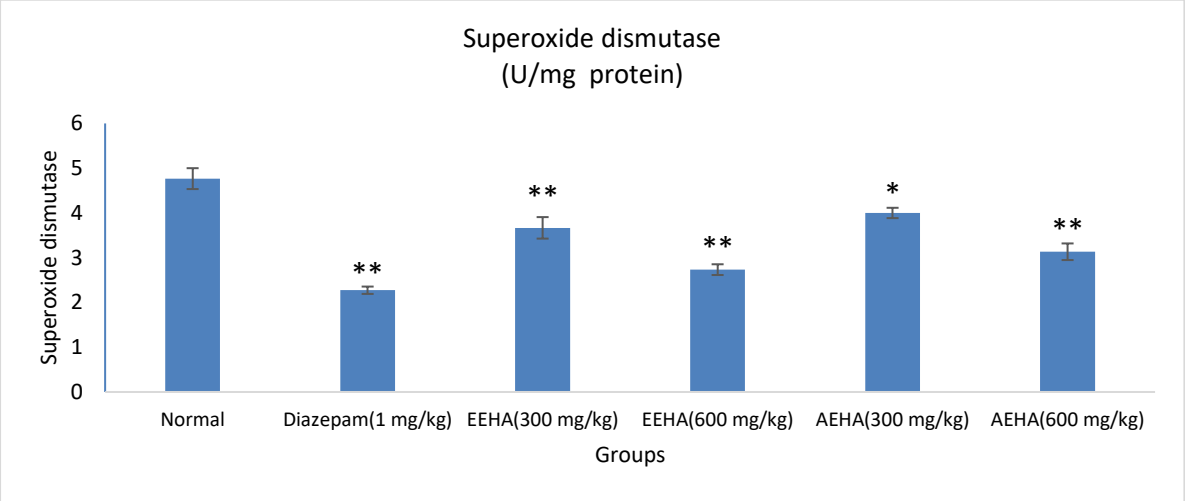
The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.:12 Effect of EEHA & AEHA in Cut off No. (Photoactometer)



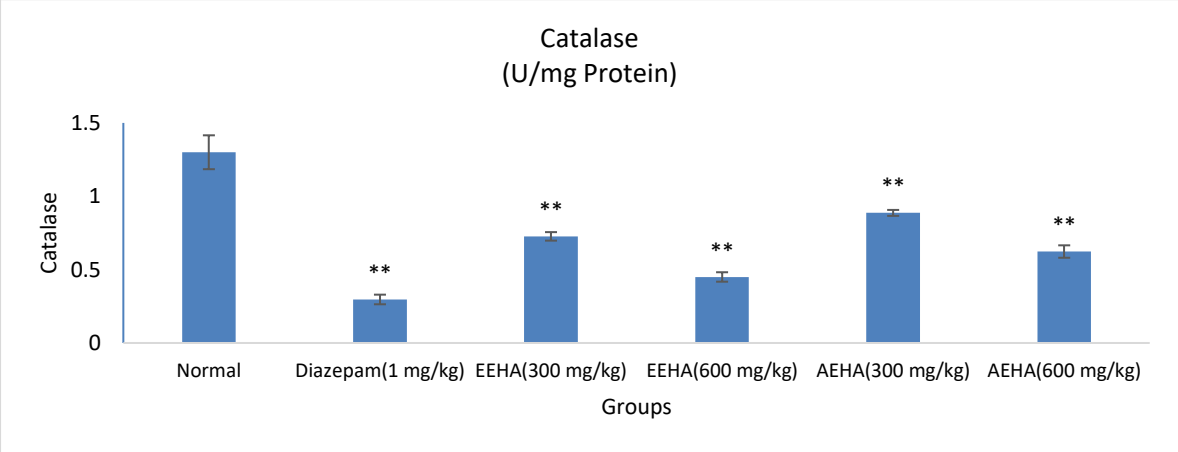
The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.:13 Effect of EEHA & AEHA in Superoxide dismutase (units/mg protein) (Antioxidant)



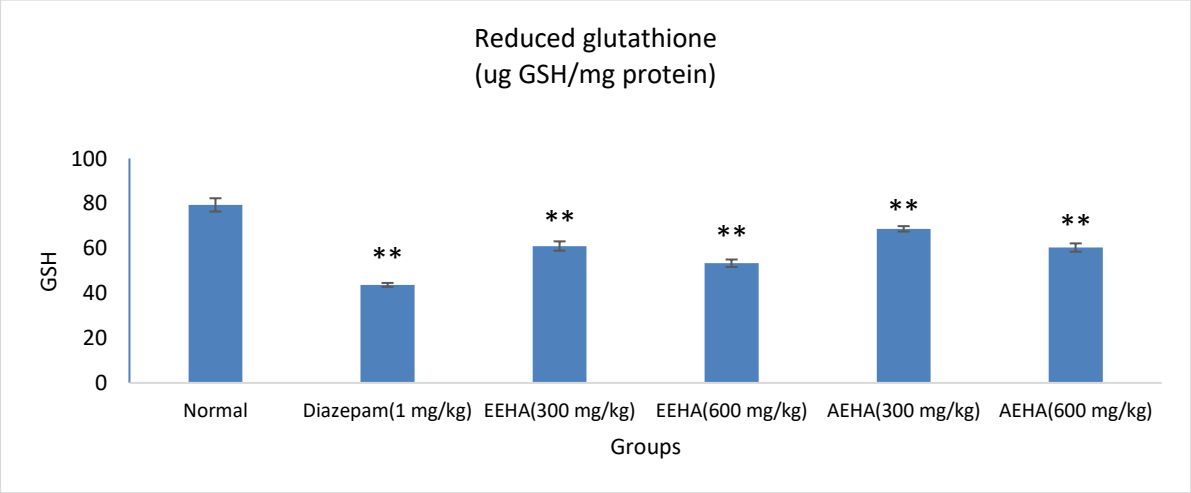
The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.:14 Effect of EEHA & AEHA in Catalase (U/mg protein) (Antioxidant)



The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

Fig.:15 Effect of EEHA & AEHA in Reduced glutathione (ug GSH/mg protein) (Antioxidant)



The signs (**) and (*) indicate values significantly different from normal group at **P<0.01 and *P<0.05 respectively.

DISCUSSION:

Anxiety is an unpleasant state of tension, apprehension, or uneasiness a fear that seems to arise from a sometimes-unknown source. Disorders involving anxiety are the most common mental disturbances and the most reported mental illness and as a lifetime prevalence of 21% with specific phobias. The physical symptoms of anxiety are like those of fear (such as tachycardia, sweating, trembling) and involve sympathetic action. In research of anxiolytic drug are generally use animals screening models.

Nowadays, Treatment use for Anxiety disorder are psychotherapy and pharmacotherapy. In pharmacotherapy generally use medication like benzo-diazepam class of drugs, selective serotonin reuptake inhibitors and β- blocker class of drugs.

Various animal's screening models of anxiolytic activities used for research of anxiolytic drug. Important criteria to select a model include behaviour parameters, motor co-ordination and locomotor activities. Anxiolytic effect evaluates with behaviour parameters with using animal screening models like Elevated plus-maze model, Light-Dark model. And other parameter like as motor co-ordination evaluate with Rotarod model and locomotor activities evaluates with Open field test, Photoactometer.

The plant in this study, "*Hygrophila auriculata*", is said to be considered as one of the promising candidates for Anxiety disorder treatment. Its commonly known as Talimkhana in Gujarati and Hindi, Kokilaksah in Sanskrit and Marsh Barbel in English, it is commonly found in marshy places and is native to tropical Asia and Africa. It comes under the Family: Acanthaceae. The plant contains various groups of phytoconstituents, namely, phytosterols, fatty acids like myristic acid, palmitic acid, oleic acid, stearic acid, linoleic acid, minerals, polyphenols, proanthocyanins, mucilage, alkaloids, enzymes, amino acids, carbohydrates, hydrocarbons, flavonoids, terpenoids, vitamins, and glycosides. The parts of this plant are widely used in traditional medicine for the treatment of various disorders, which include anasarca, diseases of the urinogenital tract, dropsy from chronic Bright's disease, hyperdipsia, vesical calculi, flatulence, diarrhoea, dysentery, leukorrhea, gonorrhoea, asthma, blood diseases, gastric diseases, inflammation, cancer. It is also scientifically proved to have a variety of pharmacologic functions, which indicate its usefulness in the treatment of different types of diseases and disorders.

Over the years, different pharmacological models have been employed in the evaluation of medicinal plants for neuropharmacological activities towards the identification of botanicals and drugs with beneficial effects in the treatment of diverse CNS disorders. The choice of test methods not only determines effectiveness but, in some instances, also gives an indication of the mechanism of the test substance. In this study, elevated Plus maze model, Light-dark model, Open field test, Rotarod model and photoactometer were used to investigate the anxiolytic effect of the seeds methanolic and aqueous extract of *Hygrophila auriculata* plant.

The elevated plus maze is a standard pharmacological model for evaluating the anxiolytic effect of drugs and it is based on the fact that exposure of animals to an elevated plus maze evoke approach-avoidance conflict stronger than elicited by the exposure to a close arm. Anxiolytic effect is indicated by decrease in aversion to the open arms by way of increase in time spent and entries into the open arms, while anxiogenic compounds reduce the value of these parameters. In this study, *Hygrophila auriculata* ethanolic and aqueous extract at the dose of 300 mg/kg non-significantly increased the time spent in the open arms and significantly increased the number of entries into open arm compartment. As observed with diazepam (1 mg/kg), the ethanolic and aqueous extracts at dose of 600 mg/kg significantly reduced the number of entries into the closed arms. The actions of *Hygrophila auriclulata* in the elevated plus maze model in this study suggest anxiolytic property.

The anxiolytic-like activity was also observed in the light-dark box. light and dark are an ethological-based approach-avoidance conflict test and it is sensitive to drugs that affect anxiety. In this test, the number of transitions between the light and dark compartments as well as the time spent in the light side are recognized as anxiety indices, despite the transition parameter being highly dependent on locomotor activity. Mice treated with *Hygrophila auriculata* ethanolic and aqueous extract (300-600 mg/kg) showed increase in the time spent in the light compartment and no changes in the numbers of shuttle crossings, confirming the activity upon the main anxiolytic parameter. The observed anxiolytic effect of *Hygrophila auriculata* may be due to the agonistic effect on GABA/benzodiazepine receptor complex or antagonize the 5-HT1B receptor or agonize the 5-HT1A receptor.

The Open filed model is used to evaluate the animal emotional state. The open field model examines anxiety related behaviour characterized by the normal aversion of the animal to an open, brightly light area. Thus, mice removed from their acclimatized cage and placed in environment express

anxiety and fear, by showing alteration in all or some parameters. Anxiolytic treatments reduce such fearful behaviour of mice in open field. Statistical analysis of the data obtained from these experiments supported anxiolytic-like activity of *Hygrophila auriculata* ethanolic and aqueous extract at both the doses (300 and 600 mg/kg) as its effect shows significant increase in the number of squares crossed, as compared to the normal group, which indicates its anxiolytic-like effect.

Rota rod test a classical animal model used to evaluate peripheral neuromuscular blockade and the motor co-ordination. a deficit in motor coordination would very likely affect performance in the behavioural tests. Our findings showed that *Hygrophila auriculata* ethanolic and aqueous extract (300-600 mg/kg), alike diazepam (1 mg/kg), had significant effect on motor co-ordination. Plant extract showed significant decrease in the locomotory score and fall of time of the mice from the rotating rod which indicates its anxiolytic-like effect.

Photoactometer model used to evaluated locomotor activity. Mice treated with *Hygrophila auriculata* ethanolic and aqueous extract (300-600 mg/kg), alike diazepam (1 mg/kg) had significant effect on decreased of cut off number. diazepam and both extracts showed that decreased locomotor activity which indicates its anxiolytic effect.

When the production of oxygen-derived metabolites prevails over the brain defence systems, however, oxidative damage to nucleic acids, proteins and neuronal membrane lipids, which are rich in highly polyunsaturated fatty acids, can occur. oxidative stress can alter neurotransmission, neuronal function and overall brain activity. Oxidative damage in the brain causes nervous system impairment and also has been implicated in anxiety disorders and high anxiety levels. The decreased levels of GSH in brain of anxiety might be due to the excessive consumption of GSH by the system to defend oxidative damage. The production of oxygen free radicals that occurs with the development of anxiety leads to decreased GSH, CAT and SOD levels as a consequence of their consumption during oxidative stress and cellular lysis, which is evident by decreased levels of GSH, CAT and SOD in anxiety stander group. Oral administration of ethanolic and aqueous extract to the mice significantly re-established the depleted levels of GSH, CAT and SOD probably by competing for scavenging of free radicals.

CONCLUSION:

Research indicated that EEHA and AEHA (300 & 600 mg/kg) possess anxiolytic effects on different parameters evaluated as behaviour, emotional state, muscles co-ordination, locomotor activities. All these results thus predict that *Hygrophila auriculata* provide pharmacological rationale for the traditional use of the plant against Anxiety disorder.

Further research in the assessment of the anti-anxiety effect of *Hygrophila auriculata* is considered necessary specifically in the identification of the active constituent present in the seed extract that contributed to the GABA antagonist and Selective serotine selective serotonin reuptake inhibitors in anxiety state. Alongside with these, research should also include the determination of the optimal dose of *Hygrophila auriculata* as an anxiolytic agent.

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