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PHARMACOLOGICAL SCREENING TO ASSESS THE ANXIOLYTIC ACTIVITY OF *ANDROGRAPHIS ECHIOIDES* (L) NEES EXTRACT IN ALBINO RATS

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ABSTRACT

Everyone can feel anxious sometimes, but people with anxiety disorders often experience fear and worry. Anxiety disorders are a serious threat to public health, affecting a major section of the world's population. Although effective treatments exist, only a fraction of people with anxiety disorders receive treatment due to barriers such as lack of awareness, insufficient mental health services and social stigma. Plants containing flavonoids constituents are reported to be potent anti-anxiety. In this study, we evaluated the anxiolytic activity of the hydro-alcoholic extract of *Andrographis echioides* aerial parts in rats. The study involved behavioral tests, such as the elevated plus maze, open field test and marble burying test to evaluate the anxiolytic potential of the extract. Acute oral toxicity test performed to determine safety profile of the plant extract. The results showed that the time spent in the open arms of the elevated plus maze, the central zone of the open field test and reduction in the number of buried marbles. These effects were comparable to the effect produced by diazepam. All the doses of plant extract employed for acute oral toxicity studies were found to be non - toxic. Phytochemical screening of the extract revealed the presence of alkaloids, flavonoids, glycosides, tannins, steroids, carbohydrates and proteins and amino acids. From the study, it can be concluded that hydro-alcoholic extract of *Andrographis echioides* at the dose of 200mg/kg possesses marked anxiolytic activity. Since flavonoids are known to promote anxiolytic activity, the presence of flavonoids in these plant extract may be the cause of the anxiolytic activity.

KEYWORDS

Andrographis echioides (L) Nees, Hydro-alcoholic extract, Albino Rats, Elevated plus maze, Open field test and Marble burying test.

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INTRODUCTION

Everyone can feel anxious sometimes, but people with anxiety disorders often experience fear and worry. That is both intense and excessive. These feelings are typically accompanied by physical tension and other behavioural and cognitive symptoms. They are difficult to control, because significant distress and can last a long time if untreated[1]. Despite significant advances in the understanding and management of neuropsychiatric disorders during the past few decades, anxiety and depression still remain leading causes of mortality. According to the World Health report, approximately 450 million people suffer from serious brain / mental or behavioral disorder, yet only a few of them receive even the most basic treatment. Anxiety is an emotional state, unpleasant in nature, Associated with uneasiness, discomfort and concern or fear about some defined or undefined future threat. Some degree of anxiety is a part of normal life. Treatment is needed when it is disproportionate to the situation and excessive. Some psychotic and depressed patients also exhibit pathological anxiety. Anxiety can affect anyone, whatever their age, gender etc. It affects our thoughts, physical reactions, moods and behaviours. Anxiety can also cause us to feel panicky and frightened and prevent us from doing things. Too much stress in our lives can result in higher levels of anxiety[2].

Major drug classes for the treatment of anxiety disorders are benzodiazepines and selective serotonin-reuptake inhibitors (SSRIs). However, these compounds have a number of undesirable effects such as insomnia, muscle relaxation and hepatotoxicity. These considerations implicate the search for new anxiolytic compounds that have a fast onset of action present with less side effects and a wider safety margin. Medicinal plants are a good source to find new remedies for these disorders. Plants have long been used to treat central nervous system (CNS) disorders. Folk medicines have particular values, for example, plants that “calm down”, tranquilize and raise mood, such as *Passiflora coerulea*, *Valeriana officinalis*, *Matricaria recutita*, *Jatropa cilliata*, *Salvia guaranitica*, *Tilia tormentosa*, and *Tilia europeae*[3].

Acanthaceae is a family (the acanthus family) of dicotyledonous flowering plants containing almost 250 genera and about 2500 species. Most are tropical herbs, shrubs, or twining vines; some are epiphytes. *Andrographis* is a genus of flowering plants in the family *Acanthaceae*. They may be generally known as the False Water Willows[4] and several are called Periyanagai[5]. The species are native to the Indian subcontinent (including Myanmar, Sri Lanka and the West Himalaya region)[6,7]. Many are endemic to India. They may be herbs or shrubs[5]. In traditional Indian medicine, several *Andrographis* species have been used in the treatment of Dyspepsia, Influenza, malaria and respiratory infections and as astringent and antidote for poisonous stings of some insects[8,9]. *Andrographis echioides* (L.) Nees is an important medicinal plant and widely used around the world. *Andrographis echioides* (L) Nees also known as *Indoneesiella echioides* (L) Nees. This is commonly known as False Water Willow, is an abundantly growing in South India. *Andrographis echioides* plants are seen mostly in dry places, such as India, Sri Lanka and South Asian countries. The species of Indoneesiella is used in Goitre, liver diseases[10], Fertility problems, Bacterial[11], Malarial and fungal disorders. The leaf juice is mixed and boiled with coconut oils used to control falling and greying of hair[12]. It is reported that *Andrographis echioides* contain different classes of chemical constituents including flavonoids, alkaloids, essential oils, fatty acids, steroids, terpenoids, polyphenols, glycosides together with a several medicinal benefits such as anti -oxidant, anti-inflammatory, anti-diabetic, hepatoprotective, anti-microbial and anticancer[13].

An assessment of *Andrographis echioides* revealed that it has a significant number of phytochemical components. *Andrographis echioides* (L.) Nees was chosen for this study due to its rich flavonoid content and historical use. Flavonoids have been shown to have potential effects on the central nervous system.

MATERIAL AND METHODS

Collection and Authentication of the Plant Specimen

The collected plant specimen was authenticated as *Andrographis echioides* (L.) Nees by Dr.S.S.Hameed Scientist ‘F’ and Office in Charge, Botanical Survey of India, Southern Regional

Centre, Coimbatore. (Authentication No.BSI/SRC/5/23/2025-26/Tech./549)

Preparation of Extract

Andrographis echioides aerial parts (leaves, flowers, stems) were collected and then cleaned, shade dried, mechanically grinded. The crude extracts are prepared by Maceration technique. Coarsely powdered plant materials were separately soaked in extraction solvent hydro-alcoholic (ethanol: water (50:50)) followed by shaking periodically for seven days and then filtered. The extract are then dried in hot air oven and transferred into well labelled vials and kept in a refrigerator until required for use. The resulting dry extract was weighed and provided a percentage yield of 18.75% (w/w).

Phytochemical Screening

The hydroalcoholic extract of *Andrographis echioides* aerial parts were screened for their phytochemical composition in order to determine their qualitative chemical components. The components were screened using conventional protocol, procedure and reagents, as described in Trease and Evans[14] and sofowora[15].

Pharmacological Screening Studies

Animal Subjects employed in the study

For this investigation, 180 ± 20g of male albino rats, six weeks of age, were employed. The rats were kept in groups of six animals per cage in a temperature controlled environment (21-22°C) with a reversible light-dark cycle (12 h / 12 h) had normally to fed and water ad libitum. The Institutional Animal Ethics Committee, which was established to oversee and regulate animal experimentation, gave its approval to the animal studies. Animal care was taken as per the guidelines of CPCSEA, Govt. of India.

Acute Toxicity Test

The animals were divided into five consecutive groups containing five animals in each[16-18]. Animals were kept in close observation for 24h and for a total of seven days following the oral treatments with vehicle or extract at Control, 5, 50, 300, 2000mg/kg doses (adjusted volume 0.1ml / animal) to check any allergic reaction, swelling, vomiting, diarrhoea and mortality induced by extract. In the meantime, they were allowed to have access to food and water ad libitum.

Anxiolytic Studies

Pharmacological assays play a crucial role in identifying compounds with anxiolytic properties in animal models, offering a window into potential therapeutic interventions for anxiety disorders. These assays typically involve subjecting animals, often rodents like mice or rats, to various behavioural tests designed to mirror anxiety-like responses observed in humans. Among the commonly employed assays are the Elevated Plus Maze (EPM), Open Field Test (OFT), Marble Burying Test (MBT), we can gauge the efficacy of potential anxiolytic compounds, offering valuable insights into their mechanisms of action and therapeutic potential. These assays serve as indispensable tools in the preclinical evaluation of novel pharmacotherapies aimed at alleviating anxiety-related symptoms.

Protocol for evaluation of the anxiolytic activity of Hydro-Alcoholic Extract of *Andrographis echioides* (L.) Nees Aerial Parts in EPM, OFT and MBT

The following pharmacological evaluation studies were employed for the determination of anxiolytic activity of Hydro-Alcoholic Extract of *Andrographis echioides* (L.) Nees.

Elevated Plus Maze Test (EPM)

Open field test (OFT)

Marble burying Test (MBT)

The Acute toxicity test also employed for the study.

After administering different dosages of diazepam (standard), extract, and control intraperitoneally (i.p) or orally (p.o), the animals were individually assessed for anxiolytic behaviour in EPM, OFT, and MBT. The grouping pattern of the animals used to assess anxiolytic activity is displayed in the following table.

Elevated Plus Maze Test (EPM)

The anxiolytic activity of plant extracts was evaluated using the EPM test. The apparatus was situated 40cm above the floor, consisting of two open arms (5 × 10cm) and two closed arms (5 × 10

× 15cm) radiating from a platform (5 × 5cm) to form a plus-sign figure. The open arms edges were 50cm in height to keep the mice from falling and the closed-arms edges were 15cm in height. Sixty minutes after the administration of the test drug, each animal was placed at the center of the maze facing one of the enclosed arms. During the 5-minute test period, the number of open and enclosed arms entries, plus the time spent in open and enclosed arms, was recorded via the method of Pillow and File. Entry into an arm was defined as the point when the animal places all four paws onto the arm. The procedure was conducted in a sound attenuated room; observations were made from an adjacent corner[19].

Open Field Test (OFT)

This test is one of the most frequently used methods to evaluate the locomotor activity and emotionality of rodents. The apparatus is a square box consisting of a 50cm high wall and a wooden floor with a series of squares alternatively painted in black and white. Animals were administered with the vehicle, extract, or diazepam and placed in the middle of the open field allowing free exploration. The animals were then scored with the number of squares they visited for 3 min before and at 30, 60, 90 and 120 min post treatments. The percentage of inhibition was calculated for each time point as described in the hole cross-test[20].

Marble Burying Test (MBT)

A normal glass cage with bedding materials was used in this experiment. Before testing, the individual animal was acclimatized in one cage for 30 min. After removal of the animal, 25 glass marbles were uniformly distributed on top of the 4cm layer of bedding materials. Following extract, vehicle, or diazepam treatment, each animal was placed in the cage for 30 min. The number of buried marbles was then counted as a score of anxiety[21]. The percentage of inhibitions was calculated as follows

Percentage of Inhibition = [(Control – Treatment) / Control] × 100

Statistical Analysis

The results are presented as Mean ± SEM. The statistical analysis was performed using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test, for these tests, One way, two-way ANOVA followed by Bonferroni's post hoc tests was adopted. In all the cases $P < 0.05$ was considered as significant. All statistical analysis was performed using SPSS software. Moreover, graphical presentations made by using Graphpad Prism software version 10.2.0 (392)[22,23].

RESULTS AND DISCUSSION

Preliminary phytochemical analysis of the extract

Preliminary phytochemical evaluation of crude hydro-alcoholic extract of *Andrographis echioides* (L.) Nees aerial parts revealed the presence of Alkaloids, flavonoids, glycosides, tannins, steroids, carbohydrates and proteins and amino acids.

Pharmacological screening studies

Acute Oral Toxicity Test

HAEAE was subjected to acute oral toxicity test following OECD guidelines 425. All the doses (5, 50, 300, 2000mg/kg, p.o) of HAEAE employed for acute oral toxicity studies were found to be non – toxic. The HAEAE did not produce any mortality even at the highest dose (2000mg/kg) employed. In addition, the general behaviour remained unaltered, and similar observations were recorded for the controls. Two arbitrary doses 100mg/kg and 200mg/kg were selected for the study.

Anxiolytic Studies

Anxiolytic Effect of HAEAE in the Elevated Plus Maze Using Rats

The results showed that HAEAE at both doses 100mg/kg and 200mg/kg treated rats significantly increased the time spent and the number of entries in open arms. Still, they decreased the time spent and the number of entries in close arms depending on dose when compared to Diazepam as the standard drug reflects plants and Anxiolytic properties.

The HAEAE Decreased the Frequency and Amplitude of Movements in the Open Field Test (OFT)

Rats that were given HAEAE (100 and 200mg/kg) showed a substantial increase ($p<0.0001$) in the amount of time spent in the central compartment and the number of crossings of the open field.

Effect of HAEAE on Marble Burying Test (MBT)

HAEAE (100 and 200mg/kg, i.p.) dose - dependently reduced the Marble-burying behaviour in rats (Table No.6) and the reduction was significant ($p<0.001$) compared with the control group. Diazepam (1 mg/kg) also showed significant ($p<0.001$) Reduction in the number of buried marbles (Table No.6).

Discussion

The hydro-alcoholic extract of *Andrographis echioides* aerial parts were screened for their phytochemical composition in order to determine their qualitative chemical components. The components were screened using conventional protocol, procedure and reagents, as described in Trease and Evans and sofowora. Preliminary phytochemical evaluation of crude hydro-alcoholic extract of *Andrographis echioides* (L.) Nees aerial parts revealed the presence of Alkaloids, flavonoids, glycosides, tannins, steroids, carbohydrates and proteins and amino acids.

Pharmacological assays to identify anxiolytic behaviour in animals typically involve various behavioural tests designed to assess anxiety-like responses. We employed following assays, Elevated Plus Maze Test (EPM), Open field test (OFT), Marble burying Test (MBT) and Acute toxicity study also included in the study. Rats were chosen for the evolution of anxiolytic activity in an experimental model because mice exhibit robust anxiety-like behaviour when exposed to stressors (for example, novelty, bright light, or social confrontation). These phenotypes have obvious utility in testing the effects of psychotropic drugs.

HAEAE was subjected to acute oral toxicity test following OECD guidelines 425. All the doses (5, 50, 300, 2000mg/kg, p.o) of HAEAE employed for acute oral toxicity studies were found to be non – toxic. The HAEAE did not produce any mortality even at the highest dose (2000mg/kg) employed. In addition, the general behaviour remained unaltered and similar observations were recorded for the controls. Two arbitrary doses 100mg /kg and 200mg/kg were selected for the study.

In the EPM Study, rats were individually placed on the center of the maze facing an open arm, and the number of entries and the time spent in closed and open arms were recorded during a 5 min observation period. Arm entries were defined as the entry of all four paws into the arm. The procedure was conducted preferably in a sound-attenuated environment. Anxiolytic Compound Containing HAEAE expected to increase the the time spent in the open arms, reflecting reduced anxiety-like behavior, while anxiogenic compounds have the opposite effect. The results showed that HAEAE at both doses 100mg/kg and 200mg/kg treated rats significantly increased the time spent and the number of entries in open arms. Still, they decreased the time spent and the number of entries in close arms depending on dose when compared to Diazepam as the standard drug reflects plants Anxiolytic properties

The Open Field Test (OFT) is a widely used behavioural assay to assess general locomotor activity and anxiety-like behaviour in rodents, particularly mice and rats. The test is conducted in a square or circular arena with defined zones, typically divided into a central region and a peripheral region. Animals are placed in the centre of the open field, and their spontaneous exploration of the environment is observed and recorded the results were shown in table no-4.The time spent In the central versus peripheral areas, as well as other behaviours such as rearing and grooming, can provide insights into the animal emotional state. The present study was conducted to explicate central nervous system (CNS) depressant activities of the HAEAE n rats. The HAEAE decreased the frequency and amplitude of movements in the open field test in rats in a dose dependant manner as shown in the Figure No.5. The number of squares crossed by the animal which shows the percentage of inhibition of locomotor activity. The results of these study provided evidence that the extract reduced locomotor activity confirming its CNS depressant effects.

Marble Burying Test (MBT) is a behavioural assay commonly used to assess anxiety-like and compulsive behaviours in rats. In this test, rats are placed in a cage containing a layer of marbles evenly spaced across the bedding. The natural digging and burying behaviour of rodents lead them to interact with the marbles. An increase in the number of marbles buried is considered indicative of higher anxiety or compulsive-like behaviour. The response of HAEAE was studied with 100 and 200mg/kg doses and compared with the standard drug of Diazepam 1mg/kg, HAEAE (100 and 200mg/kg, i.p.) dose - dependently reduced the Marble-burying behaviour in rats (Table No.5) and the reduction was significant ($p<0.001$) compared with the control group. Diazepam (1mg/kg) also showed significant ($p<0.001$) Reduction in the number of buried marbles (Table No.5). The percentage of inhibition was shown in the Figure No.7. it was clearly noted that the HAEAE extracts effectively control anxiety or compulsive-like behaviour. And also it was found to be HAEAE 200mg/kg showed 78.479 % of inhibition when compared to the standard drug Diazepam 1mg/kg which was nearly same 87.339 %.

The efficacy of most herbal remedies is attributed to various active principles in combination, in the present study HAEAE containing significant amounts of flavonoid components. Which are probably more responsible for the anxiolytic activity of HAEAE. Recently some natural and synthetic flavonoids have been found to bind specifically and competitively to benzodiazepine receptors and to possess anxiolytic effects. Therefore, the Anxiolytic activity may be due to the phytoconstituents present in the HAEAE Extract.

Table No.1: Grouping of Animals for Evaluation of the Anxiolytic activity of Hydro-Alcoholic Extract of *Andrographis echioides* (L.) Nees Aerial Parts

S.No	Group	Number of Animals	Treatment
1	Group I	6	Control (Vehicle 10ml/Kg)
2	Group II	6	Positive Control (Diazepam 2Mg/Kg IP.)
3	Group III	6	Low Dose (Hydro-alcoholic Extract of *Andrographis echioides* Aerial Parts)
4	Group IV	6	High Dose (Hydro-alcoholic Extract of *Andrographis echioides* Aerial Parts)

Table No.2: Preliminary Phytochemical analyses of hydro-alcoholic extract *Andrographis echioides* (L) Nees Aerial parts

S.No	Phytoconstituents	Hydro-alcoholic Extract
1	Alkaloids	+
2	Glycosides	+
3	Flavonoids	+
4	Tannins	+
5	Steroids	+
6	Carbohydrates	+
7	Proteins and Amino Acids	+
8	Fats and Fixed oils	-
9	Vitamin C	-
10	Phenols	+

Table No.3: Effect of HAEAE at doses of 5, 50, 300, 2000mg/kg and Control on Toxic Symptoms and Mortality in Rats

S.No	Drug	Dose (mg/kg)	Mortality	Symptoms of Toxicity
1	Control	10ml/kg	0	None
2	HAEAE5	5mg/kg	0	None
3	HAEAE50	50mg/kg	0	None
4	HAEAE300	300mg/kg	0	None
5	HAEAE2000	2000mg/kg	0	None

Table No.4: Effect of HAEAE on Rats EPM Test

Group	Treatment	Dose	Number of entries (counts)		Time spent	
			Open Arm	Closed Arm	Open Arm	Closed Arm
Group I	Control	10ml/kg, i.p	6.30±0.46	15.30±0.64	68.3±0.54	231.2±0.53
Group II	Diazepam	10ml/kg, i.p	10.50±0.43*	7.30±0.46*	181.65±0.65*	118.30±0.64*
Group III	HAEAE 100	100mg/kg, p.o	7.64±0.30ns	10.2±0.40*	93.15±0.58*	206.80±0.57*
Group IV	HAEAE 200	200mg/kg, p.o	8.64±0.31b	9.30±0.46*	135.15±1.06*	164.80±1.05*

Data expressed as mean ± SEM (n=6). The data were analysed by one-way ANOVA followed by and Dunnett's test. a- P<0.05; b- P<0.01, #- P<0.001; *- P<0.0001; ns- not significant when compared with control group

Table No.5: Effect of HAEAE on Open Field Test

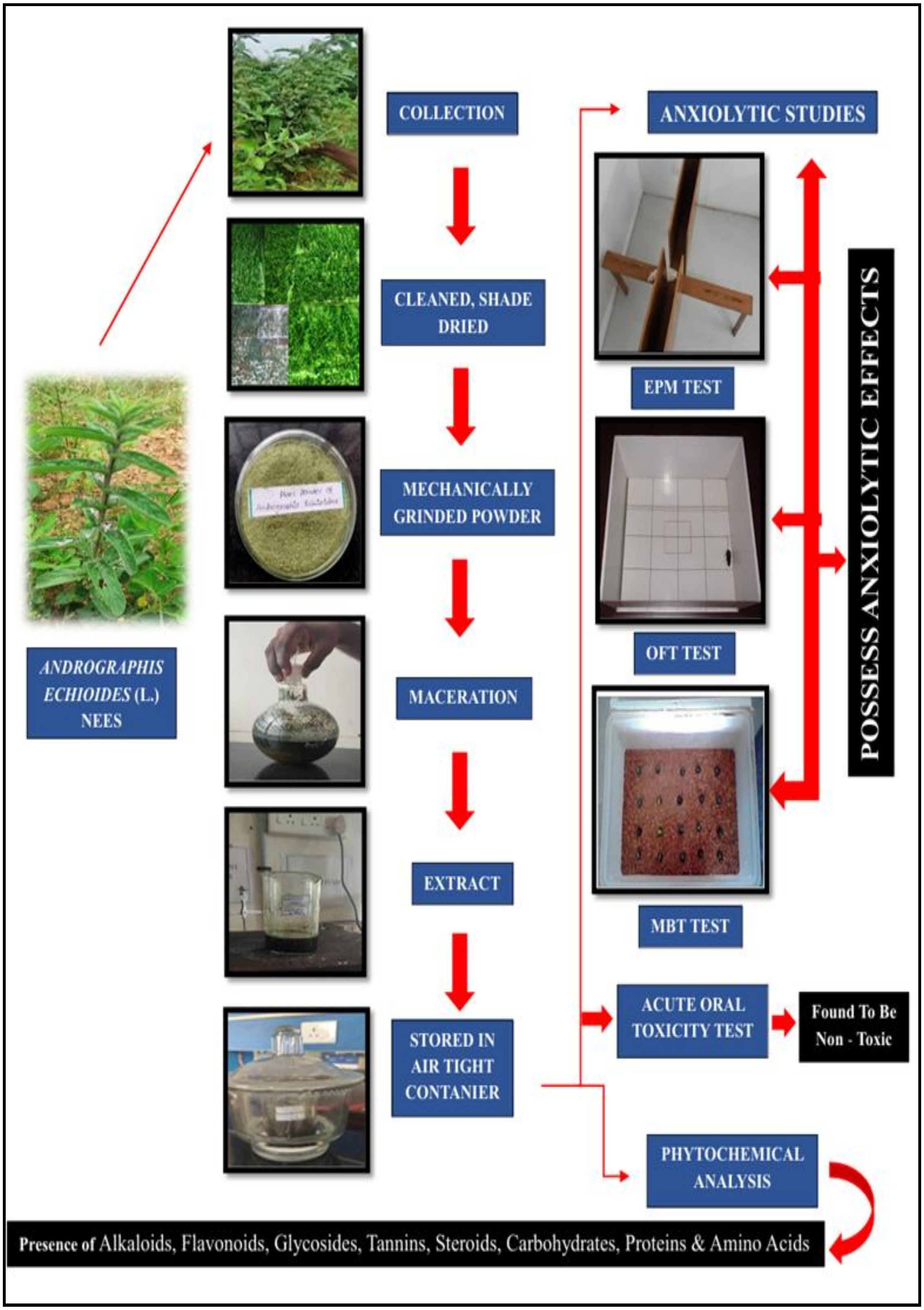
Treatment	Dose (mg/kg)	Number of Squares Crossed (% of Inhibition)				
		Pretreatment	30 min	60 min	90 min	120 min
Control	10ml/kg (p.o)	96.78±3.19	96.79±3.09	95.86±3.39	96.78±3.89	96.78±3.26
Diazepam	2mg/kg (p.o)	98.66±3.39	52.35±2.95	45.24±3.01	33.78±2.19**	16.78±2.25**
HAEAE100	100mg/kg (i.p)	101.38±3.72	67.50±2.32	52.43±2.52	17.16±2.98	17.16±2.96
HAEAE200	200mg/kg (i.p)	99.58±3.17	58.26±2.52*	42.12±2.87**	15.76±0.56***	15.57±2.49***

Data expressed as mean ± SEM (n=6).HAEAE = Hydro-alcoholic extract of andrographis echioides; **P<0.001, ***P<0.0001compared with control group (two-way ANOVA followed by Bonferroni's test)

Table No.6: Effect of HAEAE on marble burying test

S.No	Treatment	Dose (mg/kg)	Responses	
			Number of Marbles Buried	% Inhibition
1	Control	0.1ml/animal (i.p)	9.86±2.318	0
2	Diazepam	1mg/kg (i.p)	1.20±0.240*	87.339
3	HAEAE100	100mg/kg (p.o)	3.50±0.468	62.867
4	HAEAE200	20 mg/kg (p.o)	2.02±0.498	78.479

Data expressed as mean ± SEM (n=6).HAEAE = Hydro-alcoholic extract of andrographis echioides; *P<0.05, **P<0.01; compared with control group (one-way ANOVA followed by Barlett's test)



Graphical Abstract

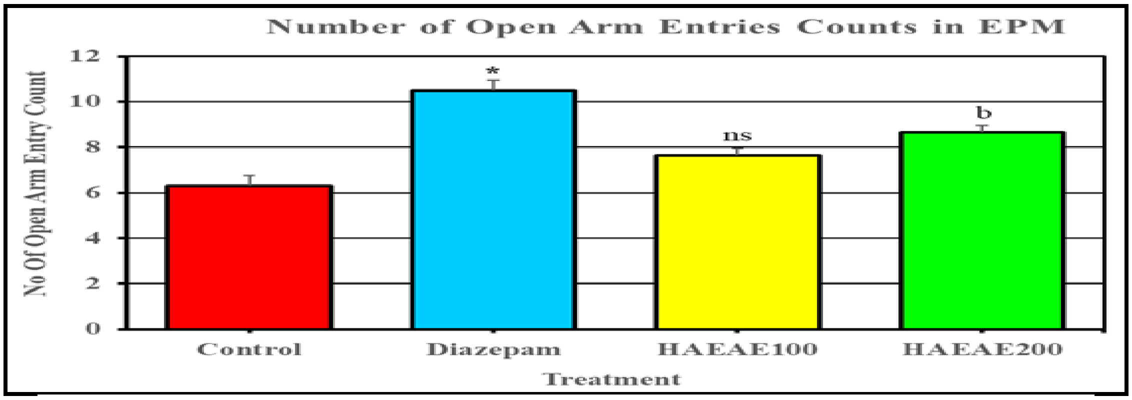


Figure No.1: Results of EPM Test of HAEAE (Number of Open Arm Entries)

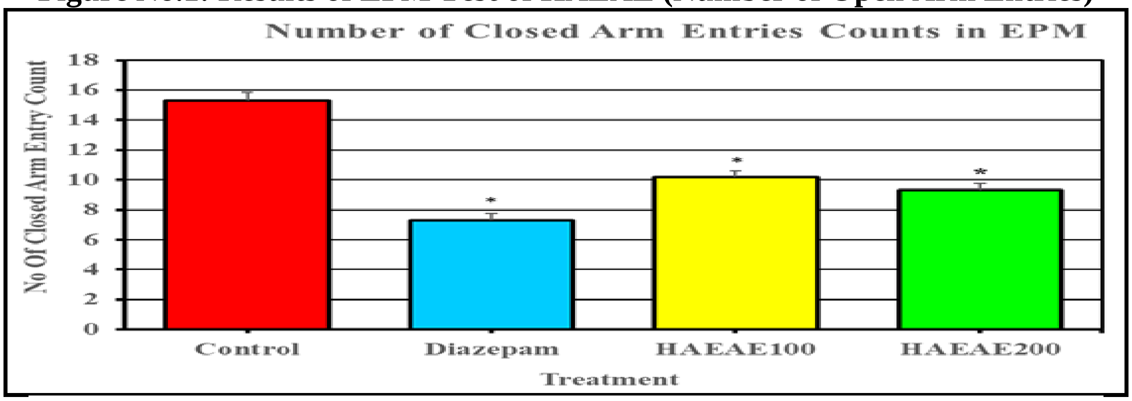


Figure No.2: Results of EPM Test of HAEAE (Number of Closed Arm Entries)

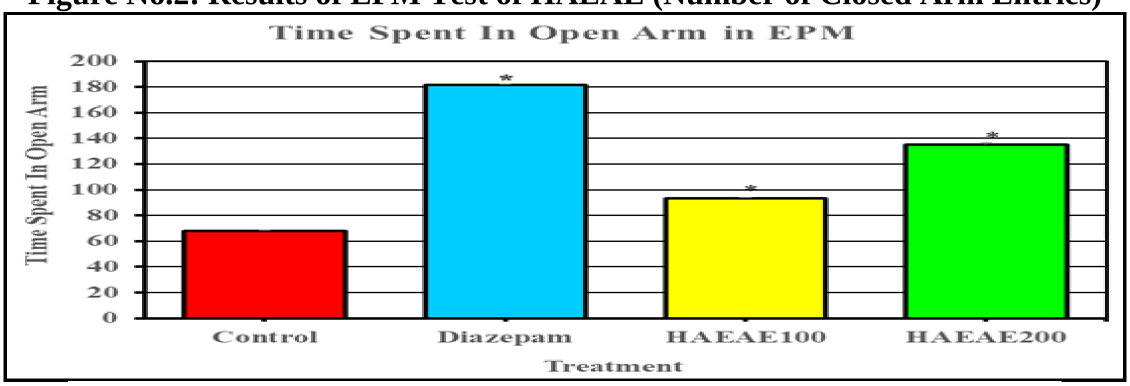


Figure No.3: Results of EPM Test of HAEAE (Time Spent in Open Arm)

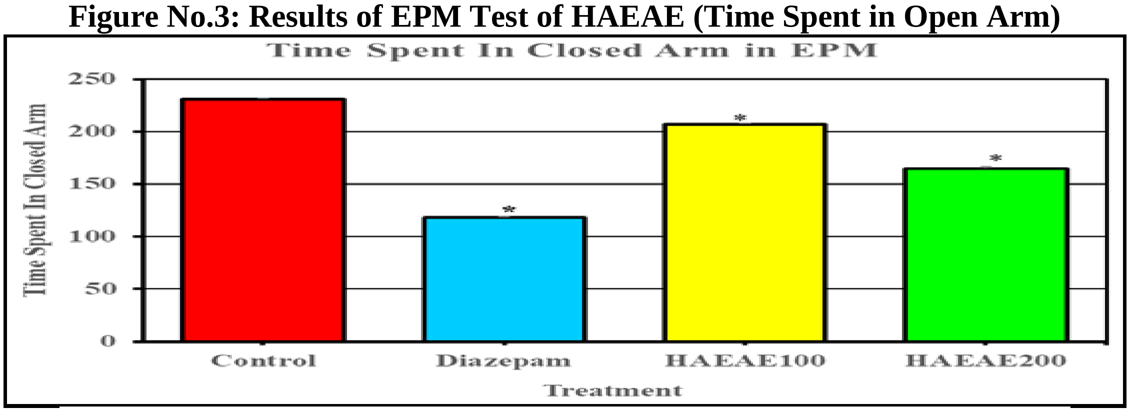


Figure No.4: Results of EPM Test of HAEAE (Time Spent in Closed Arm)

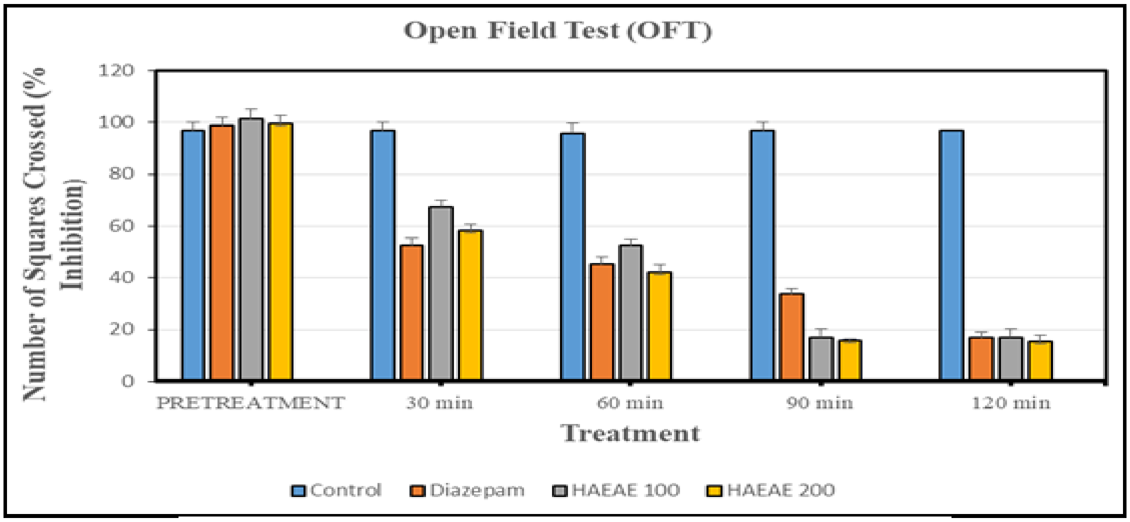


Figure No.5: Results of HAEAE on open field test in rats

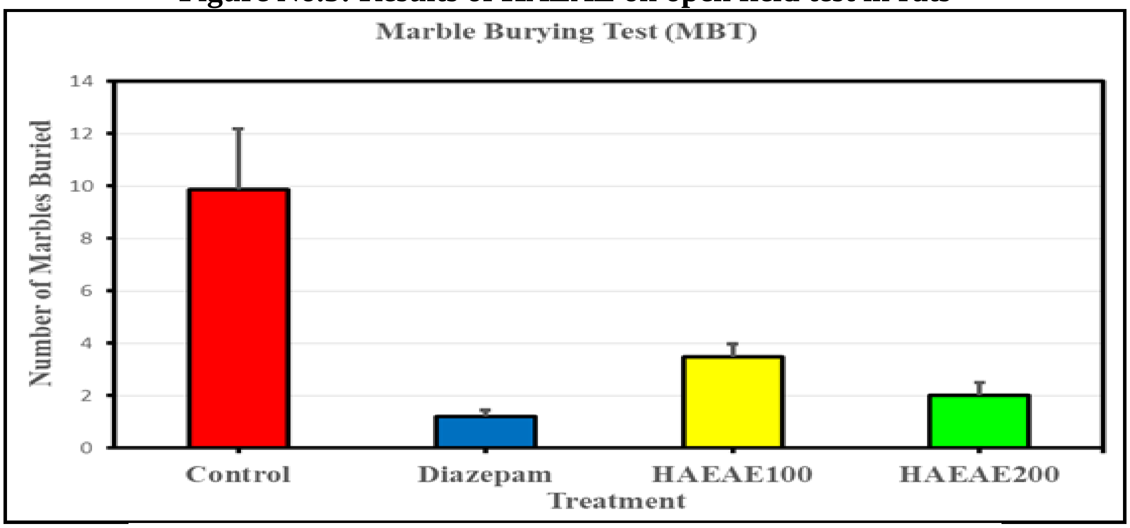


Figure No.6: Marble Burying Test – Number of Marbles Buried

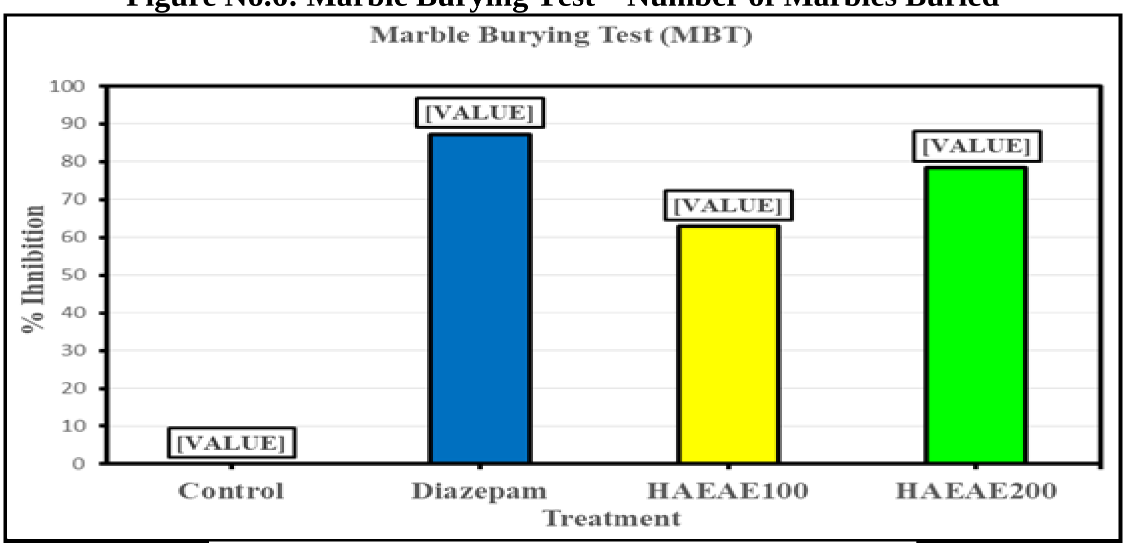


Figure No.7: Marble Burying Test - % of Inhibition

CONCLUSION

The dried plant material was successfully extracted using ethanol and water solvent by cold maceration technique - hydro-alcoholic extract yield: 18.75% (w/w). Preliminary phytochemical evaluation of hydro-alcoholic extract of *Andrographis echioides* (L.) Nees Aerial parts revealed the presence of Alkaloids, flavonoids, glycosides, tannins, steroids, carbohydrates and proteins and amino acids. The study assessed the anxiolytic effects of the hydro-alcoholic extract using established EPM, OFT, MBT and did acute toxicity studies. The study compared the anxiolytic activity of the hydro-alcoholic extract with that of standard anxiolytic drug diazepam. The effects of drugs or other interventions on anxiety-related responses and studied the underlying neurobiology of anxiety disorders and obsessive-compulsive behaviours in rat with the EPM, OFT, MBT tests.

In conclusion, the results of the behavioural tests in selected animal models clearly demonstrate that the hydro-alcohlic extract of *Andrographis echioides* aerial parts shows distinct potential for being developed as an anti-anxiety drug. Since flavonoids are known to promote anxiolytic activity, the presence of flavonoids in these plant extract may be the cause of the anxiolytic activity.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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