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Evaluation of Anti-anxiety Activity of Ethanolic Leaves Extract of Terminalia Arjuna in Wistar Albino Rats

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KEYWORDS

Anti-anxiety, Terminalia Arjuna, Leaves Extract, Wistar albino rats, TLC, Elevated plus maze (EPM), Light/dark exploration, Histopathology.

ABSTRACT:

Present study evaluated the anti-anxiety activity of leave extract of Terminalia Arjuna in experimental rats. Terminalia Arjuna is the well known herbal plant used for treatment of various diseases such as hypertension, heart disease, chest pain, asthma, wound healing and also CNS disorders. Arjuna plant is the richest source of Alkaloids, glycosides, flavonoids and tannins which has major role in treatment of various diseases. Present study is carried out by ethanolic extraction of Arjuna leaves by soxhlation method. Preliminary phytochemical analysis of leaves extract was carried out to confirm the presence of such metabolites. All the animals were divided into five groups; normal control, disease control, standard, test-1 and test-2 groups. Diazepam 2mg/kg was used as standard treatment drug. Test 1 group received Arjuna leave extract 200mg/kg oral and test group 2 was treated with Arjuna leaves extract 400mg/kg. Anti-anxiety activity in experimental animals had been evaluated by Elevated plus maze test and Light/dark exploration method. The result obtained from experiments was concluded that, the ethanolic leaf extract of Terminalia Arjuna having significant activity by EPM test (188±9.1) for open arms and Light/dark exploration test (239.68±9.2) for light box as compared to other animal groups ($P<0.05$). Histopathological findings showed the normal morphological structures of hippocampal sections of normal control animal group and test 2 animal groups. The result obtained from study was concluded that, the Arjuna leaves extract having significant anti-anxiety activity.

INTRODUCTION

Plants are the basic need and part of life provided by nature and these are the richest sources of medicinal constituents such as alkaloid, glycosides, tannins, flavonoids, phenolic compounds etc. which is widely used as major natural source for medicinal values of for therapeutic purpose to prevent and treat a large numbers of diseases [1].

Anxiety is a multifaceted reaction that includes behavioral, physiological, and cognitive alterations to perceived dangers. It sets off the hormone and chemical messenger known as the fight-or-flight response in the brain, which can happen during stressful social circumstances or in the lead-up to significant occasions or choices [2],[3]. The most prevalent category of mental disease is anxiety disorders. Anxiety disorders include panic disorder and agoraphobia (which manifest mainly in adults, starting at age 18), specific phobias, social anxiety disorder, and generalized anxiety disorder (which manifest primarily in childhood, between the ages of 4 and 18), as well as separation anxiety and selective mutism [4],[5].

Terminalia Arjuna, also known as Arjuna plant is member of the *Combretaceae* family. Several ancient Indian medical writings, such as the Charaka Samhita, Sushruta Samhita, and Astang Hridayam, have referenced this ayurvedic medicine since the vedic era. Vagabhatta was the one who initially recommended using stem bark powder to treat cardiac conditions [6],[7].

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MATERIAL AND METHODS

Collection and Authentication of Plant

The leaves of Arjuna plant were collected in February, 2024 from a local garden at rural area of Jaunpur, Uttar Pradesh. Arjuna leaves were dried in shade for 5-7 days and stored in a paper bag for further use. The Arjuna plant was authenticated on the basis of a herbarium file submitted with plant sample at regional centre of Botanical survey of India, Prayagraj, Uttar Pradesh. Authentication had been done by Mr. Vinay Ranjan (Head of Office & Scientist E) and certificate along with reference number BSI/CRC/2024-25/207 had been issued.

Chemicals and Reagents

Loba Chemicals in Mumbai, India, provided all of the chemicals used in this investigation. Analytical-grade substances were employed in the investigation. Glenmark Pharmaceuticals provided the standard medication, diazepam, as a gift sample.

Physicochemical Analysis

Dried powder of Terminalia Arjuna leaves were employed for evaluation of various physicochemical properties such as ash value, total ash, acid insoluble ash, moisture content etc. for evaluation of qualitative parameters for crude drugs.[8]

Extraction of Plant Materials

250 grams of dried Arjuna leaves were powdered in a mechanical grinder into coarse powder. Powdered leaves had been extracted with 95% ethanol in a soxhlet apparatus (Figure-5.2). The solvent containing extract were evaporated in a Rotatory evaporator at 45^{o}C and concentrated over a waterbath to obtain a semisolid mass. The extract was collected in a container and stored in a deep freezer until utilized for experiment [9].

Preliminary Phytochemical Analysis

Ethanolic leaves extract of Arjuna had been examined for presence of various active secondary metabolites such as alkaloids, glycosides, saponins, tannins, phenolic compounds etc.

Thin Layer Chromatography

To identification and separation of active compounds present in plant extract thin layer chromatography were performed by methods described by Nahari et al. 2019. Precoated TLC plate of silica gel G-60 F-254 was used. Mobile phase consist of Chloroform: Ethyl acetate: Ethanol (20:30:20) was used to run through stationary phase. The sample was spotted 1cm from edge through a capillary tube. TLC plate was placed in the development chamber consist of mobile phase. The solvent starts run through TLC plate. The spots had been visualized under UV light at 365nm [10].

Selection of Animals

Healthy Wistar Albino Rats (150-180gm) of either male or female sex were selected for evaluation of anti-anxiety activity. Total 30 animals were used in the study. All animals were caged in a clean propylene cage at 23±2^{o}C with 12:12 hours light/dark cycles. Animals were feeded by standard pallet food with clean water ad libitum until completion of experiment. Animals were acclimatized before experiment for 1 hour at laboratory conditions. The experiments were carried out according to Institutional Animal Ethics Committee (IAEC) guidelines with approval number SIP/IAEC/011/03/24.

Acute oral toxicity study

Acute toxicity was commonly used to determine the LD50 value in experimental animals. The LD50 was determined in mice using OECD guidelines no- 423. The goal of completing an acute toxicity study is to determine a drug's therapeutic index and assure its safety in vivo [11]. Mice were selected for acute oral toxicity test. All animals were fasted overnight before treatment and administered 1 hour following the ethanolic Arjuna leaves extract 200 and 500mg/kg treatment. General behavior was monitored after 1, 4, and 12 hours after administration. Every day for 10 days, the mortality rate of animals were recorded [12].

Grouping of Animals

After acclimatization of animals for seven days, all the animals were divided into four groups; negative control, positive control and group III and IV will be considered as test groups (Figure-5.1). Negative control animals received only normal saline. Positive control animals treated with Diazepam 2 mg/kg p.o. Group III and IV animals received extract solution at dose of 200mg/kg and 400mg/kg respectively.

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EXPERIMENTAL PROTOCOL

Elevated Plus Maze Test (EPM)

Elevated plus maze test is a widely used procedures used for monitoring neurological behavior in rats [13]. It consists of four arms 50-60cmlong and 10cm width, arranged in a plus sign; two open arm and two closed arm. Open arm consist of a platform without side wall while closed arm platform consist of side wall [14]. The centre area of platform was rectangular. The animals were placed individually after 30 minutes of administration in the centre of maze with head facing towards open arm.

The following parameters were recorded:

- Preference of rats to open or closed arm.
- Number of entries in open arm.
- Average time spend in open arm.

Average time = total time spent in the arm

Number of entries

Lastly, we contrasted the percentage of entry in open arms in each group, the average amount of time spent in open arms, and the animals' preferences for open or enclosed arms [15].

Light/Dark Exploration Test

The apparatus used for assessment of neurological behavior by light/dark test procedures consisting of 2 plastic boxes (20×10cm); consist of one which painted with white and brightly illuminated with 500 lux with additional light source and another one was painted with black with dimly illuminated with red light at 50 lux (Arrant et al. 2013). An open door was made between both boxes. Animals were allowed to move towards one compartment to other through open door. The mice were kept in the centre or one box facing door and movement of animal and time spent in the light/dark box were recorded using a video camera for 5 minutes [17].

Histopathological evaluation

At the end of experiments, all the animals were sacrificed under light chloroform anesthesia. The brain part of rats was dissect out carefully with the help of surgical scalpel and preserved in formalin buffer for histopathological study. Any morphological changes in the brain of experimental animals were observed under microscope at suitable magnifications and photographs were taken [18].

Statistical Analysis

Data collected from the study were expressed as Mean± Standard Error of Mean (SEM). Data were analyzed by ANNOVA single factor followed by Tukey's Post-hoc multiple comparison tests to describe difference among groups. The Probability value ($P<0.05$) was considered as significant.

RESULT AND DISCUSSION

Physicochemical Analysis

Physiochemical Analysis

Dried leaves of Arjuna plant were grounded coarsely in a mechanical grinder and their physicochemical examination was carried out. Extraction was done in a soxhlet apparatus and ethanol was used as solvent. A semisolid mass of yellowish green colored was obtained after evaporation of solvent containing extract. The extractive value of leaves was found to 13.4% w/w. The result found after examination is listed below

S.No.	Parameter	Result (%w/w)
1	Ash Value	13.8±1.2
2	Water soluble Ash	6.4±0.2
3	Acid insoluble Ash	2.12±0.3
4	pH	5.4±0.02
5	Moisture content	9.6±0.3
6	Extractive Value	13.4
7	Foreign materials	1.22

Fluorescence analysis of leaf powder

Before extraction process, fluorescence analysis of leaves powder was carried out. Powder was treated with different solvents and observed under different wavelength of ultraviolet light. The results of fluorescence analysis of powder were listed in (table-6.2).

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Table-2: Result of fluorescence analysis of dried Arjuna leaf powder.

S.No	RF values

Treatments	Visible light	UV 254nm	UV 365nm
Methanol	Pale green	Black	Pale green
Ethyl alcohol	Green	Dark Brown	Dark green
Dil. NaOH	Brown	Black	Light green
Dil. $H2SO_4$	Green	Black	Dark green
Dil. Hcl	Pale green	Dark brown	Dark green

Preliminary Phytochemical Analysis

The ethanolic leaf extract of Arjuna was employed for identification of various secondary active metabolites. Different phytochemical tests and their results was listed below in table-00. The result suggested that, the extract is rich source of alkaloids, tannins, flavonoids and saponins.

Table-6.3: Phytochemical tests of extract.

Phytochemicals	Test	Result
Alkaloids	Mayer's test	++
	Dragendorff's test	++
Tannins	$FeCl_3$ test	++
	Lead acetate test	++
Proteins	Biuret test	--
Flavonoids	Shinoda test	++
	Lead acetate test	++
Saponins	Foam test	++
Carbohydrates	Molisch test	--
	Benedicts test	++

6.1.1 Thin layer chromatography

For identification of active constituents present in extract thin layer chromatography was used. Chloroform: Ethyl acetate: Ethanol (20:30:20) was used as mobile phase solvent system. The TLC plate was visualized under UV light at 254nm and 365nm respectively for identification of spots. Spots developed in TLC plate were listed in figure-6.1.

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1.	0.22
2.	0.41
3.	0.52
4.	0.88
5.	0.92

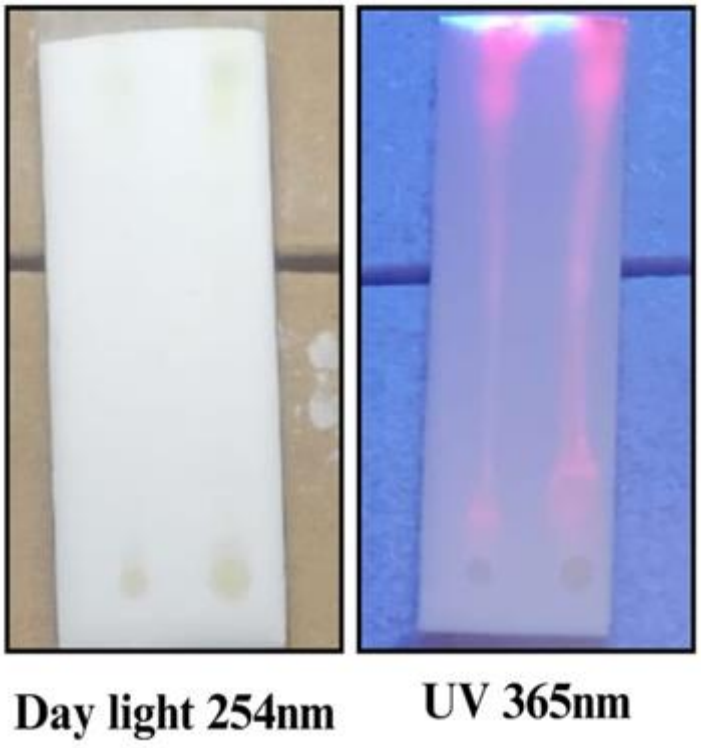

Figure-6.1: Thin layer chromatography of extract

Acute Oral toxicity study

For determination of safety profile of ethanolic Arjuna leaf extract, acute oral toxicity study was performed. The test was done according to guidelines provided by OECD. The study declare that, Arjuna leaf extract at 200 and 400mg/kg was found safe after oral administration. All the animals was examined for mortality and observed that, there was no mortality of animals were observed.

ANTI-ANXIETY ACTIVITY

Elevated plus maze test (EPM)

All animal group was received their respective treatment and after 30min placed one by one in the centre of the maze. The movement and entries of animals into different arms recorded ($P<0.05$) was mentioned in Table-6.5. There is less number of disease control animal group entries into open arm was observed (3.8±0.3), while maximum time was spent into closed arm (240±7.8).

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Table-6.5: Elevated plus maze test results in wistar albino rats. Where, NC= normal control, DC= disease control, STD= standard group, Test-1= anima received 200mg/kg extract and Test-2= animal treated with 400mg/kg extract.

Animal Group	Number of open arm entries	Time spent in open arm	Number of closed arm entries	Time spent in closed arm	Time spent in neutral zone
NC	8.8±1.4	165.3±13.3	6.3±0.7	110±14.3	49.5±8.8
DC	3.8±0.3	31.3±5.9	12±0.7	240±7.8	28.6±6.6
STD	10.8±0.7	166±11.36	5.1±0.5	97.83±7.9	36.1±6.5
Test 1	9.0±1.0	108.6±17.8	9.0±1.0	148±18.4	43.3±10.6
Test 2	9.8±1.3	188±9.1	7.1±0.4	82.16±3.8	29.8±7.8

Standard group animals treated with Diazepam 2mg/kg markedly showed maximum number of entries (10.8±0.7) and time spent into open arm (166±11.36) as compared to other animal groups. Test-2 animal group received extract 400mg/kg had been maximum time spent into open arm while less time spent into closed arm as compared to test group-1, normal control and disease control animals ($p<0.05$).

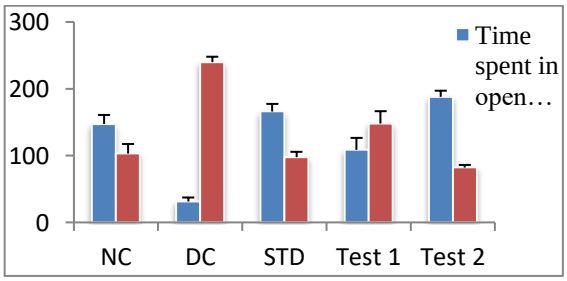

Figure: Total time spent by animals in open and closed arm during EPM test.

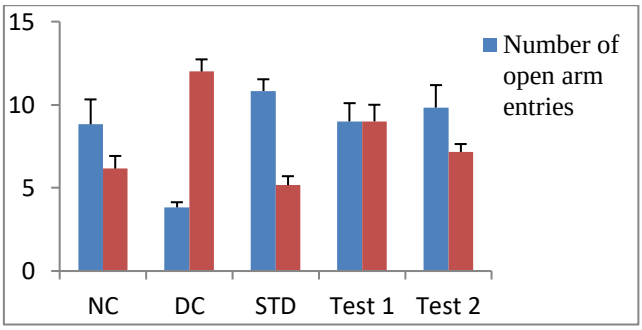

Figure: Total number of entries of different animal group into open and closed arm in EPM test.

Light and dark exploration test

All the animals were received their respective treatments and after 30 minutes later was placed into centre point of the box. After experiments the data was collected as mentioned in table-7.3.

Table: Light and dark exploration test result of different animal groups. Where, NC= normal control, DC= disease control, STD= standard group, Test-1= anima received 200mg/kg extract and Test-2= animal treated with 400mg/kg extract.

Animal Group	Time spent into light box	Number of entries into light box	Time spent into dark box	Number of entries into dark box
NC	232.16±13.8	5.4±0.3	67.84±5.7	2.6±0.03
DC	47.12±6.7	3.6±0.2	252.88±8.6	4.1±0.3

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STD	246.34±11.2	4.8±0.3	53.66±4.2	3.0±0.17
Test 1	176.66±7.4	3.7±0.2	123.34±10.3	4.0±0.19
Test 2	239.68±9.2	4.0±0.1	60.42±4.7	3.3±0.13

Data obtained from experiment suggested that, standard animal group spent maximum time into light box compartment (246.34±11.2) with 4.8±0.3 times entries as compared to all other animal groups. Test group-2 animals received 400mg/kg extract having significantly spent maximum time into light box (239.68±9.2) as compared to all other animal group except standard group animals with a significant difference ($p<0.05$).

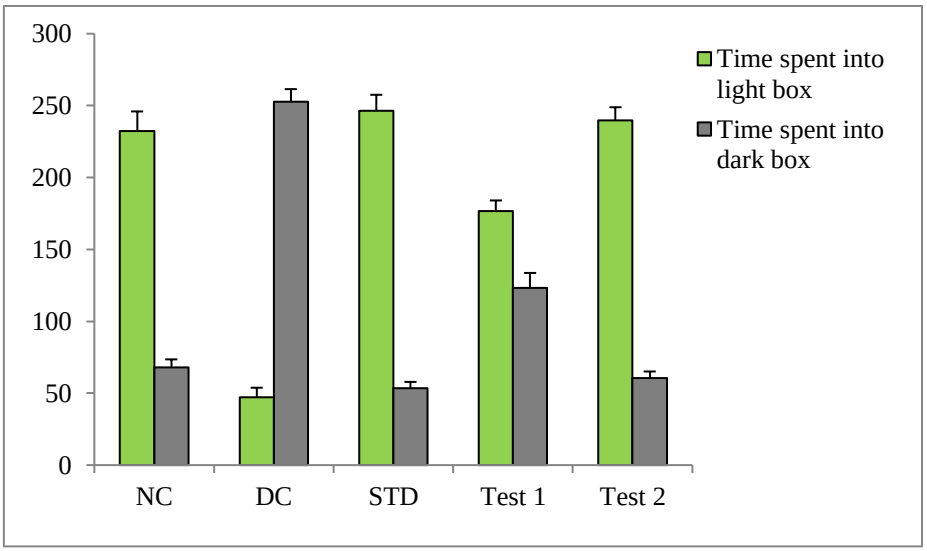

Figure: Total time spent by animals into light and dark box.

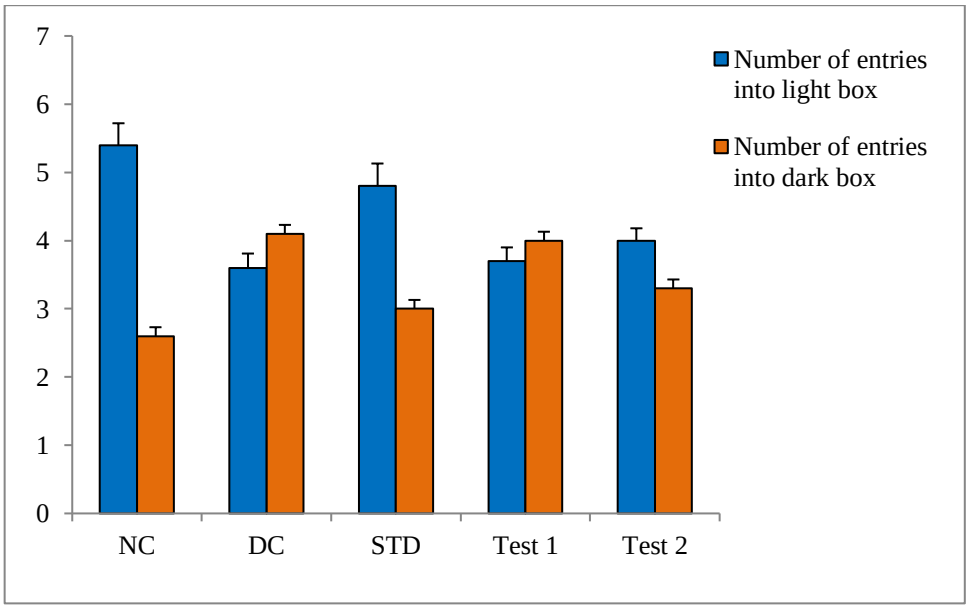

Figure: Total number of entries of different animal groups in light/dark exploration test.

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The number of entries into box was also evaluated during experiment and maximum number of entries into light box was found for normal control group animals, while fewer entries for disease control animal group was observed. Figure-7.6 described the entries of all animal group into light and dark box. Test-2 and normal control animal group showed less number of entries into dark box and produces significant reduced anxiety symptoms.

Histopathological Evaluation

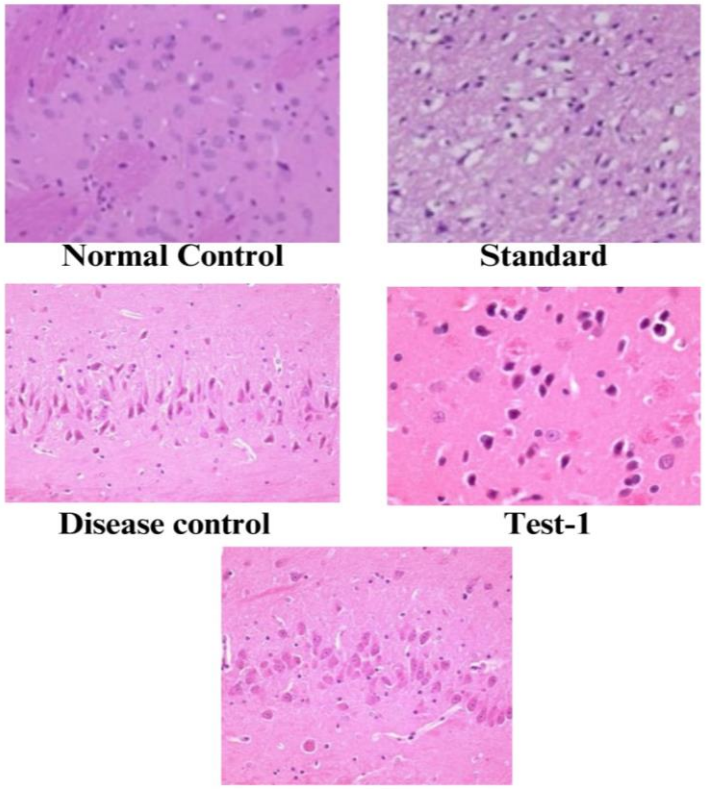

Figure: Histopathology of brain of different animal group.

The histopathological sections of Hippocampal of rats were examined under microscope with suitable magnification and recognized the normal shape and regular and closed arrangement of pyramidal cells in the normal control group animals are described in figure-6.7. Test-1 group animals treated with Arjuna leaves extract at 200mg/kg was observed that, there are a vacuolization with loosely arranged boundries and irregular arrangement of hippocampal cells as compared to normal control, test-2 animal group and standard treatment received animals. Standard group animals showed reduced number of vacoulized Hippocampal cells with regular arrangements. Disease control animals brain tissue having loose and irregular cellular arrengemt was seen and there was vacuolization of cells with their cyroplasmic boundries and their morphology was not regular. The above histological sections attachments concluded that, the the standard group and test-2 animal groups having normal cellular morphology was seen and there are no pathological changes was observed. While, disease control animals group showing diseased morphology and pathologically changed Hippocampal cells was observed.

DISCUSSION

T. Arjuna has a large number of secondary metabolites that are employed as medical treatments. Numerous biogenic qualities, including antiviral, anticancer, antibacterial, anti-malarial, anxiety and other CNS conditions, antithrombotic, anticoagulant, anti-

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mutagenic, antherogenic, and wound healing, are present in this plant. Because of its antioxidant qualities Thus, some food items employ plant components including tannins, polyphenols, and ascorbic acid as antioxidants. Plant leaves include flavonoids, whereas the bark of the plant contains phytochemicals such as tannins, arjunic acid, and arjunetin, among others. It is well known that T. arjuna can cure heart conditions.

Anxiety is a serious mental disorder. Generalized anxiety disorder (GAD), panic disorder, obsessive-compulsive disorder (OCD), post-traumatic stress disorder (PTSD), and specific phobias (such agoraphobia, social anxiety, etc.) are some of the names given to this mental condition.

Present study was based on evaluation of anti-anxiety effects of Arjuna leaves in wistar albino rats. Elevated plus maze test and light and dark box exploration test was used to explore the behavioural activity of experimental animals. The test group animals were treated with Arjuna leaves extract at 200 and 400mg/kg orally found effective for reduced anxiety symptoms. The anti-anxiety effect of Arjuna leaves may be due to increased GABA activity in the brain and by reduced the activity of 5HT and norepinephrine. Several other mechanisms may also be involved in the pathway to produce anti-anxiety effects. To understanding the cellular and molecular mechanism of action of Arjuna leaves for anti-anxiety effect, further research needed to explain.

CONCLUSION

Experimental data obtained from the study was concluded that, ethanolic leaves extract of Terminalia Arjuna at 400mg/kg significantly reduced the anxiety symptoms and restores normal mental health of experimentally induced anxiety in wistar albino rats. The thin layer chromatography of Arjuna leaves extract concluded the presence of alkaloids, tannins, flavonoids. These bioactive components are responsible to produce sufficient CNS effects to reduce anxiety.

Acute oral toxicity study concluded that, oral administration of Arjuna leaves extract at 200 and 400mg/kg was safe and does not produce any toxic symptoms. The elevated plus maze test and light and dark box exploration test was used to evaluate anti-anxiety activity and concluded that, Arjuna leaves extract at 200mg/kg and 400mg/kg significantly produce anti-anxiety effects.

The present study concluded that, ethanolic Arjuna leaves extract at dose dependent manner significantly produced anti-anxiety effects and reduced abnormal behavioural symptoms in wistar albino rats in EPM and light and dark exploration tests. Arjuna leaves extract can be used to treat anxiety and may be an alternative cost effective options in management of anxiety disorder.

Future research should be carried out to explore the molecular mechanism involved to produce effects or any adverse effect produced by the Arjuna leaves extract and drug interaction should be focused. The study also is focused on the safety and efficacy of extract. Present outcomes concluded that, the Arjuna leaves can be used for treatment of anxiety disorder and as alternative options today.

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