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Research Article

Preclinical Evaluation of an Indian Herbal Plant for its Antidepressant Action using Laboratory Animals

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Abstract: Present investigation is focused on the "Preclinical evaluation of antidepressant activity of ethanolic extract Tribulus terrestris fruits and n- butanol fractions of ethanolic extract of Tribulus terrestri fruits (NETT) were evaluated for antidepressant activity using in laboratory animals using different preclinical models of antidepressant activity such as forced swim test, tail suspension test and spontaneous locomotor activity. The preliminary phytochemical analysis revealed the phytoconstituents present in the extracts. Ethanolic extract of Tribulus terrestris fruits (ETT) showed the presence of steroids, saponins, tannins, terpenoids, flavonoids, alkaloids. n-butanol fractions of ethanolic extract of Tribulus terrestris fruits (NETT) were subjected to the High-Performance Thin Layer Chromatography (HPTLC) studies Ethanolic extract of Tribulus terrestri fruits (NETT) and their fractions showed potent antidepressant activity. The multistep putative action of these plants may be attributed to the prominent phytoconstituents namely 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-2,3-dihydrochromen-4-one from n-butanol fractions of ethanolic extract of Tribulus terrestris fruits (NETT). As a result of these studies two different phytoconstituents were isolated i.e., 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-2,3-dihydrochromen-4-one from n-butanol fractions of ethanolic extract of Tribulus terrestris fruits (NETT). The isolation of these phytoconstituents is an important concluding result of the study and cardioprotective activity of the extracts may be attributed to these Phytoconstituents.

Keywords: Tribulus terrestri fruits, CNS, Dysthymia, ANOVA, HPTLC

INTRODUCTION

The central nervous system (CNS) associated diseases are appearing as a major threat because of increasing mental stress[1], workload and strain which have become inherent to the present-day competitive world. Depression is a frequent and serious psychological illness ranging from mild to severe form [2]. It is characterized by negative impact on thought processing by persistent sadness, loss of interest or pleasure, low energy, worse appetite and sleep, suicide, extreme exaggeration, lack of interest and mood disturbance, disrupting daily activities and psychosocial functions, which adversely affect cognition and psychomotor function. Epidemiology of depression is a multifactorial, chronic, and life-threatening disease with globally high prevalence[3]. The world health report shows signs of psychological or behavioural syndromes in about 50 million people worldwide.3 It accounts for 15.3% of the world's affliction of disease and is anticipated to rise to 20% by 2025. Depression has an extreme global economic burden and has been listed as the third largest cause of disease burden by the World Health Organization since 2008, and is expected to rank the first by 2030. Depressive disorders are mood disorders that come in different forms, just as do other illnesses, such as heart disease and diabetes. Three of the most common types of depressive disorders are discussed below[4,5].

Today, a large variety of synthetic drugs are used as standard treatment for clinically depressed patients[6]. Regardless of the medication that may be used to treat depression, practitioners have become more aware that different ethnic groups may have different responses and have different risks for side effects than others[7].

Antidepressant medications Selective serotonin reuptake inhibitors (SSRIs) are medications that increase the amount of the neurochemical serotonin in the brain[8]. (Remember that brain serotonin levels are often low in depression.) As their name implies, the SSRIs work by selectively inhibiting (blocking) serotonin reuptake in the brain[9,10]. This block occurs at the synapse, the place where brain cells (neurons) are connected to each other. Serotonin is one of the chemicals in the brain that carries messages across these connections (synapses) from one neuron to another[11].

The SSRIs work by keeping serotonin present in high concentrations in the synapses. These drugs do this by preventing the reuptake of serotonin back into the sending nerve cell[12,13]. The reuptake of serotonin is responsible for turning off the production of new serotonin. Therefore, the serotonin message keeps on coming through. It is thought that this, in turn, helps arouse (activate) cells that have been deactivated by depression, thereby relieving the depressed person's symptoms[14]. SSRIs have fewer side effects than the tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs), which are discussed below. SSRIs do not interact with the chemical tyramine in foods, as do the MAOIs, and therefore do not require the dietary restrictions of the MAOIs[15]. Also, SSRIs do not cause orthostatic hypotension (sudden drop in

blood pressure when sitting up or standing) and heart-rhythm disturbances, like the TCAs do. Therefore, SSRIs are often the first-line antidepressant drugs[16].

Since all the synthetic drugs available for the treatment of depression have various adverse effects and drug interactions. Such conditions create opportunities for alternative treatment of depression treatment. Currently, researchers are seeking out more specific drugs with higher safety and lower cost[17]. Traditional medicine offers several treatment options for mood disorders, most of them based on plant products which are empirically tested and proved to be safe in the past for human consumption. Medicinal plants have attracted the attention of the researchers working in this field because these plants have long been used to treat different diseases, including psychiatric disorders and have less adverse effect than synthetic and chemical medicines [18]. Several herbal drugs have been introduced during the recent past for decreasing depression in many emotional and physical disorders. Medicinal herbs are still the preferred remedy for nearly 80% of people around the world, mainly in the developing countries to cure and improve the general health.[24] This is primarily due to the common belief that plant derived drugs are without any adverse effects along with being economical and locally accessible[19,20].

In light of this, the present investigation is focused on the evaluation of antidepressant activity of "Preclinical evaluation of Indian medicinal plant for its antidepressant activity in laboratory animals" To explore and validate traditional medicine using principles of modern medicine[21], to provide more effective and patient friendly alternative, to isolate phytoconstituent/s responsible for antidepressant action.

MATERIALS AND METHODS

Materials

Collection, identification and authentication of selected plant material Fruits of Tribulus terrestri were procured from Local area of Bhopal and were authenticated from Saifia Science College, Bhopal MP, All the drugs and chemicals are AR grade and All the equipments/instruments used for the study were calibrated and validated.

Animals

Swiss albino mice (20-40gm) and Wistar albino rats (180-220 gm) of either sex was procured from animal house as CCSEA reg 1587/PO/RE/S/11and were maintained at 25 ± 2°C and relative humidity of 45 to 55% and under standard environmental conditions (12 h light: 12 h dark cycle) at animal house of 100 . The animals had free access to food and water throughout study. Institutional Animal Ethical Committee approved the protocol. All the experiments were carried out between 9:00- 16:00 hour [23, 23].

Methods:

Extraction of selected plant materials

Both the powdered drugs (100 gm. each) were separately extracted successively using a Soxhlet extractor with hydroalcoholic solvent system is used in the ratio 1:1 and the temperature was set at 60-70 °C[24]. Extracts were filtered, concentrated and after complete solvent evaporation, each of these solvent extracts was weighed and preserved at 5°C in an airtight bottle until further use. Ethanolic extract of *Tribulus terrestri* fruits was prepared and labeled as ETT[25].

Preliminary phytochemical analysis of extracts[26,27,28]:

The selected plant extracts were subjected to preliminary phytochemical analysis for the presence of prominent chemical constituents using following methods: (Khandelwal, 2006).

- ***Test for steroids***
  - **Salkowaski test**
- 2 ml of each Chloroform and concentrated sulphuric acid were added to this 2 ml of test solution, shaken and allowed to stand. Change in the colour of lower chloroform layer to red and acid layer to greenish yellow fluorescence which indicates the presence of steroids was observed.
- **b. Liebermann-Burchard test**
- 2 ml test solution was mixed with chloroform (2 ml). To this solution, 2 ml of acetic anhydride and 2 drops of concentrated sulphuric acid from the side of test tube were added. Change in colour as, first red, then blue and finally green which indicates the presence of steroids was observed.
- ***Test for triterpenoids***
  - **Salkowaski test**
- 2 ml of concentrated sulphuric acid was added to 2 ml of test solution. This mixture was shaken and allowed to stand. Change in the colour of lower layer to yellow which indicates the presence of triterpenoids was observed.
- **b. Liebermann-Burchardt Test**
- The 3 ml of test solution was mixed with 3 ml of acetic anhydride, and then 2 ml of concentrated sulfuric acid was added to it from the sides of the test-tube. The development of deep red colour which indicates the presence of triterpenoids was observed.
- ***Test for glycosides***
  - **Balget's test**
- 2 ml of the test solution was treated with 2 ml of sodium picrate solution. The development of yellow to orange colour which indicates the presence of cardiac glycosides was observed.
- **b. Keller-Killiani test**
- 3-5 drops of glacial acetic acid, 1 drop of 5% $FeCl_3$, conc. sulphuric acid were added to the test tube containing 2 ml of test solution. Appearance of reddish-brown color at the junction of two layers and bluish green in the upper layer which indicates the presence of glycosides was observed.
- **c. Legal's test**
- To 2 ml of test solution, 1 ml of pyridine and 1 ml of sodium nitroprusside was added. Change in color to pink or red which indicates the presence of cardiac glycosides was observed.
- **d. Borntrager's test**
- 2 ml of dilute sulphuric acid was added to 2 ml of test solution, boiled for a few minutes and filtered. To the filtrate 2 ml of benzene or chloroform was

added and shaken well. The organic layer was separated and ammonia was added. The change in colour of ammoniacal layer to pink-red which indicates the presence of anthraquinone glycosides was observed.

- ***Tests for saponins***
    - **Foam Test**
- 10 mg of extract was shaken vigorously with 1 ml water. Development of persistent foam which is stable at least for 15 minutes which indicates the presence of saponins was observed.
- ***Tests for carbohydrates***
    - **Molisch's test**
- 3 ml of Molisch's reagent was added to the 3 ml of test solution, and was shaken for few minutes. Then 2 ml of concentrated sulphuric acid was added slowly from the sides of the test tube. The development of a purple ring at the junction of two liquids which indicates the presence of carbohydrates was observed.
- **b. Barfoed's test**
- 1 ml of Barfoed's reagent and 1 ml of test solution were mixed in a test tube, heated in boiling water bath for 2 minutes and then cooled. The appearance of red precipitate which indicates the presence of monosaccharides was observed.
- **c. Fehling's test**
- Fehling's A and B solutions (1 ml each) were added to the test tube and boiled for 1 minute. To this, 2 ml of test solution was added and heated in boiling water bath for 10 minutes. Appearance of yellow and then brick red precipitate which indicates the presence of reducing sugars was observed.
- **d. Benedict's test**
- Benedict's reagent (1 ml) and test solution (1ml) were mixed in a test tube and heated in boiling water bath for 10 minutes. Change in colour to yellow, green or red which indicates the presence of reducing sugar was observed.
- ***Tests for alkaloids***
- 2 ml dilute hydrochloric acid was added to the 20 mg of dry extract, shaken well and filtered. With filtrate the following tests were performed.
- **Mayer's test**
- To the 3 ml of filtrate, 3 drops of Mayer's reagent (potassium mercuric iodide) was added. Appearance of reddish brown or cream precipitate which indicates the presence of alkaloids was observed.
- **Hager's test**
- To 3 ml of filtrate, 4-5 drops of Hager's reagent (saturated picric acid solution) was added. Appearance of yellow precipitate which indicates the presence of alkaloids was observed.
- **Dragendorff's test**
- 3 ml of the filtrate was mixed with Dragendorff's reagent (potassium bismuth iodide). Appearance of reddish-brown precipitate which indicates the presence of alkaloids was observed.
- ***Tests for Flavonoids***
    - **Ferric-chloride test**
- Few drops (2-4) of ferric chloride solution were added to the test solution and appearance of intense green color which indicates the presence of flavonoids was observed.
- **b. Shinoda test**
- 5 ml of ethanol (95%), 3 drops of hydrochloric acid and 0.5 gm magnesium turnings were added to 10 mg of extract. Change of color of solution to pink which indicates the presence of flavonoids was observed.
- ***Tests for tannins***
    - **Ferric-chloride test**
- 3 ml of test solution was treated with 4-5 drops of ferric chloride solution. Development of dark color which indicates the presence of tannins was observed.
- ***Tests for proteins***
    - **Millon's test**
- Test solution (3 ml) and Million's reagent (5 ml) were mixed in a test tube. The appearance of white precipitate changing to brick red or dissolved and gave red color to solution on heating which indicates the presence of proteins was observed.
- **b. Xanthoproteic test**
- To the test tube containing 3 ml test solution, 1 ml of conc. sulphuric acid was added. Appearance of white precipitate which turns yellow on boiling and orange on addition of NH_4OH which indicates the presence of tyrosin and/or tryptophan containing proteins was observed.
- **Biuret test**
- 3 ml of the test solution was treated with 4% sodium hydroxide (3-5 drops) and 1% copper sulphate solution (3-5 drops). The appearance of blue colour which indicates the presence of proteins was observed.
- **Ninhydrin test**
- Test solution (3 ml) and 3 drops of 5% lead acetate solution were boiled on water bath for 10 min. Change in the color of solution to purple or blue which indicates the presence of amino acid was observed.

Pharmacological screening of selected plant extracts

Acute toxicity study of ethanolic extract of Tribulus terrestri fruits (ETT) as per OECD guideline 425.

Acute toxicity study was performed in healthy adult male albino mice (18-22 gm) as per guidelines (AOT 425) suggested by the Organization for Economical Co-operation and Development (OECD). Hydroalcoholic extract of *Diospyros melanoxylon* fruits (HEDM) were administered separately at the doses of 175, 550 and 2000 mg/kg in mice for oral toxicity study [29]. Mice were then observed for incidence of mortality or any sign of toxicity up to 24 hours after oral administration [30]. The dosing schedule as per the OECD (guideline 425) and Survived animals were observed for outcomes for a period of 24 hours (AOT425 Guidelines).

Evaluation of antidepressant activity of ethanolic extract of Tribulus terrestri fruits (ETT) using Forced swim test

[31]
24 Swiss albino mice of either sex are divided into four groups and given the respective treatments for 14 days as follows.
Group-1: Animals of this group received distilled water (1 ml/kg, p.o.)
Group-2: Animals of this group received ETT (100 mg/kg, p.o.)
Group-3: Animals of this group received ETT (200 mg/kg, p.o.)
Group-4: Animals of this group received ETT (400 mg/kg, p.o.)

Rats individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm) containing 15 cm of water at 25±1°C. All animals are forced to swim for 5 min and the duration of immobility is observed and measured. Each rat judged immobile when it ceased struggling and remained floating motionless in the water, making only those movements to keep its head above water. A decrease in the duration of immobility is indicative of an antidepressant like effect (Porsolt et al.. 1977).

Evaluation of antidepressant activity of ethanolic extract of Tribulus terrestri fruits (ETT) using Tail suspension test *[32]*
24 Swiss albino mice of either sex are divided into four groups and given the respective treatments for 14 days as follows.
Group-1: Animals of this group received distilled water (1 ml/kg, p.o.)
Group-2: Animals of this group received ETT (100 mg/kg, p.o.)
Group-3: Animals of this group received ETT (200 mg/kg, p.o.)
Group-4: Animals of this group received ETT (400 mg/kg, p.o.)

On 14th day, one hour after the last dosing, mice were individually suspended on the edge of the table, 50 cm above the floor for the period of 10 minutes, with the help of adhesive tape placed approximately 1 cm from the tip of the tail. The total duration of immobility induced by tail suspension was recorded during a 6 min of the 10 min period. Mouse was considered immobile when it did not show any body movement, hanged passively and completely motionless [33].

Evaluation of antidepressant activity of ethanolic extract of Tribulus terrestri fruits (ETT) using spontaneous locomotor activity model
24 Swiss albino mice of either sex are divided into four groups and given the respective treatments as follows.
Group-1: Animals of this group received distilled water (1 ml/kg, p.o.)
Group-2: Animals of this group received ETT (100 mg/kg, p.o.)
Group-3: Animals of this group received ETT (200 mg/kg, p.o.)
Group-4: Animals of this group received ETT (400 mg/kg, p.o.)

On 14th day all the animals were subjected to assessment of locomotor activity using digital actophotometer[34]. Then animals were placed in the digital actophotometer for 5 minutes and the locomotor activity counts displayed by interception of photo beams by movement of animals were be recorded and compare against control mice. Imipramine (5 mg/kg i.p.) was used as reference standard [35].

Evaluation of antidepressant activity of n- butanol fractions of ethanolic extract of Tribulus terrestri fruits (NETT) using Forced swim test
24Swiss albino mice of either sex are divided into three groups and given the respective treatments for 14 days as follows.
Group-1: Animals of this group received distilled water (1 ml/kg, p.o.)
Group-2: Animals of this group received NETT (20 mg/kg, p.o.)
Group-3: Animals of this group received NETT (40 mg/kg, p.o.)
Group-6: Animals of this group received imipramine (5 mg/kg, p.o.)

Rats individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm) containing 15 cm of water at 25±1°C. All animals are forced to swim for 5 min and the duration of immobility is observed and measured[36]. Each rat judged immobile when it ceased struggling and remained floating motionless in the water, making only those movements to keep its head above water. A decrease in the duration of immobility is indicative of an antidepressant like effect.

Evaluation of antidepressant activity of n- butanol fractions of ethanolic extract of Tribulus terrestri fruits (NETT) using Tail suspension test
24 Swiss albino mice of either sex are divided into four groups and given the respective treatments for 14 days as follows.
Group-1: Animals of this group received distilled water (1 ml/kg, p.o.)
Group-2: Animals of this group received NETT (20 mg/kg, p.o.)
Group-3: Animals of this group received NETT (40 mg/kg, p.o.)
Group-6: Animals of this group received imipramine (5 mg/kg, p.o.)

On 14th day, one hour after the last dosing, mice were individually suspended on the edge of the table, 50 cm above the floor for the period of 10 minutes, with the help of adhesive tape placed approximately 1 cm from the tip of the tail. The total duration of immobility induced by tail suspension was recorded during a 6 min of the 10 min period[37]. Mouse was considered immobile when it did not show any body movement, hanged passively and completely motionless.

Evaluation of antidepressant activity of n- butanol fractions of ethanolic extract of Tribulus terrestri fruits (NETT) using spontaneous locomotor activity model
24 Swiss albino mice of either sex are divided into four

groups and given the respective treatments for 14 days as follows.
Group-1: Animals of this group received distilled water (1 ml/kg, p.o.)
Group-2: Animals of this group received NETT (20 mg/kg, p.o.)
Group-3: Animals of this group received NETT (40 mg/kg, p.o.)
Group-4: Animals of this group received imipramine (5 mg/kg, p.o.)

On 14th day all the animals were subjected to assessment of locomotor activity using digital actophotometer. Then animals were placed in the digital actophotometer for 5 minutes and the locomotor activity counts displayed by interception of photobeams by movement of animals were be recorded and compare against control mice. Imipramine (5 mg/kg i.p.) was used as reference standard [38].

Phytochemical qualitative analysis of extracts
The selected plant extracts were subjected to phytochemical qualitative analysis for presence of various phytoconstituents of extracts.

High Performance Thin Layer Chromatography (HPTLC) studies of Tribulus Terrestris Fractions
HPTLC system of Shimadzu consisting of sample applicator (Linomat 5), Twin trough chamber with lid {5×10 cm, CAMAG, Muttenz, Switzerland), UV cabinet (Aetron, Mumbai) with dual wavelength (254/366 nm) and the HPTLC photo documentation (Aetron, Mumbai) was used for study[39].

Chromatographic Conditions
The samples were applied in the form of bands of width 6 mm with space between bands of 5 mm, with a 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) on precoated silica gel aluminium plate 60 F254 (10 × 10, 5 × 10) with 250 µm thickness (E. MERCK, Darmstadt, Germany) using a CAMAG Linomat 5 sample applicator (Switzerland). The plates were prewashed with methanol and activated at 110^{0}C for 10 minutes, prior to chromatography. The optimized chamber saturation time for mobile phase was kept 30 min. The length of chromatogram run was 8 cm. HPTLC plates were dried in a current of air with the help of a hair dryer. The slit dimensions of 5× 0.45 mm and scanning speed of 20 mm/sec were employed in analysis. Mobile phase composition of mobile phase was Toluene: Ethyl Acetate: Methanol [5:3:2]

Calculation of Rf Values:
Plate was observed in the daylight, under UV light (254 and 366 nm). After each observation the central points of spots appeared on chromatogram were marked with needle[40]. Retention factor (Rf) was calculated by following formula (Chatwal and Anand, 2004; Sethi and Charegaonkar, 1999). Rf = A/B

A = distance between point of application and central point of spot of material being examined.
B = distance between the point of application and the mobile phase front.

RESULTS AND DISCUSSION

Preliminary Phytochemical analysis of extracts:

Table 1: Preliminary phytochemical evaluation of extracts

Plant constituents	Tests performed	ETT
Test for Steroids	1. Salkowaski Test 2. Liebermann-Buchard Test	++ ++
Test for Triterpenoids	1. Salkowaski Test 2. Liebermann-Buchard Test	++ ++
Test for Glycosides	1. Balget's test 2. Keller-Killiani test 3. Legals test 4. Borntrager's test	- - - -
Tests for Saponins	1. Foam Test	++
Tests for Carbohydrates	1. Molisch's test 2. Barfoed's test 3. Fehling's test 4. Benedict's test	- - - -
Test for Alkaloids	1. Mayer's Reagent 2. Hager's Reagent 3. Dragendorff's Reagent	++ ++ ++
Tests for Flavonoids	1. Ferric-chloride test 2. Shinoda test	++ ++
Test for Tannins	1. $FeCl_3$ Solution 2. Gelatin test	++ ++
Test for Proteins	1. Millon's test 2. Xanthoproteic test 3. Biuret test 4. Ninhydrin test	- - - -

+ Present, - Absent (+ Small, ++Moderate, +++High)

The preliminary phytochemical analysis revealed the phyto-constituents present in the extracts. Ethanolic extract of Tribulus terrestri fruits (ETT) showed the presence of steroids, saponins, tannins, terpenoids, flavonoids, alkaloids.

Pharmacological screening of selected plant extracts

Acute toxicity study of ethanolic extract of Tribulus terrestri fruits (ETT) as per OECD guideline 425.

There were no signs of morbidity nor mortality and any type of adverse effects found up to the dose of 2000 mg/kg p.o. for both the extracts.

Table 2: Evaluation of antidepressant activity of ethanolic extract of *Tribulus terrestri* fruits (ETT) using Forced swim test

Sr. No.	**Treatment**	Immobility time (secs) Mean±SEM
1	Normal control	75.78±2.63
2	ETT (100 mg/kg)	70.39±1.38
3	ETT (200 mg/kg)	64.59±2.44*
4	ETT (400 mg/kg)	50.91±2.50**
5	Imipramine (5 mg/kg, p.o.)	53.48±2.25**

Values are mean ± S.E.M., n=6; Statistical analysis by one-way ANOVA followed by Dunnett's test using Graph pad Instat software; p<0.05*, p<0.01**and p<0.001*** compared to control group.

In this study, ETT 200 and 400 mg/kg showed dose dependent decrease in the immobility time as compared to normal control.. Higher doses of the extracts were found to be potent to that of reference standard i.e. imipramine.

Table3: Evaluation of antidepressant activity of ethanolic extract of *Tribulus terrestri* fruits (ETT) using Tail suspension test

Sr. No.	**Treatment**	**Immobility time (secs) Mean±SEM**
1	Normal control	72.04±1.73
2	ETT (100 mg/kg)	69.49±1.24**
3	ETT (200 mg/kg)	58.14±1.42**
4	ETT (400 mg/kg)	45.52±1.24**
5	Imipramine (5 mg/kg, p.o.)	51.64±2.73**

Values are mean ± S.E.M., n=6; Statistical analysis by one-way ANOVA followed by Dunnett's test using Graph pad Instat software; p<0.05*, p<0.01**and p<0.001*** compared to control group.

In this study, all the doses of the extracts have shown significant decrease in the immobility time.

Table 4: Evaluation of antidepressant activity of ethanolic extract of *Tribulus terrestri* fruits (ETT) using spontaneous locomotor activity model

Sr. No.	**Treatment**	Locomotor score Mean±SEM
1	Normal control	42.00±2.73
2	ETT (100 mg/kg)	44.83±2.97
3	ETT (200 mg/kg)	41.66±03.06
4	ETT (400 mg/kg)	47.50±2.04
5	Imipramine (5 mg/kg, p.o.)	32.33±2.06

Values are mean ± S.E.M., n=6; Statistical analysis by one-way ANOVA followed by Dunnett's test using Graph pad Instat software; p<0.05*, p<0.01**and p<0.001*** compared to control group.

In this study, no treatment including Reference standard has shown any significant change in the locomotor activity. However, reference stander has shown trend of reducing in the score.

Table 5: Evaluation of antidepressant activity of n- butanol fractions of ethanolic extract of *Tribulus terrestri* fruits (NETT) using Forced swim test

Sr. No.	**Treatment**	Immobility time (secs) Mean±SEM
1	Normal control	64.16±1.63
2	NETT (20 mg/kg)	59.00±1.89
3	NETT (40 mg/kg)	55.83±1.87**
6	Imipramine (5 mg/kg, p.o.)	42.68±0.80**

Values are mean ± S.E.M., n=6; Statistical analysis by one-way ANOVA followed by Dunnett's test using Graph pad Instat software; p<0.05*, p<0.01**and p<0.001*** compared to control group.

In this study, NETT-20 was not significant but NETT-40 mg/kg, and NETT (20 mg/kg) showed significant decrease in the immobility time as compared to normal control. Furthermore, all these doses were equipotent to reference standard Imipramine.

Table 6: Evaluation of antidepressant activity of n- butanol fractions of ethanolic extract of *Tribulus terrestri* fruits (NETT) using Tail suspension test

Sr. No.	**Treatment**	Immobility time (secs) Mean±SEM
1	Normal control	77.88±2.79
2	NETT (20 mg/kg)	70.98±1.98*
3	NETT (40 mg/kg)	67.95±1.17**
4	Imipramine (5 mg/kg, p.o.)	63.11±1.77**

Values are mean ± S.E.M., n=6; Statistical analysis by one-way ANOVA followed by Dunnett's test using Graph pad Instat software; p<0.05*, p<0.01**and p<0.001*** compared to control group.

In this study, the extracts have shown significant dose dependent reduction in immobility time. NETT extracts were equipotent to each other. The reference standard has shown significant action, which was statistically equal as that of higher dose of both fractions.

Table 7: Evaluation of antidepressant activity of n- butanol fractions of ethanolic extract of *Tribulus terrestri* fruits (NETT) using spontaneous locomotor activity model

Sr. No.	**Treatment**	**Locomotor score Mean±SEM**
1	Normal control	49.66±1.47
2	NETT (20 mg/kg)	47.50±2.01
3	NETT (40 mg/kg)	48.50±1.43
4	Imipramine (5 mg/kg, p.o.)	43.66±1.43*

Values are mean ± S.E.M., n=6; Statistical analysis by one-way ANOVA followed by Dunnett's test using Graph pad Instat software; p<0.05*, p<0.01**and p<0.001*** compared to control group.

In this study, no treatment including Reference standard has shown any significant change in the locomotor activity. However, reference standard Imipramine has shown significant reduction.

Phytochemical Qualitative Analysis of Extracts

The selected plant extracts were subjected to phytochemical qualitative analysis for presence of various phytoconstituents of extracts.

High Performance Thin Layer Chromatography (HPTLC) studies of n- butanol fractions of ethanolic extract of *Tribulus terrestri* fruits

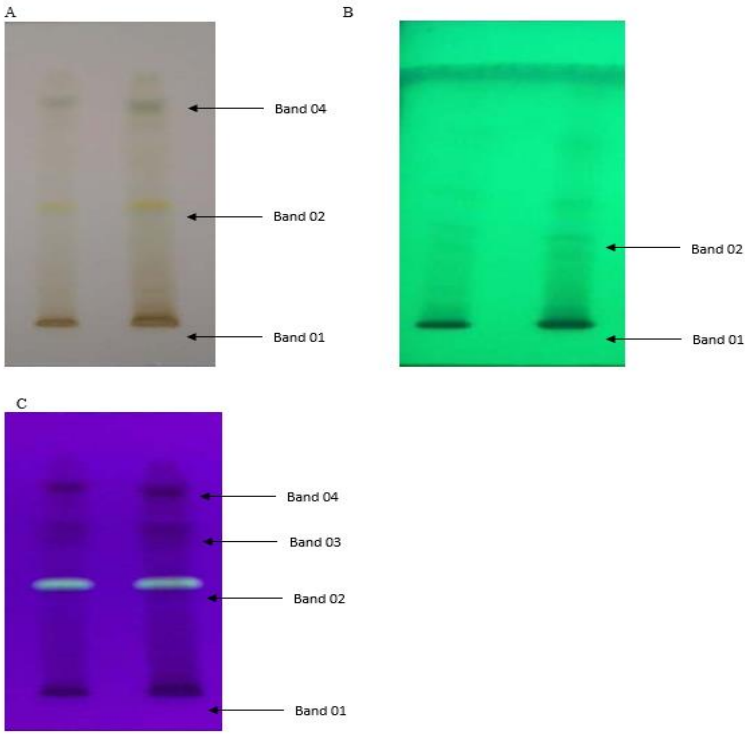

Figure 1: n-Butanol IIb fraction of hydroalcoholic extract of *Tribulus terrestris* A) Visible light B) at 254 nm and C) at 366 nm and acid reagent, Volume applied 10 µl and 20 µl.

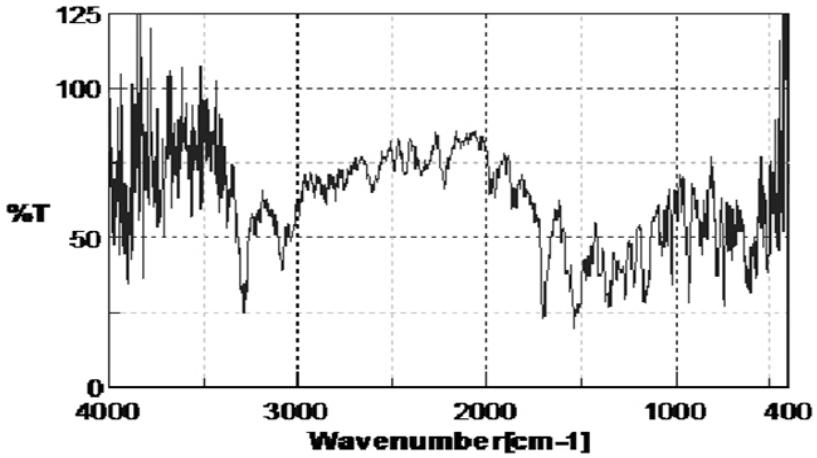

Figure 2: FT-IR Spectrum of compound in n-butanol fraction Band 2 (Rf. 0.48)

Table 8: IR data interpretation (Rf: 0.48)

Sr. No.	Part of molecule	Vibration	Frequency (cm^{-1})
1	Benzopyran ring and Aromatic Ring	a) C-H str b) C=C str c) C-H bend d) C-O str	3081 1537 745 1352
2	CH_2 Protons	a) C-H str b) C-H bend	2980 1539
3	-OH	a) O-H str	3284

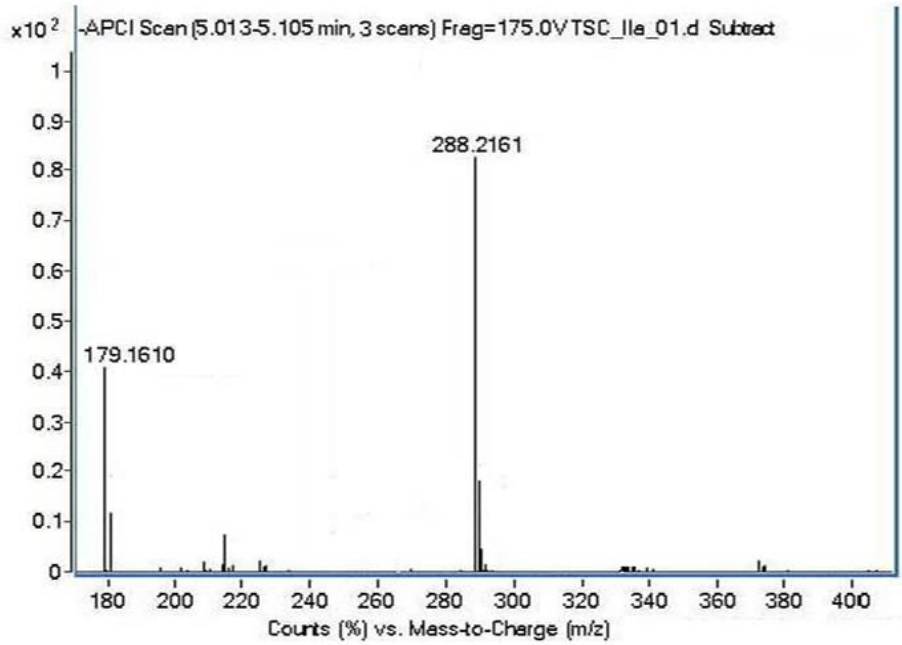

Figure 3: Mass Spectrum of compound in n-butanol fraction Band 2 (Rf. 0.48)

Table 9: NMR data of compound in n-butanol fraction band 2 (Rf. 0.48)

Sr No.	δ	Protons	Type
1	3.234-3.385	1 +1 (d)	CH_2 Protons on ring of fused Benzopyran ring
2	5.422	1 (m)	CH Protons on ring of fused Benzopyran ring
3	5.934-6.032	1 +1 (d)	Aromatic Protons on ring of fused Benzopyran ring
4	6.325-6.616	3 (m)	Protons on Benzene ring attached to Benzopyran ring
5	9.853	4 (s)	OH Protons

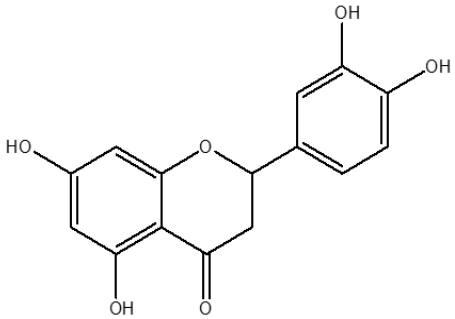

Figure 4: 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-2,3-dihydrochromen-4-one

Probable structure of the compound from IR, Mass and NMR is

DISCUSSION

Recently, herbal medicines have received much attention as alternative treatments for depression because of their fewer side effects and lower costs.[7] The description of the antidepressant activity of selected plant-derived compounds involves several mechanisms, including inhibition of monoamine reuptake; enhanced serotonin receptor binding and sensitization; monoamine oxidase inhibition; GABAergic effects (especially for plants exhibiting sedative and anxiolytic effects accompanying the antidepressant effect); complex, excitatory or inhibitory effects on various receptors (N-methyl-D-aspartic acid (NMDA), GABA, cholinergic, adrenergic, serotonergic, dopaminergic and opioid ones); and cannabinoid system effects.[7-11]

Tribulus terrestris herbal medicines with wide range of pharmacological actions. These plants have been

traditionally claimed to be effective in relieving depressed mood and anxiety and other such depressive disorders but have not been scientifically studied for their antidepressant activity.

Hence the present investigation "Preclinical evaluation of Indian medicinal plants *Tribulus terrestris* for their antidepressant activity in laboratory animals" was carried out.

The antidepressant activity of herbal medicine and the mechanisms therein depends on the phytoconstituents present in them. The actual therapeutic results are closely related to the conventional wisdom, scientific findings, and phytochemical presence. The most trustworthy way to determine whether phytoconstituents are present is by a preliminary phytochemical examination, followed by a comparison of the results with existing literature. So as to assess the phytochemical profile of plant, the ethanolic extract of *Tribulus terrestris* fruits were subjected to preliminary phytochemical analysis. The preliminary phytochemical analysis revealed the phytoconstituents present in the extracts. Ethanolic extract of *Tribulus terrestris* fruits (ETT) showed the presence of steroids, saponins, tannins, terpenoids, flavonoids, alkaloids. These phytoconstituents have been reported to possess antidepressant effects.

Natural products may be a source in the quest for antidepressant medicines due to the evaluation and confirmation of these activities of several phytochemical ingredients in preclinical models. The plants quality of the aforementioned plants, particularly in relation to the phytoconstituents responsible for their toxicity profile and antidepressant activity, has not yet been properly proven. Toxicology profile testing becomes extremely important to ensure its safety before moving forward with the actual exploration of its pharmacological activity after the phytochemical profile and its pharmacological importance have been confirmed. To confirm the extent of a therapeutic drug's therapeutic use, toxicity profile testing is crucial for all therapeutic drugs. Moreover, in the present investigation, the results of preliminary phytochemical analysis suggested further pharmacological exploration of extracts, hence it was essential to assess the extracts for their toxicity profile and thereby confirmation of their safety (OECD 2008)[41]. Hence the acute toxicity study of ethanolic extract of Tribulus terrestris fruits (ETT) was carried out as per OECD guideline 425. The results of acute oral toxicity studies revealed that extracts are safe with no signs of morbidity nor mortality and any type of adverse effects up to 2000 mg/kg which is highest prescribed limit as per this test. Thus, both the extracts fulfilled the safety criteria before the assessment of preclinical activity (OECD guidelines 425)[42]. Based upon these findings and available literature, the three different doses i.e. 100, 200 and 400 mg/kg of each extract were selected for the further preclinical investigations.

In the present study, the antidepressant effects of the extracts were evaluated using three widely used tests i.e. forced swim test, tail suspension test and spontaneous locomotor activity test. These models are known to be of highly predictive relevance with respect to the clinical spectrum of activity.

In forced swim test, ETT 200 and 400 mg/kg showed dose dependent decrease in the immobility time as compared to normal control. Higher doses of both the extracts were found to be equipotent to that of reference standard i.e. imipramine. In the tail suspension test, all the doses of the extracts have shown significant decrease in the immobility time. In spontaneous locomotor activity, no treatment including reference standard showed any significant change in the locomotor activity. However, reference standard Imipramine showed a non-significant reduction in locomotor activity. After the pharmacological activity of individual extracts, the fractionation of the extracts was carried out and n- butanol fractions of ethanolic extract of Tribulus terrestri fruits (NETT) were prepared. In order to assess the antidepressant activity of these fractions, they were also subjected to the same preclinical models of antidepressant activity at the doses of 20 mg/kg and 40 mg/kg. In the forced swim test, NETT-40 mg/kg, significant decrease in the immobility time as compared to normal control. Furthermore, all these doses were equipotent to reference standard Imipramine. In the tail suspension test, the fractions showed significant and dose dependent reduction in immobility time at the doses. The reference standard has shown significant action, which was statistically equal as that of higher dose of fractions. In the spontaneous locomotor activity, none of the fractions showed any significant change in the locomotor activity. However, reference standard Imipramine showed significant reduction in locomotor activity.

Many of the pharmaceuticals that are currently on the market have either been directly or indirectly produced from plants, which have traditionally been exceptional sources of drugs. These phytopharmaceuticals have offered many advantages over the current drugs from modern medicine however availability of drug and variation regarding the yield as well as quality with respect to cultivation from time to time, season, region etc. cannot be ignored. The requirement of large amount of crude drug to obtain the desired quantity of extract is another major drawback of herbal medicine. Utilizing medicinal biodiversity to its fullest extent in order to search for phytochemicals with antidepressant properties is a difficult task. Additionally, administration of phytoconstituents without desired pharmacological activity led to precipitation of adverse effects or reduced therapeutic efficacy.

This problem can be overcome by the isolation of most active phytoconstituent responsible for the desired pharmacological activity from the extract. This not only identifies and isolates the active phytoconstituent but also provides an important input to the synthetic chemistry regarding the structure of patient friendly bioactive constituent with required pharmacological action.23 HPTLC technique is most simple and fastest separation technique available today which gives better precision and accuracy with extreme flexibility for various steps.21,22 In

light of this, n-butanol fractions of ethanolic extract of Tribulus terrestris fruits (NETT) were subjected to the High-Performance Thin Layer Chromatography (HPTLC) studies.

As a result of these studies two different phytoconstituents were isolated i.e., 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-2,3-dihydrochromen-4-one from n-butanol fractions of ethanolic extract of Tribulus terrestris fruits (NETT).

The isolation of these phytoconstituents is an important concluding result of the study and cardioprotective activity of the extracts may be attributed to these phytoconstituents.

CONCLUSION

The present study antidepressant activity of the ethanolic extract of *Tribulus terrestris* fruits and n- butanol fractions of ethanolic extract of Tribulus terrestri fruits (NETT) were evaluated for antidepressant activity using in laboratory animals using different preclinical models of antidepressant activity such as forced swim test, tail suspension test and spontaneous locomotor activity. Ethanolic extract of Tribulus terrestri fruits (NETT) and their fractions showed potent antidepressant activity. The multistep putative action of these plants may be attributed to the prominent phytoconstituents namely 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-2,3-dihydrochromen-4-one from n-butanol fractions of ethanolic extract of *Tribulus terrestris* fruits (NETT).

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