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Evaluation of *in-vivo* analgesic activity in mice using methanolic extract of *Tribulus terrestris* leaf

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Abstract
This study was carried out to evaluate the effect of *Tribulus terrestris* methanolic extract of leaf for analgesic activity by using eddy's hot plate method. Here the extract group and control group animals were six which shows the different responses like 2 sec, 5 sec, 6 sec 7 sec at different time intervals like 0 min, 15min, 30 min, 60 min and 90 min etc. are recorded and observed the a response by avoiding the injury to the paws. The standard drug collected is diclofenac sodium 25mg of 5ml with calculated quality. Selected animals are albino mice having10mg/kg/body weight. Six animals are selected. Take the standard drug diclofenac sodium and calculate respectively depending upon the body weight of animals and standard drug is also taken as such the calculated dose. Observe the basal reactions of standard drug at different time intervals of 0, 15, 30, 60, and 90 min in each animal. Observe the paw licking or jumping response according to the time intervals given we observe the jumping response of the mice in sec i.e 3, 5,8,9,10,15 sec. The *in-vivo* analgesic activity was evaluated from methanolic extract of leaves of *Tribulus terrestris* in mice. The basal reaction response of control, standard and test animals were recorded in seconds, in which cut off period is 15 seconds, to avoid paw injury to the mice because eddys hot plate maintained at 55 °C temperature. The test samples were shown the significant analgesic activity when compared to the standard samples.

Keywords: *Tribulus terrestris*, analgesic activity, Eddy's hot plate, cut-off period, basal reaction response

Introduction
Tribulus terrestris is a hardy, herbaceous plant belonging to the family Zygophyllaceous. It is widely distributed across Southern Europe, Asia, Africa, and Australia. The plant is characterized by its small yellow flowers, pinnate leaves, and it has ability to puncture tires and footwear. *Tribulus terrestris* has a long history of use in traditional systems of medicine, particularly in Ayurveda, Traditional Chinese Medicine, and Unani. It has is employed for a wide range of health issues including urinary disorders, reproductive problems, and fatigue.
Tribulus terrestris L. is an annual plant of the family Zygophyllaceous, and has been used for ages in traditional medicine in Greece, China, and India to energize, vitalize. It causes improvement sexual function and physical performance in humans. It is also used individually as a single therapeutic agent or sub ordinate component of many compound formulations and food supplements. It is a shrub found in Mediterranean, subtropical, and desert climate regions around the world, including India, China, southern US, Mexico, Spain, and Bulgaria [1-2].

Various types of compounds with a variety of biological properties and chemical structures have been identified from T. terrestris, including steroidal saponins, tannins, phytosterols, terpenoids, amide derivatives, amino acids, and protein. The whole plant of TT has been explored exhaustively for its phytochemical and pharmacological activities, such as diuretic, aphrodisiac, anti urolithic, immunomodulatory, antihypertensive, antihyperlipidemic, antidiabetic, hepatoprotective, anticancer, anthelmintic, antibacterial, analgesic, and anti-inflammatory.
Tribulus terrestris (TT) is a flowering herb found in temperate climates all over the world.

The plant and its extracts have traditionally been used as medicines in Asia and India to treat urinary, cardiovascular, and gastrointestinal disorders as well as being used as a sexual stimulant [3-5]. The aphrodisiac properties are due to the steroidal saponin known as protodioscin (PTN). In a 2002 study by Gautama, aphrodisiac properties of TT extract containing PTN were tested on normal and castrated rats fed either water, testosterone (10 mg/kg), or PTN (castratedonly) (5 mg/kg). The aphrodisiac effects are thought to be due to increases in androgen levels in the PTN-fed groups. The results of this study were confirmed by Gautama's group in 2003 using non-castrated rats at various TT concentrations (2.5, 5, 10 mg/kg). The study also confirmed an increase in intra cavernous pressure (ICP), which is a widely accepted index of penile erection.

TT has been used medicinally to cure a range of health-associated problems such as high blood pressure, stomach problems, and urinary infections. The plant has also been used to boost male sexual desire and mating behavior investigated the androgenic properties of TT extract in primates, testosterone, dihydrotestosterone, and dehydroepiandrosterone sulphate, especially at a dose of 7, 5 mg/kg body weight. The authors concluded that TT may be safe and effective for use in the alleviation of sexual desire and erectile function. However, further clinical trials with larger sample sizes are needed to evaluate its safety and efficacy before it can be recommended to patients for Treatment. Tribulus is composed of the biologically active compounds sapiens and tannins. Saponins are known to increase NO activity. Tribulus has also been reported to increase libido and serum testosterone in rat models of sexual dysfunction. Fewer studies on the effects of Tribuluson erectile function have been conducted in humans [6-8].

*Tribulus terrestris*is commonly known as Gokshur. It is distributed along a wide geographic perimeter. It is found all over India up to 11,000 ft in Kashmir, Ceylon, and all warm regions of both hemispheres. It is a common weed of the pasturelands, road sides, and other waste places, chiefly in hot, dry, and sandy regions including West Rajasthan and Gujarat in India.

Tribulus terrestris, flowers are yellow in color, its carpal fruits are of characteristic, satellite shape, somewhat round-shaped, compressed, five cornered, and covered with prickles of very light yellow color. There are several seeds in each crocus with a transverse partition between them. The seeds are oily in nature. When fresh, the root is slender, fibrous, cylindrical, frequently branched, bearing a number of small rootlets and is of light brown color. Fruits and roots are mainly used as a folk medicine for the treatment of various ailments.

Tribulus terrestris leaves are small, pinnately compound leaves, typically with 5-8 pairs of leaflets, arranged opposite each other. They are elliptical in shape, and can be described as lanceolate. The leaves are unequal in size within a pair, and the leaflets are mucronate. The leaves are hairy structures (hirsute). Arrangement of leaves are opposite and they are divided into smaller leaflets arranged along a central stalk. Leaflet Shape is typically oblong or elliptical, sometimes described as oblong-lanceolate, and they are usually mucronate [13-15].

Biological source

The biological source of *Tribulus terrestris* is the dried fruits, roots, and aerial parts of the plant, which belongs to the family Zygophyllaceous.

- **Botanical name:** *Tribulus terrestris*
- **Family:** Zygophyllaceous
- **Partused:** Fruits(commonly), alsorootsandwhole plant
- **Commonnames:** GoksHura (Ayurveda), Puncturevine, Caltrop, Devil'sweed

Cultivation and collection of *Tribulus terrestris*

Cultivation of *Tribulus terrestris*: limate: Preferswarm, dry, and sunny climates; thrives in subtropical and tropical regions.

- **Soil:** Grows well in well-drained sandy or loamy soils with neutral to slightly alkaline ph. It tolerates poor and dry soils.
- **Propagation:** Primarily by seeds.
- **Sowing time:** Usually sown during spring or early summer (March–June).
- **Spacing:** About30cm x 30cm between plants.
- **Irrigation:** Requires moderate watering; drought-tolerant once established.
- **Fertilization:** Requires minimal fertilizers; organic compost can be used to improve oil fertility.

Collection

- **Harvesting time:** The fruits are collected when they are fully matured and dried, typically 3–4months.
- **Method:** Fruits are hand-picked or collected manually from the ground.
- **Post-harvest processing:** The collected plant material is cleaned, dried in the shade, and then stored in airtight containers away from moisture and light to preserve active constituent.

Chemical constituents

The preliminary phyto chemical study of TT revealed the presence of saponins, flavonoids, glycosides, alkaloids, and tannins. According to literature data, the saponin composition and the saponin content of TT from different geographic regions is different. Kostova *et al*. studied the chemistry and bioactivity of saponins in TT. They reported that fur stanol and Spiro stanol saponin of tigogenin, neo tigogenin, gitogenin, neogitogestin, hecogenin, neohecogenin, diosgenin, chlorogenic, ruscogenin, and Sars sapogenin types of constituents were found in this plant.

In addition, four sulfated saponin tigogenin and diosgenin types were also isolated. Majorly present are furstanol glycosides including protodioscin and protogracillin, of which protodioscin is the most dominant saponin and Spiro stanol glycosides are present in small quantities that the quantity of main flavonoids is about 1.5 times that of main saponins. Isolated kaempferol, kaempferol-3-glucoside, kaempferol-3-rutinoside, and tribuloside from leaves as well as fruits and identified them by spectroscopic analysis. Detected 18 flavonoids using high-performance liquid chromatography (HPLC) in four Tribulus species leaf extracts. Optimized the extraction condition using orthogonal experiment. Matin Yekta *et al*. isolated three flavonoid glycosides such as quercetin 3-O-glycoside, quercetin 3-O-rutinoside, and kaempferol 3-O- glycoside from the aerial parts of T. terrestris in the northeast of Iran. Identified flavonoids from the petroleum ether and

chloroform extracts of fresh fruits of TT from India using ethyl acetate: benzene (1:9) solvent system [16-18].
These flavonoids were not detected in the fruit extracts of other varieties, namely Talatus. Hence, presence of such pharmacognostic constituents can be used as a diagnostic tool in the identification of the species and study of contamination/ adulteration. The β carboline alkaloid, tribulusterine, is present in minor quantities in fruits. Gas chromatography-mass spectrometry analysis of methanolic extract of the whole plant of TT revealed the presence of α-Amyrin as the major constituent and seven minor constituents, which are 3,7,11,15- tetramethyl-2-hexadecen-1-ol, n-hexadecatrienoic acid, hexadecadienoicacid ethyl ester, phytol, 9,12- octadecadienoic acid, 9,12,15-octadecatrienoic acid, and 1,2-benzenedicarboxylic acid diisooctyl ester. Sterols such as β-sitosterol and stigmasterol were also found to be present 1, 2 benzene dicarboxylic acid [18-20].

Aim and objectives

Aim

Evaluation of analgesic activity in mice using methanolic extract of leaves of Tribulus terristris

Objectives

1. Collection and preparation of dried leaves of Tribulus terristris plant
2. Cold maceration is the extraction procedure to get final product
3. By using end product, carry out the acute toxicity studies for dose determination on mice (CPCSEA approval certificate added in thesis project.
4. Evaluation of analgesic activity by using eddy's hot plate method on mice.

Plan of work

- Selection of plant and method of extraction**,**
- Analysis of activity on the basis of literature reviews availability of solvent and then proceeds for pharmacological activity.

Materials and Methods

Procedure (Cold maceration)

Coldmacerationisacommonmethodusedinherbalextractionto obtainbioactivecompound without applying heat which help preserve heat- sensitive constituents.

Materials used

1. Dried powder of *Tribulus terrestris* (leaves powder)
2. Solvent: Methanol with Distilled water
3. Glass container with tight lid
4. Filter paper or muslin cloth
5. Funnel
6. Measuring cylinder
7. Beaker
8. Mortar and pestle (for grinding)

Procedure

Preparation of plant material

a. Cleanthe *Tribulus terrestris* plant leave to remove dirt and foreign matter
b. Dry the leaves in shade at room temperature
c. Grind the dried material to coarse powder using motor and pestle

Weighing and solvent selection

a. Weigh the required amount of powder material (100 gram)
b. Choose a solvent (70% ethanol or Hcl mixture)
c. 1000 ml of solvent for100 gram of powder

Maceration procedure

a. Allow them mixture to stand at room temperature (15 –25 degree Celsius) for
b. Stir or shake the container once or twice daily to enhance maceration

Maceration

a. Transfer the powder plant material into the clean and dry glass container
b. Add measure amount of solvent to the container
c. Seal the container and shake well to ensure complete mixing

Filtration

a. After Maceration period filter the mixture through filter paper.
b. Squeeze or press the residue to recover as much as extracts possible

Concentration

Concentrate the filter using rotatory evaporator or by drying under reduced pressure if needed for any further analysis.

Methodology

Acute toxicity studies

The Acute lethal toxicity was determined by using methanolic extract of *Tribulus terrestris* leaf in mice. The animals divided into three groups, each group consists of six mice, as per the OECD guidelines 420, and different doses were used such as 5,50,300,2000 mg/kg. bodyweight. Among the doses 100 mg /kg. Bw, significantly produces analgesic activity and toxicity found at 2000 mg/kg body weight. LD50 (The lethal dose $_{50}$ was found and further testing was done).

Evaluation of analgesic activity in mice using methanolic extract of leaves of *Tribulus terrestris* leaf extract

- Take Swiss albino mice were used for experimental purpose.
- Weigh the animals and divide them into three groups. Each group consists of six animals.
- The selected animals maintained under the aseptic conditions have been maintaining feeding conditions in animal house, as per the OECD and CPCSEA guidelines, the animals are maintained at temperature 20 °C.

Group 1: control group of animals, 0.1ml/per oral.

Group 2: Standard group of animals 25 mg/kg, bwt. / per oral.

Group 3: test group of animals 100mg/kg, bwt. / per oral.

- Control group animals are treated by saline by oral, 0.1ml, per oral.

- Standard group animals administered by diclofenac sodium, i.p, 25 mg / kg/b wt. According to the standard procedure, the given dose are 25mg / kg / bwt.
- Extraction dose is 100 mg/kg. Body wt. (0.1 ml)

Group1: Evaluation of control group of animals by distilled water orally

Take the 1ml of saline in the syringe and administer it orally into Swiss albino mice. The animal response was observed on Eddy's hotplate maintained at the temperature 55ºC, in the form of jump response. Mainly jumping response observed in seconds. The basal reaction response is very less in control group animals, after administration of distilled water, keep the mice on the plate and simultaneously starts the stop watch to measure the withdrawal reflex of limbs. Stop the stopwatch after the mouse displays any reaction to heat (paw shaking, licking, or jumping) Here the control group animals were six which shows the different responds like 2 sec, 5 sec,6 sec,7 sec at different time intervals like 0 min,15min,30min,60min and 90 min etc. are recorded. Remove the animals from hot surface to avoid paw injury to the paws if animals exceeds 15sec,considered as cut off period.

Group2: Evaluation of standard group of animals

- Weigh the animals, each animal have on an average of 20gm, the standard drug taken for the study is diclofenac sodium 25 mg/kg, bw.
- Selected animals are albino mice.
- Take the standard drug diclofenac sodium and calculate respectively depending upon the body weight of animals and standard drug is also taken as such the calculated dose.
- Take the six animals and inject the, 25mg/kg.bw, i.e, 0.1ml (4IU) diclofenac sodium through intra peritoneal route.
- Place the animals on the hot plate and simultaneously start the stopwatch to measure the withdrawal latency.
- Observe the basal reaction responses of standard drug at different time intervals of 0, 15, 30, 60, and 90 and 120min for each animal.

Observe the paw licking or jump response according to the time intervals, observe the jump response ofthe mice in sec i.e.3,5,8,9,10,15sec.

Group 3: Evaluation of test group of animals from *Tribulus terrestris* leaf extract Extraction procedure of leaf as a test drug

- Collect the extract from the dried leaves by cold maceration apparatus. The obtained extract was diluted with carboxy methyl cellulose with calculate quantity.
- Collect 1000 gm of *Tribulus terrestris* leaves from the plant
- Clean the leaves and keep them for drying for about 10-20daysby natural drying process.
- After the drying process is done the quantity of the flowers are reduced then crush the leaves of *Tribulus terrestris* by using motor or pestle and filter them with the help of sieve.
- After crushing take about 500 gm of dry powder extract and keep it in air tight container.
- Now take on conical flask or a beaker and add 1000 ml of methanol solvent.
- Place the mixture in a cool temperature i.e. 20-25 c for five days keep stirring with a stirrer randomly each day.
- Now, filter the extract using the filter paper to obtain a clear solution.
- Transfer the clear solution which is filtered to the clean and dried conical flask.
- Now take the solution into the insulin syringe up to 2ml
- Switch on the hot plate apparatus and wait until the plates reaches defined temperature 52 (adjust if necessary) tore-establish the surface temperature before commencing with the test
- Inject orally about 2 ml of normal saline to the first animal and then put the first mice on the plate and simultaneously start the stop watch to measure the withdrawal latency.
- Stop the stop watch after the mice displays any reaction to heat (paw shaking, licking, jumping) If the mice does not react to heat after 30 seconds, it is removed from the hot plate and 30 second is considered as latency by default**.**
- Observe the basal reaction of test drug at different time intervals of 0, 15, 30, 60, 90.
- Observe the paw licking or jumping response according to the given time intervals. We observed the jumping response of the mice at 2, 5, 6, 7, 10, 12 sec.

Results and Discussion

Table 1: Control animals Basal reaction response in seconds

S. No	Drug	Dose (ml)	Basal Reaction Response (sec)	Basal reaction response in seconds (Jumping response) Time interval in minutes					
				0 min	15 min	30 min	60 min	90 min	120 min
1		Saline / Distilled water 0.1 ml	Paw Licking / Jumping Response	1 sec	5 sec	6 sec	6 sec	7 sec	7 sec
2				1 sec	5 sec	5 sec	5 sec	6 sec	6 sec
3				1 sec	3 sec	3 sec	4 sec	4 sec	5 sec
4				1 sec	3 sec	4 sec	5 sec	5 sec	5 sec
5				2 sec	3 sec	5 sec	6 sec	6 sec	6 sec
6				2 sec	3 sec	5 sec	6 sec	6 sec	7 sec

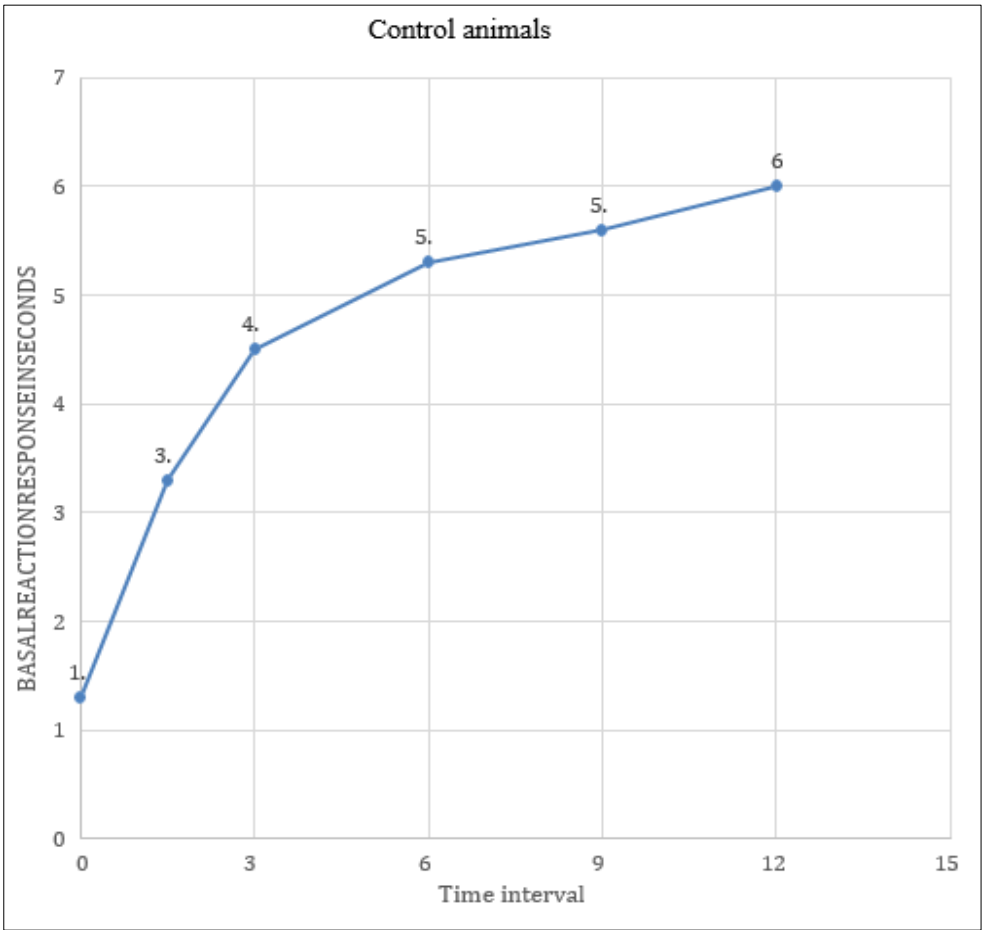


Table 2: Test group animals Basal reaction response in seconds

S. No	Drug	Dose (ml) / kg b.wt	Basal Reaction Response (sec)	Basal reaction response in seconds (Jumping response) Time interval in minutes					
				0 min	15 min	30 min	60 min	90 min	120 min
1	*Tribulus terrestris* methanolic extract (100 mg/4 µg)	100 mg/kg b.wt (0.1 ml/4 µg)	Paw Licking / Jumping Response	2 sec	4 sec	4 sec	6 sec	8 sec	12 sec
2				2 sec	5 sec	6 sec	7 sec	10 sec	12 sec
3				1 sec	6 sec	7 sec	9 sec	10 sec	14 sec
4				2 sec	6 sec	8 sec	10 sec	10 sec	15 sec
5				2 sec	6 sec	8 sec	10 sec	12 sec	15 sec
6				1 sec	5 sec	8 sec	9 sec	12 sec	15 sec

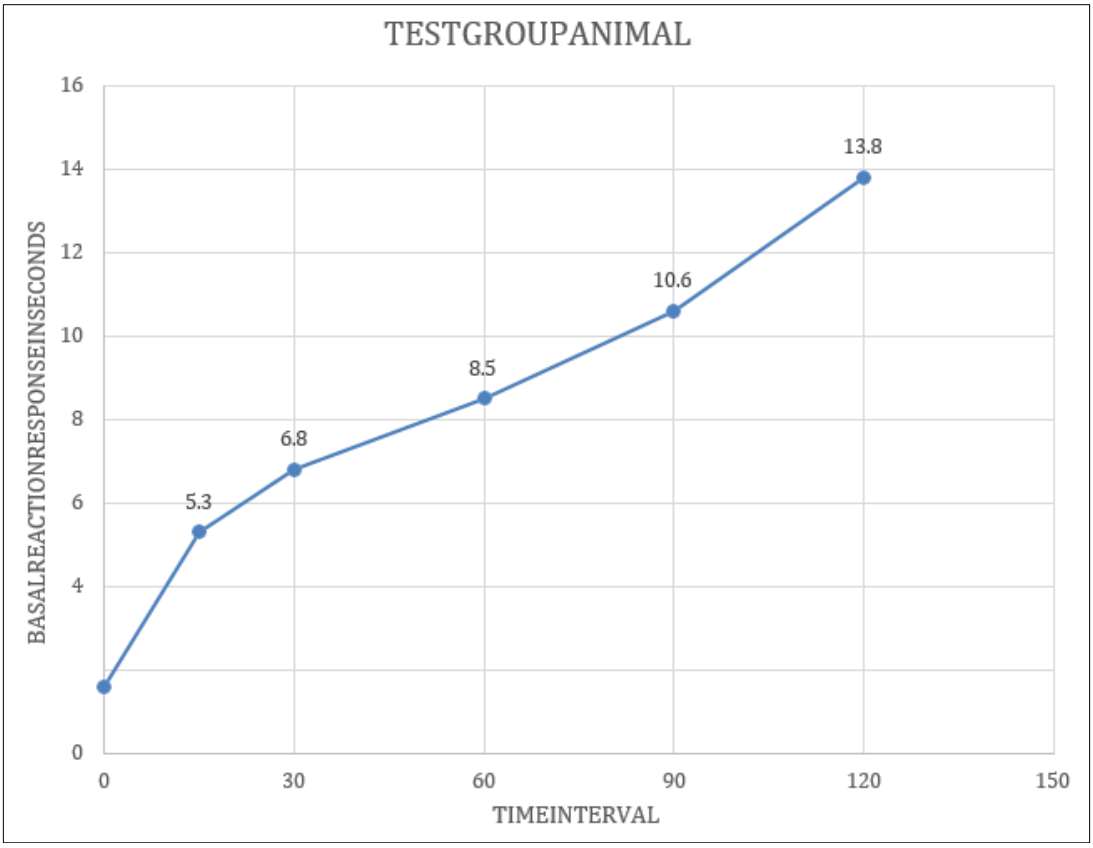
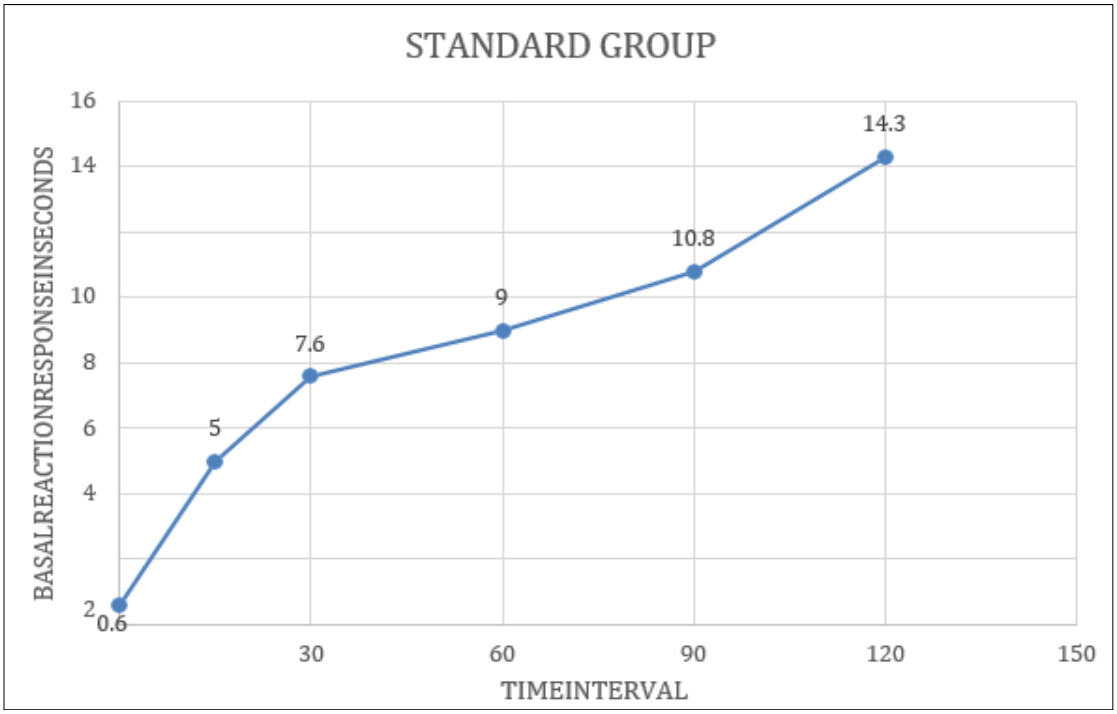
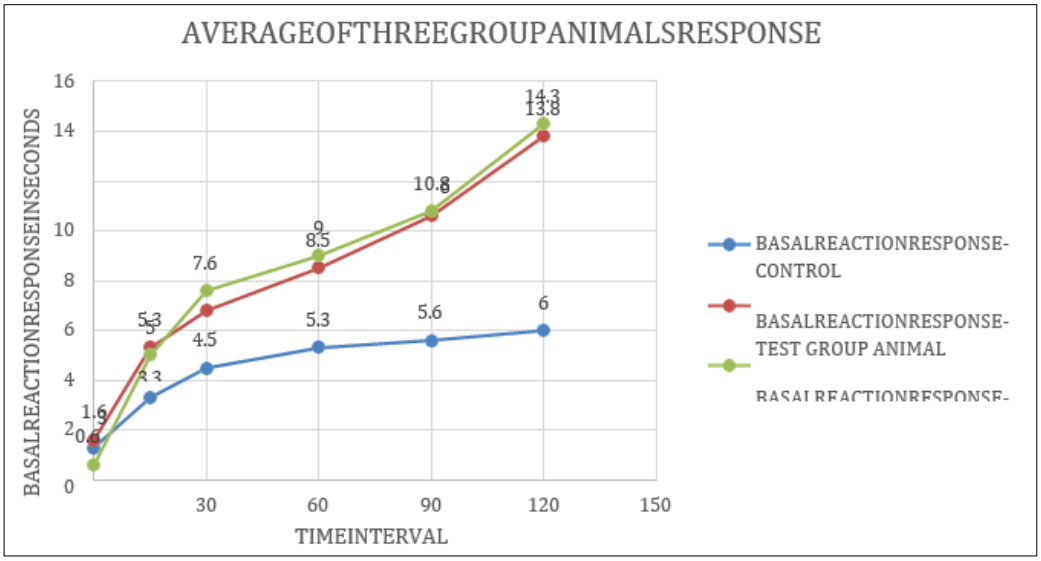


Table 3: Standard group animals Basal reaction response in seconds

S. No	Drug	Dose (ml/kg bwt)	Basal Reaction Response (sec)	Basal reaction response in seconds (Jumping response) Time interval in minutes					
				0 min	15 min	30 min	60 min	90 min	120 min
1	Diclofenac sodium 25 mg	10 mg/kg bwt (3.4 µg)	Paw Licking / Jumping Response	2 sec	6 sec	8 sec	10 sec	12 sec	15 sec
2				2 sec	3 sec	5 sec	6 sec	10 sec	13 sec
3				0 sec	5 sec	8 sec	9 sec	10 sec	15 sec
4				0 sec	6 sec	8 sec	10 sec	11 sec	15 sec
5				0 sec	5 sec	9 sec	10 sec	12 sec	14 sec
6				0 sec	5 sec	8 sec	9 sec	10 sec	14 sec





Acute toxicity studies evaluate the adverse effects of a substance after a single or short-term exposure. They are conducted to determine the potential harm caused by a substance, using a single and large dose. The important aspect of acute toxicity study is determining the LD_{50} (lethal dose for 50% of test animals) and observing for signs of toxicity within 24 hours or 14 days. These studies are crucial for regulatory requirements, like classification and labeling of industrial chemicals and pesticides, and for understanding a substance's potential for harm. Acute lethal toxicity was determined in mice by using methanolic extract of *Tribulus terrestris* leaf. The animals divided into three groups, each group consists of six mice, as per the OECD guidelines 420, different doses were used such as 5,50,300,2000 mg/kg. body weight. Among the doses 100 mg/kg. Body weight, significantly produces analgesic activity and toxicity found at 2000 mg/kg body weight. LD50 (The lethal dose 50 was found and further testing was done).

The injectable dose to the mice through intra-peritoneal route is 3.2 units. Collect the extract from the dried leaves by cold maceration process. The obtained extract was diluted with carboxy methyl cellulose with calculate quantity. Collect 1000 gm of *Tribulus terrestris* leaves from the plant. Clean the leaves and keep them for drying for about 10-20 days by natural drying process. After the drying process is done the quantity of the flowers are reduced then crush the leaves of *Tribulus terrestris* by using motor or pestle and filter them with the help of sieve. After crushing take about 500 gm of dry powder extract and keep it in an air tight container. Now take one conical flask or a beaker and add 1000 ml of methanol solvent. Place the mixture in a cool temperature i.e 20-25 c for five days keep stirring with a stirrer, each day, after filtration the extract using the filter paper to obtain a clear solution. Transfer the clear solution which is filtered to the clean and dried conical flask. Now take the solution into the insulin syringe up to 2ml.

This study was carried out to evaluate the analgesic activity by using methanolic extract of leaves of *Tribulus terrestris*. As the collection of Tribulus is done from the nursery and other materials such as methanol, distilled water, were also collected with the apparatus required for the procedure. Apparatus such as conical flask, glass rods, filter paper, Eddy's hot plate apparatus, and syringes needles were collected. By taking some dried leaves of *Tribulus terrestris* and powder them to obtain an extract from it. After extraction of Tterrestris clear solution, add methanol to the extract and make up the volume with distilled water. The solution was mixed thoroughly and kept it in a dark for 3 days. Take the 1ml of distilled water in the syringe and administer it orally into Swiss albino mice. The animal response was observed on Eddy's hot plate maintaining the temp at 55c.In the form of paw licking or jump response. Mainly jumping response observed in seconds. The basal reaction responses very less in control group animals, when salineis administered put them ice on the plate and simultaneously starts the stop watch to measure the withdrawal latency. Stop the stopwatch after the mouse displays any reaction to heat (paw licking, or jump response) Here the control group animals were six which shows the different responds like 2 sec, 5 sec, 6 sec, 7 sec at different time intervals like 0 min,15min,30min,60min and 90 min etc… are recorded and observed the a response by avoiding the injury to the paws.

The standard drug is diclofenac sodium 25mg of 5ml with calculated quality. Selected animals are albino mice having 10 mg/kg/body weight. Six animals are selected. Take the standard drug diclofenac sodium and calculate respectively depending upon the body weight of animals and standard drug is also taken as such the calculated dose. Take the six animals and inject the diclofenac sodium as standard drug given through peritoneal route. Place the animals on the hotplate and simultaneously start the stopwatch to measure the withdrawal latency. Observe the basal reactions of standard drug at different time intervals of 0, 15, 30, 60, and 90 min in each animal. Observe the paw licking or jumping response according to the time intervals given we observe the jumping response of the mice in sec i.e 3, 5,8,9,10,15 sec.

Conclusion

The in-vivo analgesic activity was evaluated from methanolic extract of leaves of *Tribulus terrestris* in mice. The basal reaction response of control, standard and test animals were recorded in seconds, in which cut off period is 15 seconds, to avoid paw injury to the mice because eddy's hot plate maintained at 55 °C temperature. The test samples were shown the significant analgesic activity when compared to the standard samples.

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