Phytochemical screening & Pharmacological Evaluation of the Methanol Extract of *Tabernaemontana divaricata* leaves.

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In the partial fulfilment of the requirements for the degree of Bachelor of Pharmacy (B. Pharm.)

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Department of Pharmacy
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APPROVAL

This Project, **Phytochemical screening & Pharmacological Evaluation of the Methanol Extract of *Tabernaemontana divaricata* leaves**, submitted to the Department of Pharmacy, Daffodil International University, has been accepted as satisfactory for the partial fulfilment of the requirements for the degree of Bachelor of Pharmacy and approved as to its style and contents.

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DECLARATION

I hereby declare that, this project report is done by me under the supervision of **Md. A K Azad**, Assistant Professor, Department of Pharmacy, Faculty of Allied Health Sciences, Daffodil International University, impartial fulfilment of the requirement for the degree of Bachelor of Pharmacy. I am declaring that this project is my original work. I am also declaring that neither this project nor any part thereof has been submitted elsewhere for the award of Bachelor or any degree.

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Susmita Saha
Author

DEDICATION

I dedicate this work first and foremost to my parents.

Abstract

This research work may help to board field to improve the medicinal uses of the *Tabernaemontana divaricata* leaves. This plant belonging to the family Apocynaceae. The methanolic extract of *Tabernaemontana divaricata* leaves chosen for the evaluation of Phytochemical screening, In-vitro Antioxidant potential. In-vivo Analgesic activity, Antidiarrheal activity & anthelmintic activity & Ex-vivo Thrombolytic activity. The Phytochemical studies were done as qualitative analysis. That means this only indicates the presence or absence of the chemical compounds not the quantity of the chemical compounds. The phytochemical studies were performed based on the colour change, precipitation & ring formation etc. The antioxidant activity was determined by using DPPH (1,1-diphenyl-2-picrylhydrazine) Radical Scavenging Assay for extract (25.93µg/ml) compared with the standard Ascorbic acid (18.40µg/ml). And also performed reducing sugar capacity, total flavonoid content (49.59mg/gm) & total phenolic content (54.89mg/gm) in the extract. Ex-vivo thrombolytic activity was determined by clot disruption method & showed % clot lysis of methanolic extract showed (48.33%) where standard streptokinase showed (75.44%). Analgesic activities were performed by in-vivo test and provided a good result. Standard showed (72.22%) protection of writhing where the extract showed (58.33%) protection of writhing. Anthelmintic effect of this extract was good and this plant extract have also some antidiarrheal activities. In my studies, I was found some chemical compounds such as Phenols, Glycosides, Flavonoids, reducing sugar, Terpinoids & Alkaloids in the methanolic extract of *Tabernaemontana divaricata* leaves. So my research work tries to show the bioactivity of the methanolic extract of *Tabernaemontana divaricata* leaves.

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Chapter One: Introduction

1.1 General Introduction:

Every plants contain some type of chemical compounds. Some compound may beneficial to health, some may cure or prevent some diseases & some may cause disease or harmful to health. The plants which have the ability to prevent diseases and cure disease this plants called medicinal plants. There are many several drugs which are found from the herbalism. Herbalism is a medical system, based on the use of plants or plants extracts that may be eaten or applied to the affect skin. Herbalism also known as herbal medicine. Science ancient time herbal medicines was most popular treatment of the diseases. In the present time some people still now use the herbal medicines. 500,000 species plants present on the earth. And only 5000 have been studied for their medicinal application. Only 25% of today's prescription drug are isolated from the medicinal plants. Such as digitalis, vincristine, morphine, ibuprofen etc. Todays also directly use as herbal medicine like Aloe vera, ginger, garlic, ginseng, feverfew leaves etc. The World Health Organization estimates that about 80% of the population living in the developing countries relies almost the herbal medicine for their primary health care needs. [1]

1.2 Phytochemical studies:

Phytochemical studies are an investigation that indicate the presence and absence of the chemical compound. This chemical compounds are naturally occurring. Every plants have some specific chemical constituents. By this studies we can find the specificity and the chemical constituents. And also some important metabolic compound can identify like glycoside, alkaloids, polyphenol, terpinoids etc. [2]

1.3 Ethnomedicine:

Ethnomedicine is the study that compare with the traditional medicine based upon the bioactive compound in the plants and animals and this done by various ethnic group especially those with little access to western medicines e.g. indigenous people. Scientific ethnomedicine studies use in the either anthropological research or drug discovery research. [2]

1.4 Herbalism:

Herbalism is a medical system, based on the use of plants or plants extracts that may be eaten or applied to the affect skin. Herbalism also known as herbal medicine. Science ancient time herbal medicines was most popular treatment of the diseases. In the present time some people

still now use the herbal medicines. World Health Organization estimates that about 80% of the population living in the developing countries relies almost the herbal medicine for their primary health care needs. The people use herbal medicine because they believe that natural means safe and have low side effect and adverse effect.[2]

1.5 History:

Science Ancient time, herbal medicine was the most popular treatment of the disease. All types of critical diseases were treated with the herbal medicine before invention of the conventional drugs. The Greek physician Hippocrates also referred to as "Father of Modern Medicine ". He used always herbal medicine in his practice and wrote this valuable word "Let your foods be your medicine, your medicine your foods". The great age of herbalism was between in the 15th and 17th centuries. Herbal books were just become in the English letter and then Latin and Greek. Day by day more improve the herbalism.[1],[3]

Today's herbalism:

World Health Organization estimates that about 80% of the population living in the developing countries relies almost the herbal medicine for their primary health care needs. The people use herbal medicine because they believe that natural means safe and have low side effect and adverse effect. 25% of today's prescription drug are isolated from the medicinal plants. Such as digitalis, vincristine, morphine, ibuprofen etc. Todays also directly use as herbal medicine like Aloe vera, ginger, garlic, ginseng, feverfew leaves etc.[1],[3]

1.6 Distribution:

Tabernaemontana divaricata is an evergreen shrub that most available in the India, Bangladesh. Now cultivated throughout in the South East Asia and the warmer region of the continental Asia. And also found in Africa, Australia, North America, South America.[4]

Figure 1.1: *Tabernaemontana divaricata* plant

1.6.1 Scientific Classification:

Kingdom: Plantae

Order: Gentianales

Family: Apocynaceae

Genus: *Tabernaemontana*

Species: *T. divaricata*

Botanical Name: *Tabernaemontana divaricata*

Synonyms:

Tabernaemontana coronaria

Tabernaemontana discolor Sw.

Tabernaemontana gratissima Lindl

Tabernaemontana dichotoma

Tabernaemontana recurve Roxb. ex Lindl [4]

1.6.2 Uses:

Tabernaemontana divaricata leaves usually work against cancer, diabetics, inflammation. It also helps to cure convulsion. This leaves also have some anti-fertility effects.[4]

1.7 Antioxidant:

Antioxidants are the compound that can prevent or inhibit oxidation. Oxidation is a chemical reaction which can produce free radical. The free radical leads to chain reaction that damages the cells of the organism. The chain reaction can initiate certain cancer. The antioxidant such as thiols or ascorbic acid can diverse the chain reaction and prevent cancer. Certain level of antioxidant vitamin in diet are required for good health. This also helps to prevent some chronic diseases. The antioxidant agents also act as an anti-aging agent. [5], [6]

1.8 Analgesic Activity:

Analgesic means pain reliever. This group of drugs are used for achieving analgesia. Analgesic also called painkiller. Analgesic drugs are acted in various ways on the peripheral and central nervous system.

Usually pain induce for the prostaglandin. Analgesic drug not only inhibit the prostaglandin synthesis but also peripheral and central mechanism. The prostaglandin synthesis by the help of the special enzyme cyclooxygenase. The analgesic drug exactly inhibit the cyclooxygenase enzyme to form prostaglandin and relief pain.[7],[8]

1.9 Antidiarrheal activity:

Antidiarrheal activity means have the ability to relief from the diarrhoea. Diarrhoea is the condition of having three or more loose or liquid bowel movement per day.

The main mechanism of the antidiarrheal agent is that it reduces the hypermotility of the stomach, increase the absorption of the fluid and electrolytes from the stool, adsorb toxin and kill the microorganism which causes diarrhoea.[10]

1.10 Thrombolysis:

Thrombolysis means breakdown of the blood clot. Thrombolytic agents are used in the treatment to dissolve dangerous clots in the blood vessels, improve blood flow, prevent damage to tissue & organs [12]. Thrombolysis is also used in the emergency treatment to dissolve blood clots that form in arteries fading the heart & brain which is the main cause of heart attacks & ischemic strokes.

The main mechanism of thrombolysis is to dissolve the blood clots by activating plasminogen that form a cleaved product called plasmin. Plasmin is the proteolytic enzyme

which is able to breakdown the cross links between fibrin that produce the structural integrity of the blood clots.[9],[11]

1.11 Anthelmintic activity:

Anthelmintic or anthelminthic are the group that are used to kill the parasitic worm and other internal parasites from the body. They work by either stunning or killing the parasites and without causing significant damage the host.

Infections with helminth are among the most widespread infections in humans and other domestic animals affecting a large number of world population. The majority of these infections due to worms are generally restricted mainly to the tropical regions and the occurrence is accelerated due to unhygienic lifestyle and poverty also resulting in the development of symptoms like anaemia, eosinophilia and pneumonia.[13]

Chapter Two: Literature Review

2.1 Effect of various extracts of *Tabernaemontana divaricata* on haloperidol induced catalepsy in rats. [19]

Parkinson disease is a neurodegenerative disease with selective loss of dopamine neuron. In this study anti cataleptic activity of *Tabernaemontana divaricata* leaves extract viz aqueous and ethanoic of different dose were studied using haloperidol induced catalepsy in rats. The both extract were found to reduce catalepsy significantly. This study reveals the anti-cataleptic activity of *Tabernaemontana divaricata*. This study concluded that the test drug can safely be replaced as an alternative agent in treating the extrapyramidal side effects of antipsychotic agents in clinical practice.

2.2 Chemical Constituents Antioxidant and Anticholinesterasic Activity of *Tabernaemontana catharinensis*. [20]

This study aimed to evaluate the alkaloid content of the ethanoic extract of the *Tabernaemontana catharinensis*. Also to evaluate their antioxidant and anticholinesterasic activity. In this plant mass spectrometry allowed to identifying the presence of the alkaloids of 16-epi-affinine, coronaridine-hydroxyindolenine, voachalotine, voacristine-hydroxyindolenine, and 12-methoxy-n-methyl-voachalotine. The extract and its alkaloid rich fractions showed antioxidant activity, specifically those which are contain 16-epi-affinine. Fractions with 16-epi-affinine combined good antioxidant and anticholinesterasic activities, representing the chance for further studies for treating neurodegenerative diseases.

2.3 Evaluation of in vitro anticancer activity of hydroalcoholic extract of *Tabernaemontana divaricata.* [21]

In this study the hydro alcoholic extract from the flower of *Tabernaemontana divaricata* plant is used to test for anticancer activity. The in-vitro anticancer studies were performed against human cancer cell line and the inhibition of cell growth was analysing by the MTT assay. The results showed that the hydro alcoholic extract of flowers of *Tabernaemontana divaricata* possessed a moderate amount of anticancer activity and the IC50 value was greater than 100µg/ml.

2.4 Antidiabetic and Cytotoxic Activities of Methanolic Extract of *Tabernaemontana divaricata* Flowers. [15],[17]

This study mainly performed to investigate the anti-diabetic activity of the methanol extract of the *Tabernaemontana divaricata* flower. The antihyperglycimic effect of the extract was compared with metformin. The extract was also tested to Brine shrimp lethality bioassay. The both result suggested that *Tabernaemontana divaricata* flower possess antidiabetic activity in mice with alloxan induced diabetes and low cytotoxicity that may provide new molecules for the treatment of diabetes.

2.5 Antimicrobial Potential and In Vitro Cytotoxicity Study of *Tabernaemontana Divaricata* (L.) Stem Bark Extract Against HEK 293 Cell Line. [15]

This study focused on identification of multi drug resistant microorganisms of clinical isolates by using methanolic extracts of *Tabernaemontana divaricata* stem bark crude for antimicrobial efficacy. In vitro cytotoxic effects of the stem bark extract was observed through MTT reduction assay on normal cell line. This study found antimicrobial activity against all test isolates. This is the first report of the presence of the said compounds with antimicrobial activity in *Tabernaemontana divaricata* stem bark crude.

2.6 Antibacterial activity of *Tabernaemontana divaricata* secondary metabolites capped silver and gold nanoparticles. [14]

In this investigation, SNPs and AuNPs were synthesized using an aqueous extract of *Tabernaemontana divaricata* leaves to study the antibacterial activity. By the UV-Vis spectrum supported the biosynthesis and characterization of SNPs and AuNPs. The SNPs when compared to AuNPs, showed the highest antibacterial activity against Gram-positive and Gram-negative bacteria. study envisions on the biosynthesis of SNPs from *Tabernaemontana divaricata* plant which are emerging as antibacterial therapy in modern medical applications.

Chapter Three:
Method and Materials

3. Methodology:

The basic methods of the project work are following some steps. These are –

- Plant leaves collection
- Identification
- Preparation of the plant leaves for extraction
- Extraction
- Phytochemical screening
- Preparation of the plant extract solution, standard solution & blank solution for the specific test.

3.1 Plant leaves collection:

Tabernaemontana divaricata is the plant that are available anywhere in the Bangladesh. This plant leaves ware collected from Manikgonj, Dhaka. It was collected at the noon time. When the leaves ware collected, all types of possible precaution was taken for preventing the chemical degradation of the plant leaves. After the collection of the plant leaves from the plants, the leaves ware cleaned simply with the dry cotton to remove dusts and unexpected materials.

3.2 Identification:

In the world there are present a lot of various species of the *Tabernaemontana* plants. So it was humble need to ensure the plant leaves ware *Tabernaemontana divarikata* leaves. This tusk was done by the Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. They confirmed the plants leaves ware *Tabernaemontana divarikata* leaves with the Herbarium sheet.

3.3 Preparation of the plants leaves:

The main purpose to collect extract from the leaves. For this the leaves need to prepare for easy extraction. So at first the leaves were dried by sun light with shed not direct sun light and for the low temperature this leaves ware dried in the morning time at 9.00 am to 10.00am. when the leaves became crispy, the leaves were preserved in the air tight container in the dark place. After that, the leaves were grinded into a powder with the help of a grinder. Then the powder was put in into air tight container in the dark, dry and cool place for further use.

3.4 Extraction:

At first, two amber glass bottle were cleaned with normal water then rinse with methanol. Then 1000 gm plant leaves powder was taken in the glass battle. Then added the methanol up to 1 inch above the leaves powder in the glass bottle. 1.750 litter methanol was used to soak the plant leaves powder. Then closed the bottle with the plastic closer.

The bottle was shaken everyday 2 or 3 times per day for proper extraction. And this process continued for 10 days.

3.4.1 Filtration:

For the collection of pure extract from the mixture of leaves and methanol, some steps ware followed. Firstly, extract was partially separated by the filtration process. The sterile cotton placed I the funnel some amount of methanol then filters the mixture.

Then final filtration was done with the filter paper. The filter paper was fitted properly in the funnel with methanol. The filtrated was collected in some glass container.

Figure 3.1: *Tabernaemontana divaricata* leaves extract filtration

3.4.2 Evaporation:

The final step of preparation of pure extract of the leaves is evaporation. The liquid extract was evaporated with the help of rotary evaporator. During evaporation of the leaves extract, then the temperature of the evaporator was fixed at 65°C and rotation was 35-40 rotation per minutes. The condensed extract then transfers in to a 50ml beaker. Then the beaker was put on the table for adjust with room temperature. When total amount of liquids was dried then the beaker closed with aluminium foil. The extract was preserved at cool, dark and dry place for further use.

Figure 3.2: *Tabernaemontana divaricata* leaves extract evaporation

3.5 Phytochemical screening: [14]

Since the phytochemical screening was qualitative test so amount was not fact. Firstly, some amount of plant extract was dissolved in the distilled water properly. Then some chemical tests ware done. These ware:

a. Glycosides test:
   - A small amount of extract was dissolved in 1ml water and added a few drop of aqueous sodium hydroxide. A yellow color was formed and present of glycosides
   - A small amount of extract was dissolved in 1ml water and added Fehling's solution then boiled. Brick red precipitation was formed. Presence of glycosides.

b. Flavonoid:
   Small amount of extract solution and added few drop of concentrated HCl. Immediately red color was formed. Presence of flavonoid.

c. Alkaloids:
   - 2ml extract solution added 0.2ml diluted HCl and also added 0.1ml Mayer's reagent and yellowish buff color precipitate was formed presence of alkaloids.
   - 2ml extract solution added 0.2ml diluted HCl and added 0.1ml Dragendroff's reagent and orange brown precipitate was formed.

d. Reducing sugar:
   - 2ml extract solution added 1ml equal volume of Fehling's solution A and B. boiled for 5 minutes in water bath. Formed brick red precipitation.
   - 0.5 ml extract solution added with 5 mi Benedict's reagent and boiled for 5 minutes. Brick red color precipitation was formed. presence of reducing sugar.

3.6 In-vitro Antioxidant Potential:

3.6.1 DPPH Radical Scavenging Assay: [24],[25]

DPPH (1,1-diphenyl-2-picrylhydrazyl) is the sable free radical that is used to determine the free radical scavenging capacity of the antioxidant. It usually shows the result by colour change from red to yellow which determine by measuring absorbance with the wave length at 517nm.

a. Apparatus:

- Test tube
- Beaker

- Spatula
- Electric balance
- Pipette
- UV-spectrophotometer.

b. Reagents:

- DPPH
- Ascorbic acid
- Methanol
- Distilled water

c. Preparation of reagent & extract solution:

- 0.004gm DPPH was dissolved in 100ml methanol in the dark place. So the concentration was 0.004% w/v
- Preparation of extract solution: 10mg extract was dissolved in 100ml methanol. So the concentration was 10mg/ml.

  Then serial dilution was done and prepared different concentration solution these ware 500µg/ml, 100µg/ml, 50µg/ml, 10µg/ml, 5µg/ml, 1µg/ml.
- Preparation of standard: 10mg ascorbic acid was dissolved in 100ml methanol & the concentration was 10mg/ml.

  Then 500µg/ml, 100µg/ml, 50µg/ml, 10µg/ml, 5µg/ml, 1µg/ml concentration ascorbic acid solution was prepared by serial dilution.
- Blank solution was prepared by adding 1ml methanol and 3ml DPPH

d. working procedure:

- After preparation of all types of solution, different concentration of extract solution was added with DPPH at the ratio of 1:3 that means 1ml extract solution and 3ml DPPH in different test tube.
- Then at same procedure the different concentration ascorbic acid solution was added DPPH at 1:3 ratios in different test tube.
- Another test tube was taken for preparing blank 1ml methanol and 3ml DPPH.
- Then 45 minutes stay in dark place.
- Then measured the absorbance of blank solution and different concentration of standard solution and extract solution, the wave length at 517nm.

Measurement of percentage (%) of inhibition activity:

[(Blank solution absorbance – extract solution absorbance) / blank solution absorbance] ×100

3.6.2 Reducing sugar assay:

3.6.2.1 Total phenolic content determination: [26]

a. Reagents:

- Folin Ciocalteu Reagent (FCR)
- Sodium carbonate
- Gallic Acid
- Extract
- Methanol
- Distilled water

b. Reagents preparation:

- 10ml FCR was mixed with 100ml distilled water. then the concentration was 10% v/v.
- 7.5gm sodium carbonate was dissolved in 100ml distilled water.
  The concentration was 7.5%
- Gallic acid solution: 10mg Gallic acid was dissolved in the 10ml methanol so the concentration was 1mg/ml
- Extract solution: 10 mg extract was dissolved in the 10ml methanol. The concentration was 1mg/ml.

c. Working procedure:

- Blank solution preparation: 0.5ml methanol was taken in a test tube. The added 5ml 10% FCR & 4ml 7.5% sodium carbonate. Then mixed in the vortex mixture. Then the mixture stays for 30 minutes at 40°C temperature.
- Standard solution preparation: From 10ml Gallic acid solution was prepared different concentration of 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml.

  From each concentration of the Gallic acid solution was taken 0.5ml in different test tube & added 5ml FCR & 4ml sodium carbonate. Mixed them in vortex mixture. Then stay the mixture for 30 minutes at 40 o c temperature.

- Extract solution preparation: 0.5 ml extract solution was taken in the test tube and added 5ml FCR & 4ml sodium carbonate and mixed them in the vortex mixture. then stay them for 30 minutes at 40°C temperature.
- Finally measured the absorbance of the blank, standard and extract solution at 765nm wave length.
- Gallic acid equivalent = value of x / concentration of extract solution.

3.6.2.2 Total flavonoid content determination: [27]

a. Reagent:

- 5% w/v sodium nitrate
- 10% w/v aluminum chloride
- 1M sodium hydroxide
- Quercetin
- Extract
- Methanol
- Distilled water

b. Reagents preparation:

- 5gm sodium nitrate dissolved in 100ml distilled water. the concentration was 5%w/v
- 10gm aluminum chloride was dissolved in the 100ml distilled water.
- For preparing 1M sodium hydroxide to take 40gm NaOH in 1000ml distilled water.
- Quercetin solution: 10mg quercetin was dissolved in 10ml methanol.
- Extract solution: 10mg extract dissolved in 10ml methanol.

c. Working procedure:

- Blank solution preparation: 0.5ml methanol was taken in a test tube and added 4ml distilled water &0.3ml sodium nitrate. after 5 minutes added 0.3ml aluminum chloride & at this 6 minutes added 2ml NaOH. Then the volume was adjusted up to 10ml by using distilled water.
- Standard solution: firstly, prepared 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml concentration of Quercetin solution. Then 0.5ml of each concentration solution was taken in the different test tube.

Added 4ml distilled water &0.3ml sodium nitrate. After 5 minutes added 0.3ml aluminum chloride solution. at this 6 minutes added 2ml NaOH & adjusted the volume up to 10ml by added distilled water.

- Extract solution: 0.5 ml extract solution was taken in a test tube. Added 4ml distilled water &0.3ml sodium nitrate. After 5 minutes added 0.3ml aluminum chloride solution. at this 6 minutes added 2ml NaOH & adjusted the volume up to 10ml by added distilled water.
- Finally measured the absorbance of blank solution, standard solution & extract solution at 510nm wave length.
- Quercetin equivalent = value of x / concentration of extract solution.

3.7 In-vivo Analgesic activity test (acetic acid induced writhing method):

[28]

This in-vivo test done by using Swiss-albino mice which were collected from the Animal Resource laboratory, Jahangirnagar University, Savar, Dhaka. To adjusted with the environment of our university's laboratory, they were keep in normal room temperature for 2 weeks with proper rodent food and water.

For analgesic activity test all mice were divided into three group and all group contained 4 mice. One group was control group 2nd group was standard group and 3rd group was sample or extract group.

Working procedure:

- At first 1% acetic acid was prepared by dissolving 1ml acetic acid in distilled water up to 100ml.
- Prepare the extract solution: since the extract group mice receive 300mg/kg, then the mice average weight was 30gm so I needed 9mg extract for each mice. For 4 mice needed 36mg extract. Then the extract was dissolved in 4ml distilled water.
- Standard group:
    - Diclofenac sodium was injecting in the mice on the intra-peritoneal route at the dose 10mg/kg body weight.
    - After 15 minutes injected 1% acetic acid at the same route at the dose 10ml/kg body weight. After 5 minutes observed the writhing for 10 minutes

- Control group:
    - Water was fed in the mice on the oral route at the dose 10ml/kg body weight.
    - After 15 minutes injected 1% acetic acid at the intra-peritoneal route at the dose 10ml/kg body weight.
    - After 5 minutes observed the writhing for 10 minutes.
- Sample or extract group:
    - Extract solution was fed in the mice on the oral route at the dose300mg/kg body weight.
    - After 30 minutes injected 1% acetic acid at the intra-peritoneal route at the dose 10ml/kg body weight.
    - After 5 minutes observed the writhing for 10 minutes

Acetic acid induced writhing was calculated by this formula

Percentage of protection =(Wc-Wt)/Wc × 100

Where, Wc = values of control group, Wt. = value of extract group

Figure 3.3: *Tabernaemontana divaricata* extract feed to the mice for in-vivo Analgesic activity test.

3.8 Antidiarrheal activity: [29]

This method will be taken form Galvez et al; (1993). the extract solution, control and standard solution were given orally by means of a feeding needle.

For antidiarrheal activity test all mice were divided into four group and all group contained five mice. One group was control group 2nd group was standard group and 3rd & 4th group were two concentration sample or extract group.

a. Extract solution preparation: To prepare the dose 250mg/kg & 500mg/kg per body weight, 250mg and 500mg extract was taken in the beaker and added small amount of tween 80 & 10ml distilled water then mixed properly. Then the concentration was 10mg/ml and 20mg/ml.
b. Standard solution preparation: 3mg of Loperamide was taken and then triturated properly then added small amount of tween 80 & 10ml distilled water and mixed. The concentration was 0.3mg/ml.
c. The amount of extract solution and standard solution to be administered to get the desired concentration = (Body weight of mice × 0.01) ml.

Working procedure:

- At first the sufficient mice were weighed with proper numbering.
- Extract group: The five mice were fed 25mg/ml & another five mice were fed 50mg/ml concentration of the extract solution with exact amount.
- Standard group: Other five mice were fed loperamide solution with exact amount
- Control group: five mice only fed distilled water.
- After 40 minutes 0.3ml castor oil was fed the each & all group of mice.
- Then each mice were kept on clear white paper .and each mice must be separated.
- Then observed the stool every 1 hour up to 4 hours.

3.9 Anthelmintic activity: [30]

Anthelmintic or anthelminthic are the group that are used to kill the parasitic worm and other internal parasites from the body. They work by either stunning or killing the parasites and without causing significant damage the host.

a. Animal collection: Live earthworm *Lumbricus terretris* were collected from earth of local area of Ashulia, Savar. They were stored in some amount of earth.

b. Extract solution preparation: to prepare the suspension of extract at the concentration 75, 100, 150 mg/ml required 0.5gm, 1gm & 1.5gm extract and triturated with small amount of tween 80 and added 10ml of distilled water.
c. Standard solution preparation: to prepare albendazole solution at concentration 15mg/ml required 150 mg albendazole was triturated with small amount of tween 80 and added 10ml distilled water.
d. Control solution: small amount of tween 80 & 10 ml distilled water

Working procedure:

- At first, 10ml of extract, standard & control solution in different petri dish.3 petri dish for three concentration of extract solution, one petri dish for standard solution and one for control solution.
- Two earthworm were put into the each of five petri dish.
- Then observed the time for paralysis the earthworm.
- And the time taken for death for each earthworm was recorded.

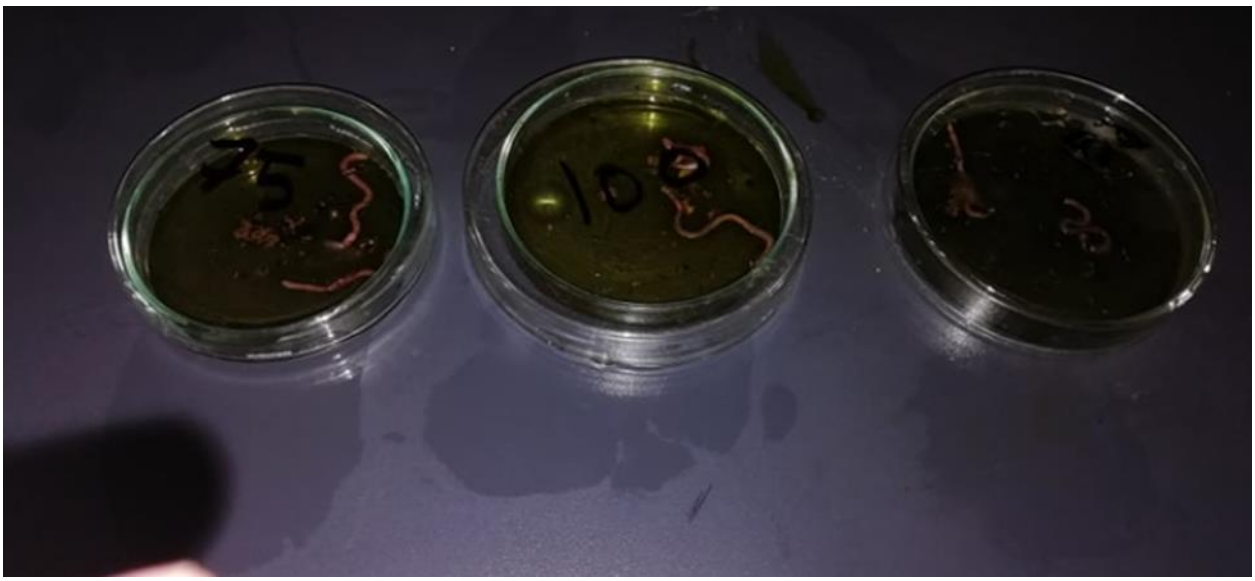

Figure 3.4: Anthelmintic activity test with *Tabernaemontana divaricata* extract

3.10 Thrombolytic activity: [31]

Thrombocytes also called Platelets are a component of blood whose function (along with the coagulation factors) is to react to bleeding from blood vessel injury by clumping, thereby initiating a blood clot. Thrombosis is the formation of a blood clot, known as a thrombus, within a blood vessel. It prevents blood from flowing normally through the circulatory system. When a blood clot forms in the veins, it is known as venous thromboembolism. This can cause deep vein thrombosis and pulmonary embolisms. When a clot forms in the arteries, it is called atherothrombosis, which can lead to heart attack and stroke.

a) Materials:
    - Micro centrifuge tube
    - Syringe filter
    - Syringe
    - Incubator
    - Micro pipette
b) Extract solution preparation: 100mg extract was suspended in 10 ml distilled water and if was kept for overnight.
c) Preparation of standard solution: Streptokinase is a lyophilized product ail of 15,00,000 I.U. was collected and 5ml sterile distilled water was added and mixed properly. This streptokinase was used as a standard.
d) Blood sample collection: 3ml blood was drawn from healthy human volunteer without history of oral anticoagulant therapy. 1ml blood was transferred into the previous weighed each 3 micro centrifuge tubes and to clot formation.

Working procedure:

- At first 1ml blood was taken in each 3 pre weighed micro centrifuge tube and incubated for 45 minutes at 37°C temperature.
- After clot formation, the serum was completely removed without disturbing the clot. Then again took the weight of each centrifuge tube for determining the only the clot weight.
- In one centrifuge tube with blood clot was added 100µl of extract solution which was filtered with the syringe filter.
- In another centrifuge tube with clot was added 100µl streptokinase solution.

- And the other tube was added 100µl distilled water as a control.
- Then the 3 tube were incubated for 90 minutes at 37℃ temperature.
- After the incubation, completely removed the released fluid and finally took the final clot weight.

The percentage of clot lysis = (weight of released clot / clot weight) × 100.

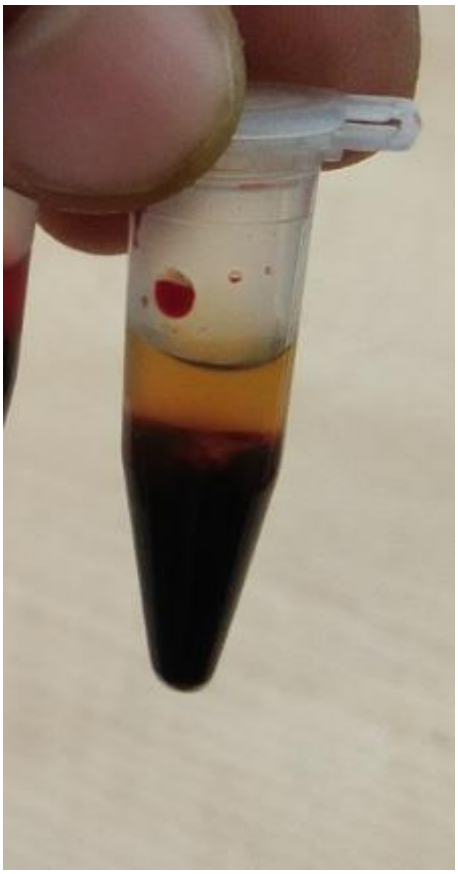

Figure 3.5: Blood clot with serum for Thrombolytic activity test with *Tabernaemontana divaricata* extract

Chapter Four:
Result and Discussion

4.1 Phytochemical test results:

Chemical groups	Results
Saponin	Absent
Phenols	Present
Glycosides	Present
Flavonoids	Present
Tannin	Absent
Terpinoids	Present
Gum	Absent
Alkaloids	Present
Reducing sugar	Present (Fehling's test & Benedict's test result was positive)

Table 4.1: The chemical compound present & absent in the methanolic extract of *Tabernaemontana divaricaka* leaves.

In my phytochemical study I found the chemical compound such as phenols, glycosides, flavonoids, reducing sugar, terpinoids & alkaloids are present.

4.2DPPH Radical scavenging assay result:

DPPH Radical scavenging at different concentration of

Standard:

Concentration (µg/ml)	Absorbance	% of scavenging	IC50(µg/ml)
1	0.698	8.5	
5	0.659	13.6	
10	0.610	20	18.40
50	0.554	27.4	
100	0.487	36.1	
500	0.388	49.1	

Table 4.2: DPPH radical scavenging of different concentration of standard.

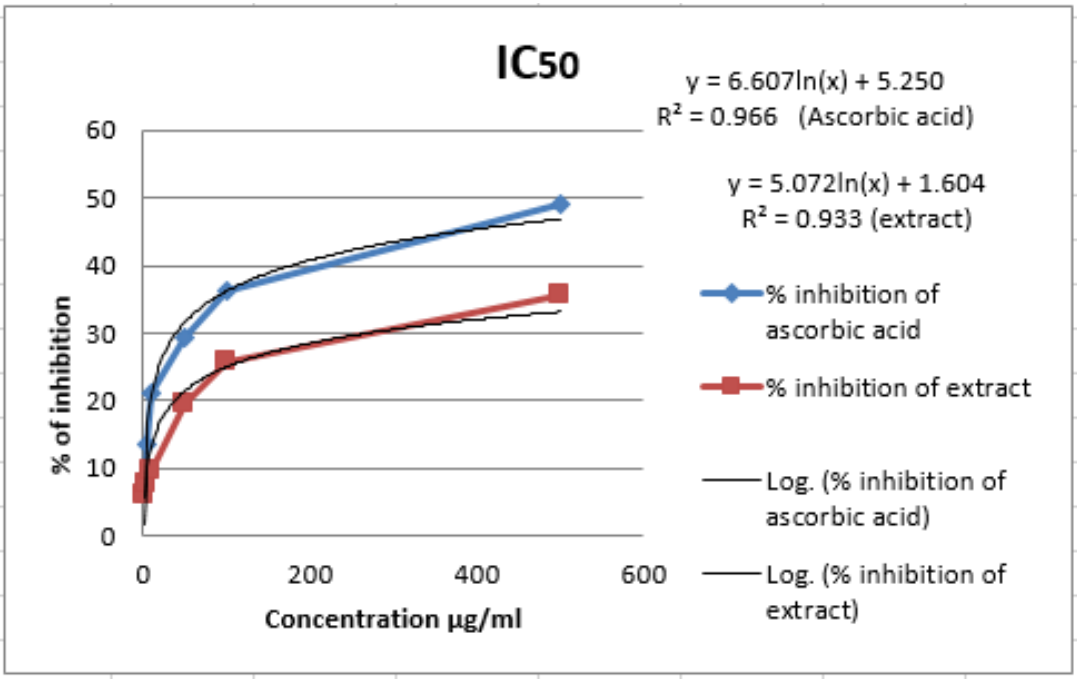

Figure 4.1: DPPH scavenging effect of standard and extract

Extract:

Concentration (µg/ml)	Absorbance	% of scavenging	IC50(µg/ml)
1	0.718	5.9	
5	0.704	7.7	
10	0.689	9.7	25.93
50	0.614	19.5	
100	0.566	25.8	
500	0.491	35.6	

Table 4.3: DPPH radical scavenging of different concentration of extract .

So, for extract IC50 39.08µg/ml compared to standard ascorbic acid IC50 23.47µg/ml.

4.3 Total phenolic content test result:

Concentration of Gallic acid (mg/ml)	Absorbance
0.1	0.724
0.2	1.54
0.3	2.376
0.4	3.539
0.5	4.621

Table 4.4: Different absorbance for different concentration of Gallic acid at the wave length 765nm

Solution	Absorbance
Blank solution	0.249
Extract solution	0.16

Table 4.5: absorbance of blank solution & extract solution at wave length 765nm.

Put this data in the Microsoft excel in X – excess the concentrations and Y – excess the absorbance of the Gallic acid.

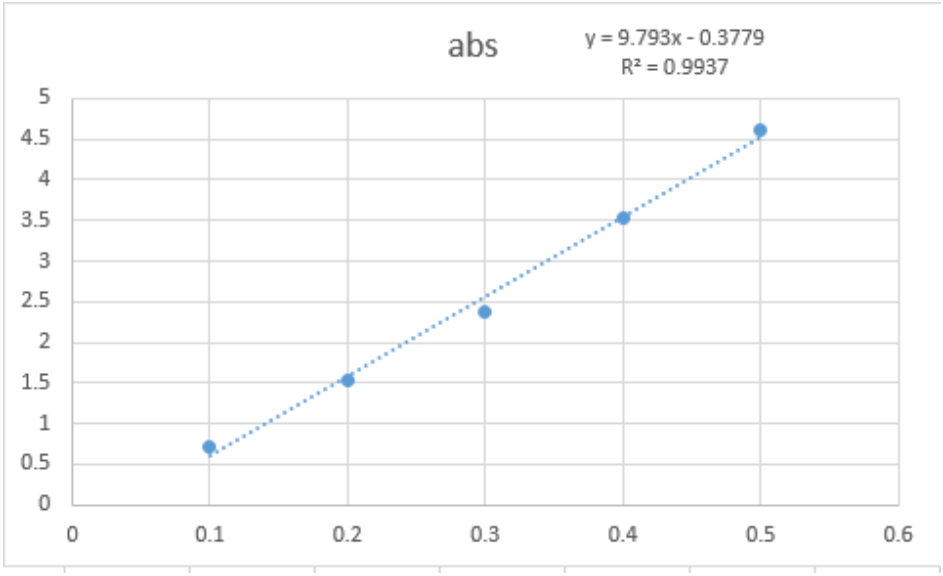

Figure 4.3: Curve of Gallic acid

And y = absorbance of extract solution.

So, x = 0.05489 mg/ml

The extract concentration was 1mg/ml

So, GAE = 54.89mg/gm

That means, 54.89mg Gallic acid equivalent present per gram of extract. Gallic acid is a type of Phenolic compound.

4.4 Total Flavonoids content test result:

Concentration of Quercetin (mg/ml)	Absorbance
0.1	0.845
0.2	1.689
0.3	2.836
0.4	3.991
0.5	5.106

Table 4.6: Different absorbance for different concentration of Quercetin at wave length 510nm.

Solution name	Absorbance
Blank solution	0.168
Extract solution	0.183

Table 4.7: Absorbance of blank solution & extract solution at 510nm wave length.

After put this data in the Microsoft excel in X – excess the concentrations and Y – excess the absorbance of the Quercetin.

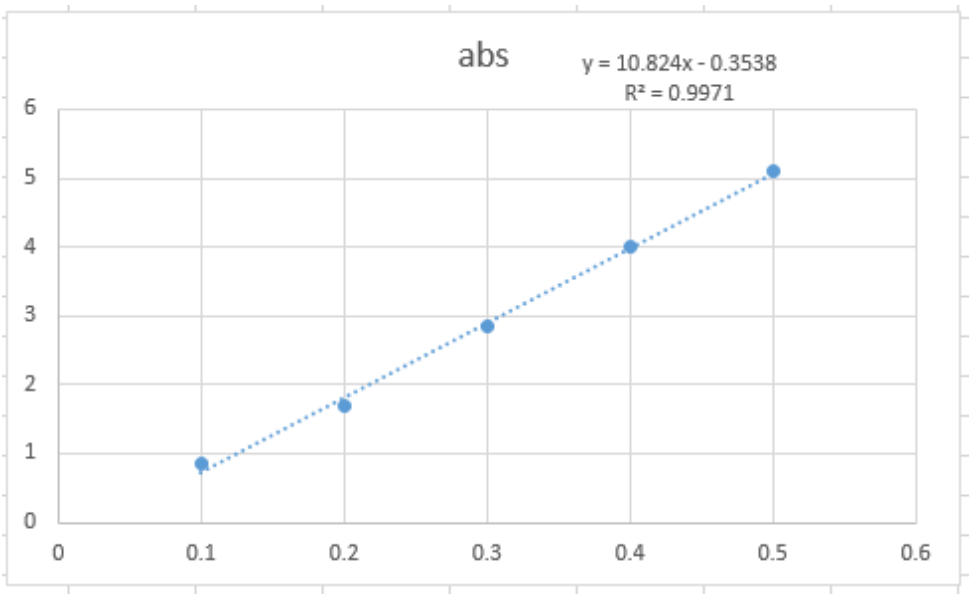

Figure 4.4: curve of quercetin

And y = absorbance of extract solution.

So, x = 0.04959 mg/ml

The extract concentration was 1mg/ml

So, Quercetin equivalence = 49.59mg/gm

That means, 49.59mg Quercetin equivalent present per gram of extract. Quercetin is a type of Flavonoids compound.

4.5 Analgesic activity test result:

Calculation of the data:

Group	Body weight (gm)	Dose (ml)	Total writhing
Standard	A) 29.3 B) 31 C) 31.4 D) 30.1	A) 0.29 B) 0.31 C) 0.31 D) 0.30	A) 8 B) 9 C) 7 D) 6
Extract	A) 30.1 B) 28.7 C) 30.3 D) 29.5	A) 0.31 B) 0.29 C) 0.30 D) 0.30	A) 11 B) 13 C) 9 D) 12
Control	A) 30 B) 32.3 C) 30.5 D) 29	A) 0.30 B) 0.32 C) 0.30 D) 0.29	A) 26 B) 25 C) 27 D) 29

Table 4.8: Total writhing count of the individual mice.

Where,(n = 4) and A= mice number 1, B=mice number 2, C=mice number 3, D=mice number 4 the separate treated group.

Animal group	Total writhing	Mean value ± SEM	% of protection
Standard	30	7.5 ± 0.65	72.22
Extract	45	11.25 ± 0.85	58.33
Control	108	27 ± 0.85	0

Table 4.9: Mean value of writhing for analgesic activity rest

So, percentage of protection of Acetic acid induced writhing for extract 58.33% and for standard 72.22% .

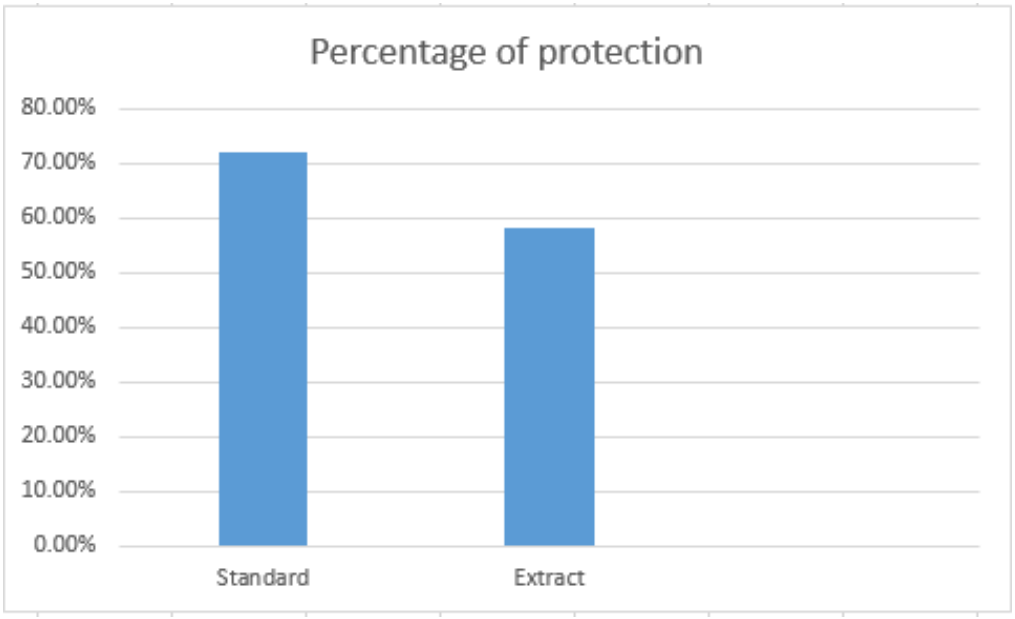

Figure 4.5: percentage of protection of writhing

4.6Antidiarrheal activity test result:

Group	Body weight	Dose	Total faeces in 1st hour	Total faeces in 2nd hours	Total faeces in 3rd hours	Total faeces in 4th hours
Standard group	A) 27.9 B) 29 C) 30 D) 29.6 E) 28	A) 0.28 B) 0.29 C) 0.30 D) 0.30 E) 0.28	A) 3 B) 3 C) 2 D) 2 E) 3	A) 2 B) 2 C) 1 D) 2 E) 4	A) 0 B) 2 C) 2 D) 0 E) 0	A) 1 B) 0 C) 0 D) 2 E) 0
Control group	A) 30.3 B) 28.2 C) 26.4 D) 29 E) 30.8	A) 0.30 B) 0.28 C) 0.26 D) 0.29 E) 0.31	A) 8 B) 7 C) 11 D) 9 E) 9	A) 9 B) 10 C) 9 D) 12 E) 6	A) 5 B) 7 C) 7 D) 6 E) 4	A) 3 B) 4 C) 4 D) 5 E) 3
Extract (25mg/ml)	A) 28.3 B) 30 C) 27.7 D) 26 E) 30.4	A) 0.28 B) 0.30 C) 0.28 D) 0.26 E) 0.30	A) 4 B) 4 C) 3 D) 6 E) 4	A) 3 B) 2 C) 4 D) 9 E) 7	A) 0 B) 0 C) 2 D) 3 E) 5	A) 0 B) 2 C) 0 D) 0 E) 1
Extract (50mg/ml)	A) 31.3 B) 31.1 C) 28.7 D) 36.2 E) 29.5	A) 0.31 B) 0.31 C) 0.29 D) 0.32 E) 0.30	A) 3 B) 2 C) 2 D) 1 E) 2	A) 4 B) 1 C) 8 D) 3 E) 3	A) 2 B) 0 C) 2 D) 0 E) 0	A) 0 B) 0 C) 0 D) 1 E) 3

Table 4.10: Total faeces count within four hours for standard, control, extract

Group	Mean value of faeces in 4 hr ± SEM	% of inhibition defecation
Standard	7.75 ± 0.30	77.37
Control	34.25 ± 0.60	0
Extract (25mg/ml)	14.4 ± 0.56	57.96
Extract (50mg/ml)	9.25 ± 0.43	72.99

Table 4.11: calculation of percentage of inhibition of defecation for standard, control & extract.

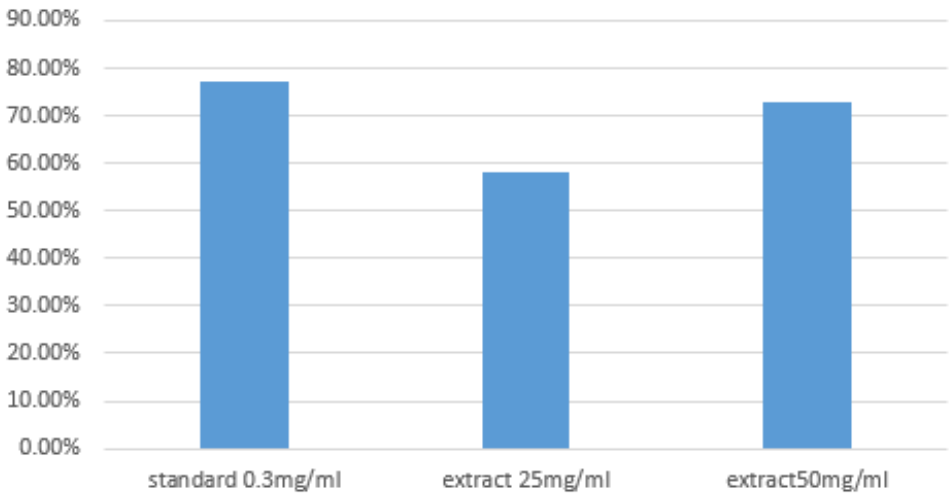

Figure 4.6: Percentage of inhibition of defecation standard & extract

4.7Anthelmintic test result:

The time was required to paralysis & death of the earthworm:

Extract:

Dose of the extract (mg/ml)	Paralysis time (minutes) (mean ± SEM)	Death time (minutes) (mean ± SEM)
75	87 ± 0.73	150 ± 0.85
100	35 ± 0.51	74 ±0.78
150	10 ± 0.66	40 ± 0.85

Table 4.12: Paralysis & death time of earthworm for extract

Where n = 3

Standard:

Dose (mg/ml)	Paralysis time (minutes) (mean ± SEM)	Death time (minutes) (mean ± SEM)
15	40 ± 0.79	90 ± 0.76

Table 4.13: Paralysis & death time of earthworm for standard

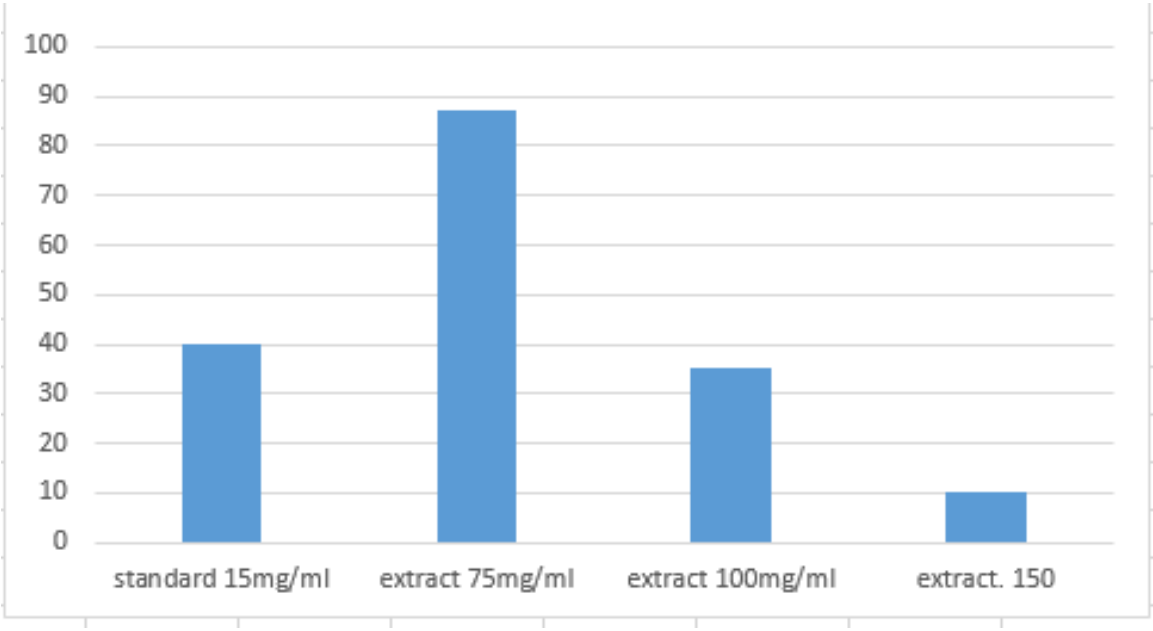

Figure 4.7: paralysis time for standard & extract

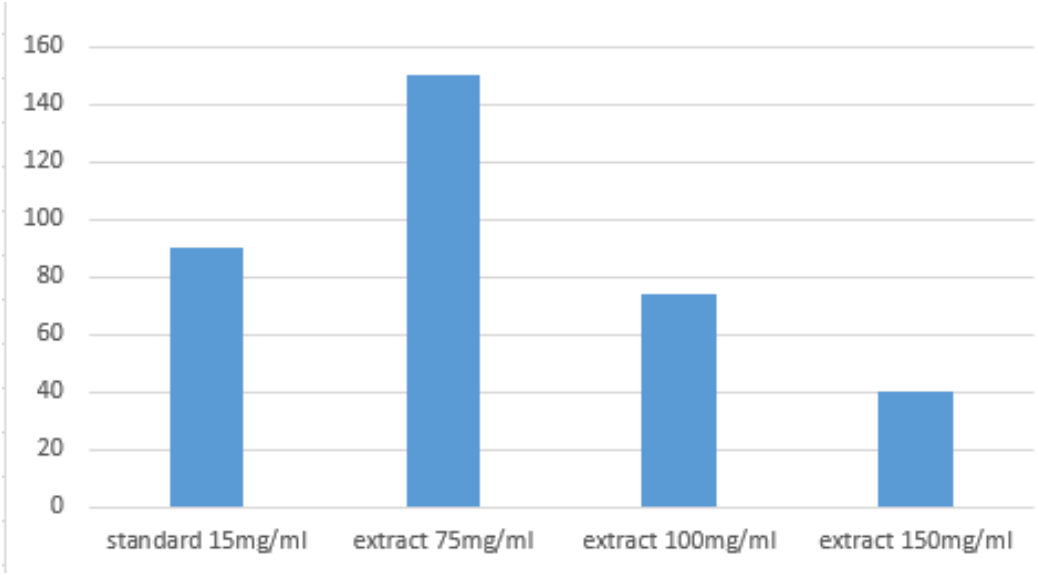

Figure 4.8: Death time for standard & extract

4.8 Thrombolytic activity test results:

Solution name	Blank micro centrifuge tube	1st clot weight with tube	2nd clot weight with tube
Standard solution	0.76	1.774	1.009
Extract solution	0.78	1.50	1.152
Control solution	0.74	1.489	1.389

Table 4.14: clot weight with micro centrifuge tube of different solution

Solution	1st clot weight without tube	2nd clot without tube	% of lysis
standard	1.014	0.249	75.44
Extract	0.72	0.372	48.33
Control	0.649	0.620	4.47

Table 4.15: percentage of lysis for standard, extract & control solution.

In my study, the standard streptokinase showed 75.44% clot lysis, extract of *Tabernaemontana divaricata* leaves showed 48.33% clot lysis & control solution showed 4.47% clot lysis.

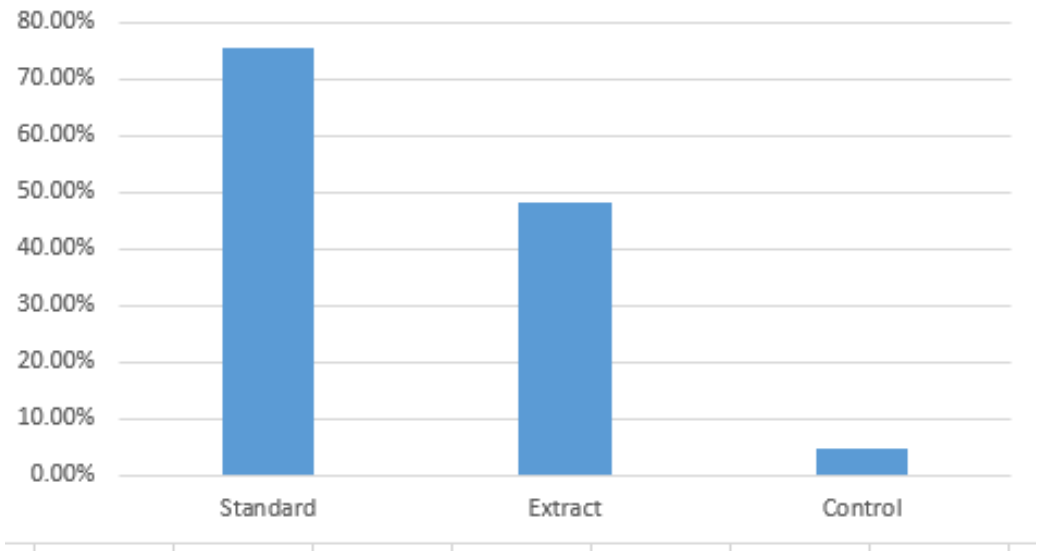

Figure 4.9: Percentage of clot lysis ability of standard extract and control solution

Chapter Five: Conclusion

Conclusion:

Tabernaemontana divaricata is the plant belonging to the family Apocynaceae. This research work conducted to find out some different activities of the methanolic extract of *Tabernaemontana divaricata* leaves.

The methanolic extract of *Tabernaemontana divaricata* leaves were subjected to phytochemical and pharmacological activities test. The chemical compound like phenols, glycosides, flavonoids, reducing sugar, tarpinoid & alkaloids were present in the methanolic extract which are responsible for the pharmacological activity.

The pharmacological activity test was carried out with in- vitro antioxidant assay, in-vivo analgesic activity, antidiarrheal activity, anthelmintic activity and ex-vivo thrombolytic activity test. All the test was done by comparing with the standard against extract sample and also control. The findings of the research work were that, this methanolic extract showed the good result of Antioxidant test. And also showed good analgesic activity, anthelminthic activity, and thrombolytic activity. The methanolic extract also showed some antidiarrheal activity respectively.

Therefore, considering the potential bioactivity, the plant can be further studied extensively to find out their unexplored efficacy and to rationalize their uses of traditional medicines.

Chapter Six:

Reference

Reference:

1. Tapsell LC , Hemphill l , Cobiac L , et al.(August 2006).“Healths benefits of herbs and spices: the past , the present , the future “ . Med. J. Aust. 185(4Suppl)s4-24. PMID 17022438 .
2. Barrett ,Stephen(23 November 2013) “ The herbal minefield”. Quackwatch Retrieved 25 February 2017.
3. Chikezie PC “Herbal Medicine :Yesterday , Today and Tomorrow” Received date: july 15, 2015 , Accepted date : August 03, 2015 , Published date : August 11, 2015.
4. “*Tabernaemontana divaticata*” Natural Resources conservation Service PLANTS Database . USDA Retrieved 7 December 2015.
5. “ Flavonoids”. Linus Pauling Institution, Oregon State University , Corvallis. 2016. Retrieved 24 July 2016.
6. Lemmo W (September 2014). “Potential interactions of prescription and over-the-counter medications having antioxidant capabilities with radiation and chemotherapy” . International Journal of Cancer. 137(11): 2525-33. 1002/ijc.29208. PMID25220632 .
7. Cashman JN. Department of Anaesthetics, St George’s Hospital, London, England. “The mechanism of action of NSAIDs in analgesia”. PMID: 8922554 DOI: 10.2165/00003495-199600525-00004.
8. Mutschler, Emst , Schafer-Korting , Monika(2001). Arzneimittwirkungen (in German)(8 ed.) Stuttgart wissenschaftliche verlagsgesellschaft. Pp. 652-4 . ISBN 3-8047-1763-2 .
9. WebMD. Thrombolysis. Available from https://www.webmd.com/stroke/guide/thrombolysis-and-facts#1
10. Awouters F, Megens A , Verlinden M, Schuuikes J , Janssen PA. “Loperamide. Survey of studies on mechanism of its antidiarrheal activity”. PMID:8508715.
11. “Indications for fibrinolytic therapy in suspected acute myocardial infraction : collaboration overview of early mortality and major morbidity results from all randomized trials of more than 1000 patients. Fibrinolytic Therapy Trialists(FTT) Collaborative Group” Lancet . 343 (8893) : 311-22. 5 February 1994. PMID: 7905143
12. Wardlaw JM , Murray V , Berge E , Del Zoppo GJ (2014). “Thrombolysis for acute ischemic stroke . Cochrane Database Syst Rev (7) :CD000213 . PMID 25072528 .

13. Prof (Dr.) Rabiul Haque. " Investigation of in Vitro Anthelmintic activity of Azadirachta Indica Leaves." International Journal of Drug Development and Research. October- December 2011, 3(4): 94-100.
14. Ayetree Rai Basumatary " Preliminary Phytochemical screening of some compounds from plant stem bark extract of Tabernaemontana divaricata Linn. Used by Bodo Community at Kkrajhar District, Assam , India". Archives of Applied Science Research , 2016, 8 (8):47-52.
15. Mrinal Kumar Baishya, Kandarpa Kr. Saikia, Naba Kr. Hazarika, Debabrat Baishya Deep Jyoti Das. "Antimicrobial Potential and In Vitro Cytotoxicity Stydy of Tabernaemontana divaricata (L.) Stem Bark Extract Against HEK 293 Cell Line." IOSR Journal Of Pharmacy(March 2018) (e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219.
16. Chanchal N.Raj, A. Balasubramaniam, Sayyed Nadeem. "Anticonvulsant activity of Tabernaemontana divaricata extract in experimental mice."Scholars Research Library J.Net. Prod. Plant Resour., 2014, 4 (1):64-68.
17. Md. Masudur Rahman, Md. Saiful Islam, Md. Sekendar Ali, Md. Rafikul Islam, Md. Zakir Hossin. "Antidiabetic and Cytotoxic Activities of Methanolic Extract of Tabernaemontana divaricata(L.) Flowers." International Journal of Drug Development & Research .[July-September 2011] Vol. 3, ISSN:0975-9344.
18. Shazid Md. Sharker, Samabesh Chakma and Ahmed Ayedur Rahman. " Phytochemical and antinociceptive study of leaves extract of Tabernaemontana divaricata(L.)Journal of Medicinal Plants Research Vol. 5(2), pp. 245-247, 18 January, 2011.
19. Chanchal N. Raj, A. Balasubramaniam, Sayyed Nadeem. "Effect of various extract of Tabernaemontana divaricata on haloperidol induced catalepsy in rats." International Current Pharmaceutical Journal, February 2014, 3(3), 240-242.
20. Carla Nicola, Mirian Salvador, Adrlana Escalona Gower,Sidnel Moura, and Serglo Echeverrlgaray. "Chemical Constituents Antioxidant and Anticholinesterasic Activity of Tabernaemontana catharinensis. The Scientific World Journal Volume 2013,Article ID519858.
21. Akhila Sravya Dantu, Shankarguru P, Ramya Devi D, Vedha Hari BN. "Evaluation of Anticancer Activity of Hydroalcoholic Extract of *Tabernaemontana divaricata*." Asian Journal of Pharmaceutical and Clinical Research Vol 5, Suppl 4, 2012.

22. Sangita Kumara, A. Mazumder and S. Bhattacharya. “Evaluation of Anti-Diarrheal and Diuretic Activity of *Tabernaemontana divaricata*(L.).” International Journal of Pharmacology .”DOI:10,13040/IJPSR,0975-8232.ijp.
23. Kannappan Poornima, Palanisamy Chella Perumal and Velliyue Kanniappar Gopalakrishnan. “Protective Effect of Ethanolic Extract of Tabernaemonata divaricata (l.)R. Br. Against DNA and Fe NTA Induce Liver Necrosis in Wister Albino Rats.” BioMed Research International volume 2014, Article ID 240243.
24. CER Handbook of Free Radicals and Antioxidants, 1989;1:209-221.
25. Antioxidants Vitamins Benefits Not Yet Proved(editorial) NEJM, 1994; 230(15): 1080-1081
26. Hemayet H, Ismet AJ, Sariful IH, Jamil AS, Shubhra KD, Arpona H, Arif A (2013) Anti-inflammatory and antioxidant of ethanolic leaf extract of Brownlowiatersa (l) Kosterm. Orient Pharm Exp Med 13(3):181-189.
27. Shah M, Behara YR, Jagadeesh B(2012) Phytochemical screening and in vitro antioxidant activity of aqueous and hydroalchololic ectract of Bacopamonnieri Linn. Int J Pharm Sci Res 3(9):3418-3424.
28. Ahmed F, Sellm MST, Das A.K., and Choudhuri MSK, Anti-inflammatory and antinociceptive activitiesof Lippa nodiflora Linn. Pharmazie, 2004;59:329-333.
29. Akter, R., Hasan, S. R., Hossain, M. M., Chowdhury, S.S.,Mazumder, M. E. H., & Rhaman, S.(2010). Antidiarrheal and antioxidant properties of Curcuma alismatifolia leaves. Australian Journal of Basic and Applied Science, 4(3), 450-456.
30. Anuj Kumar Agrahari, Ashutosh Meher, Amiya Ranjan Padhan, Srimanta Dash. “Assessment of anthelmintic activity of Jussiaes hyssopifolia G. Don. Asian journal of Plant Science and Research, 2011, 1 (4):87-91 .
31. Mohammad Imran Hossain, Md. Hossan Sakib, Asif Al Mahmood, Naymul Karim, Mahammad Shahin Alam, Md. Ariful Islam, Monalisha Sharma. “Study on in-vitro thrombolytic activity of methanolic extract of Mesua ferrea leaves.”International Journal of Medical and Health Research. Volume: 1, Issue: 2, 52-55, September 2015, ISSN: 2454-9142.