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PREPARATION OF VARIOUS SOLVENTS [ETHANOL & AQUEOUS] EXTRACTS FROM TABERNAEMONTANA DIVARICATA AND THEIR EVALUATION OF ANTIBACTERIAL ACTIVITY

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ABSTRACT
The present study describes the antibacterial activity of stem extracts from Tabernaemontana divaricata a traditional medicinal plant found in Asian countries like China, India and Myanmar.The plant is commonly called Crape Jasmine and it is belonging to the family of Apocynaceae. It is about 1.5-2.5m in height with silvery grey bark; wrinkled and milky latex exudes when wounded, and has white colored flowers. As per review of literature various parts of Tabernaemonatna divaricata have shown different pharmacological actions.The extracts (ethanol and aqueous) were found to possess maximum potency against infectious pathogens like gram (+ve) Staphylococcus aureus and gram (-ve) Pseudomonas aeruginosa. The zone of inhibition was observed after conformed with cell diffusion method with selected bacteria. Minimum inhibitory concentrations of the extracts were found to be significant. The obtained results provide a support for the use of this plant in traditional medicine and its further investigation.

KEYWORDS: Tabernaemontana divaricata, Extractive solvents, phytochemical, Antibacterial activity.

INTRODUCTION
Bacterial infections have posed significant threats to public health throughout history, leading to millions of illnesses and deaths annually. The discovery and development of antibiotics revolutionized medicine, offering effective treatments for a wide range of bacterial ailments. However, the widespread use and misuse of antibiotics have led to the emergence of antibiotic-resistant strains of bacteria, rendering many conventional treatments ineffective. Some medical plants have been used for a wide variety of purposes such as food preservation, produced naturally, rather than synthetically, will be biodegraded more easily and therefore be more environmentally acceptable. Thus, natural antioxidants, antibacterial, cytotoxic, antiviral, fungicidal agents and nutrients have gained popularity, At present condition their use and positive image among consumers are spreading. In recent years, multiple drug resistance in both human and plant pathogenic microorganisms have been developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. Naturally occurring antimicrobial compounds have enormous therapeutic potentials and advantages than similar such synthetic compounds. Synthetic antimicrobials are often found to have many biological side effects. However, plant based compounds are believed to have only limited side effects because of the fact that the cellular and molecular mechanism of plant and animal cells are similar in many ways.

PLANT PROFILE
Tabernaemontana divaricata
Tabernaemontana divaricata is an ornamental plant as well as evergreen shrub. It belongs to the family of Apocyanaceae. It is commonly known as crape jasmine. This plant is very attractive because of its silvery grey bark, milky latex, shiny deep green leaves and white coloured fragrant flowers. In traditional medicine it is very popular because this plant have huge amount of phytochemical compounds constituents such as alkaloids, terpenoids, steroids, flavonoids, phenyl propanoic acids and several plant enzymes.

Fig. 1: Entire plant with different parts of Tabernaemontana divaricate.

Table 1: Taxonomical description of Tabernaemontana divaricate.

Kingdom	Plantae
phylum	Tracheophyta
Class	Magnolipsida
Order	Gentianales
Family	Apocynaceae
Genus	Tabernaemontana
Species	divaricata
Botanical name	Tabernaemontana divaricata

Table 2: Vernacular names.

English	Crepe jasmine, pinwheel flower
Sanskrit	Nandivarsha
Hindi	Chandni, Tagar, Tagari
Assamese	Kathanda
Kannada	Nandibattalu Nanjubattalu, nadyaavarta
Mizo	Par-arsi, kelte-benbeh Battalu
Gujarathi	Sagar
Bengali	Tagar, kath Mallika
Marathi	Ananta, Tagar
Malayalam	Kuntampale

MATERIALS AND METHODS

PLANT COLLECTION

Tabernaemontana divaricata (Crape jasmine) were collected from our college garden.

CHEMICALS

All the chemicals to be used in the present studies are as follows

- Ethanol
- Aqueous

REQUIREMENTS

- Tabernaemontana divaricate (stems)
- Gram positive: Staphylococcus aureus.
- Gram negative: Pseudomonas aeruginosa

INSTRUMENTS

- Macerator
- Incubator
- Laminar airflow
- Autoclave
- Inoculator
- Petri dishes
- Nutrient agar media
- Sterilised inoculating loop.

QUALITATIVE ANALYSIS (Phytochemical screening)

- The ethanol and aqueous extracts were subjected to preliminary phytochemical screening such as flavonoids, alkaloids, glycosides, saponins, tannins, carbohydrates.

Table 3: Preliminary phytochemical screening of the stem of tabernaemontana diavaricata Linn. Using various solvents.

S. no.	Plant constituents	Ethanol extract	Aqueous extract
1	Alkaloids	+	+
2	Carbohydrates	-	-
3	Glycosides	+	-
4	Tannins	+	+
5	Flavonoids	+	+
6	Saponins	+	+

(+) = Positive & (-ve) = Negative

PROCEDURE

1. Preparation of Nutrient Agar Plates

- Mixed 2.8 grams of nutrient agar with 100ml of water, then autoclaved at 121°C and 15lbs pressure.
- Pour onto sterilized Petri plates and let to solidify.

2. Preparation of Bacterial Culture

- Using sterile disposable loops, inoculated bacterial strains(Staphylococcus aureus and Psudemonous aeruginosa) onto nutrient agar plates.
- Incubated the plates at 37°C to promote bacterial growth.

3. Preparation of Test Samples

- Using an ethanol & aqueous solvents, diluted the test samples to concentrations of 100 to 1000ug/ml. Prepare a series of dilutions for each testsample[100,200,300,400,500, 600,700,800,900, &1000ug/ml].

4. Sterilization of Cork Borer

- With a Bunsen burner, heat the cork borer until it glows red hot.
- Let the cork borer cool down before using.

5. Creation of Wells

- Use the sterilised cork borer to make wells in the agar plates for the bacterial lawn.
- Press the cork borer gently into the agar to make evenly spaced wells.

6. Addition of Test Samples

- Using micropipettes to precisely added small amount of each dilution of the test samples to t he wells. Ensured that each well receive same volume of samples .

7. Incubation

- For 18-24 hours, incubated the plates at 37°C to allow for bacterial growth and test sample diffusion.

8. Evaluation of Zones of Inhibition

After incubation, checked the plates for zone of inhibition surrounding the wells.

- Use a ruler or calipers to measure the diameter of any clear zones that have developed around the wells.

9. Data Analysis

- To measurement relative antibacterial activity, compare the diameters of the zone of inhibition for each test sample dilution.
- Larger zones imply stronger suppression of bacterial growth.

10. Statistical Analysis

A statistical analysis was carried out and data was established to check the significance of detected changes i.e antibacterial activity between test samples.

ANTIBACTERIAL ACTIVITY

SUBCULTURING OF BACTERIA IN LIQUID BROTH

- Inoculums of gram +(ve) bacillus subtilis, staphylococcus strains: and gram- (ve) pseudomonas aeruginosa, Ecoli are collected from St Francis college Begumpet.
- 0.3gms of beef extract, 0.5gms of peptone and 0.5gms of Nacl are dissolved in 100ml of distilled water in a conical flask.
- Heat the medium prepared and filter it to remove particulates and undissolved substances and sterilize it in autoclave for 15 minutes at 121C with 15lb pressure and PH adjust to 7.
- Sterile liquid broth is divided into four equal portions and transferred into sterile boiling tubes.
- Loopful of bacteria from each bacteria inoculum is inoculated in each boiling tube and incubated in incubator for microbial growth.

DEFINITION OF ANTIBACTERIAL ACTIVITY

- Antibacterial activity refers to chemicals that kill or limit the growth of bacteria on a local level while remaining non-toxic to surroundings tissue. Antimicrobials are therapeutic substances used to prevent or treat infections. They include antiseptics, antibiotics, antivirals, antifungals and antiparasitic.

INTRODUCTION

- The ability of antibacterial activity synthesized using stem extracts of Tabernaemontana divaricata to kill or limit the growth of bacteria**.**
- They offer a wide range of possible applications including health care, food processing and dental restorative materials. Recent advances in this field include the production of hydrogel-based antimicrobial coatings that contains antimicrobial polymers are pesticides.
- Most infectious are resistant to a minimum of one of the antibiotics that are generally used to eliminate the infection. This problem motivates the study of new agents that can efficiently inhibit the growth of microorganisms

INTRODUCTION ABOUT ORGANISMS USED FOR TESTING

The antibacterial activity of the compound [flavonoid] was carried out against the pathogenic bacteria; viz.

1. staphylococcus aureus [Gram +ve] ATCC-25923
2. Pseudomonas aeruginosa [Gram -ve] ATCC-27853.

DESCRIPTION OF THE ABOVE ORGANISM AS FOLLOWS

1. **Staphylococcus aureus:** They are spherical, cocci, approximately 1 micron in diameter, arranged in grape like clusters. They may also found in ring form, in pairs and short chains. They are non- motile, non sporing and non – capsulated and are uniformly gram positive. They grow readily on ordinary media at optimum temperature 37^0c,

P^H 7.4 to 7.6 on nutrient agar media. After incubation for 24 hours the colonies were found to be large [2-4 mm diameter], circular, convex, smooth shinning and easily emulsifiable. Most of the stains produce golden yellow pigments, though some may be white, orange or yellow. Uniformity turbidity is produced in the liquid media.

2. Pseudomonas aeruginosa: Cells are monotrichous, lopotrichous and non- motile, gram negative and the size is about 1.5 to 3.4 x 0.5 microns. It frequently develops fluorescent. Many strains are found in water and soil including sea water or even heavy water. Many are plant pathogens, very few strains are animals pathogens. Growth occurs at wide range of temperature 5 to42^0c, but the optimum is 37^0c. It grows well on ordinary media. The characters of the colonies are observed to be large, opaque, irregular with a distinctive musty, mawkish or eathy smell.

MATERIALS USED

1. Sterile petri dishes.
2. Sterile inoculum loops.
3. Sterile hallow cylinder.
4. Sterile graduated pipettes.
5. Sterile conical flasks, glass rods, boiling tubes, beakers.
6. Sterile test tubes for preparation of subculture.
7. Sterile test tubes for preparation of test compounds.
8. 18 – 24 hours old growth cultures on nutrient agar media.
9. Sterile cotton wool.
10. Sterile cotton swabs.

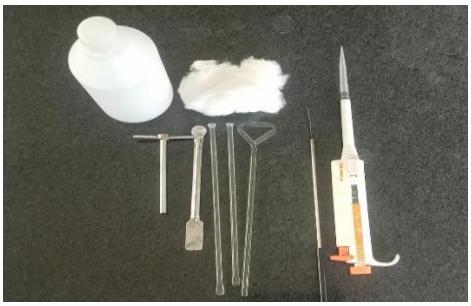

Fig. 2: Materials Used.

PROCESS OF SUBCULTRING

[a] Preparation of subculture: Liquid broth was prepared by dissolving following constituents in 100ml of distilled water.

1. peptone [Bacteriological] 20gms
2. Beef extract [Bacteriological] 05gms
3. Sodium chloride [Bacteriological] 05gms
4. Distilled water……..upto 1000ml

After dissolving, the media was steamed for about 2hrs and adjust the P^H of the reaction mixture to about 7.2 Then the media was sterilised by auto claving at 15 lbs pressure for 20 minutes.One day prior to the test, previously mentioned stock cultures were aspetically inoculated in 5ml of sterilised nutrient broth and incubated for 18 – 24hrs at 37^0c.

[b] preparation of nutrient agar media

Compostion

1. Peptone [Bacteriological] 20gms
2. Beef extract [Bacteriological] 05gms
3. Sodium chloride [Bacteeriological] 05gms
4. Agar [Bacteriological] 20gms
5. Distilled water …… upto 1000ml.

Nutrient agar medium served as the basal medium and it was prepared by dissolving the above mentioned constituents in distilled water in the following manner. Weighed quantities of peptone and beef extract were dissolved in distilled water by genius warming. Then specified amount of agar was dissolved by heating on boiling water bath. Then the volume was made upto 1000ml with distilled water and the P^H of the solution was adjusted to 7.2 by adding sodium hydroxide solution [approximately 40%, 1.25ml for 100ml of the nutrient agar]. Then the media was sterilised by autoclaving at 121^0c for 20 minutes, at 15lbs pressure.

METHOD EMPLOYED

Method employed was **AGAR – WELL DIFFUSION** method **{ Nathan et al. 1978}**

Principle: In this method, assay of antibiotic potency is based on the measurement of the diameter of zones of inhibition of growth, surrounding cylindrical wells containing various dilutions of standard and test compounds. These solutions were poured into the wells of the nutrient medium previously inoculated with a culture of a suitable organism. Zones of inhibition produced by the test compounds is compared with that produced by known concentration of a standard. A very sensitive organism can be used to measure the concentration of anti microbial drug present in given sample solution.

EXPERIMENTAL SECTION

[a] Preparation of standared solution: Standared drugs solution were prepared by dissolving them in distilled water. Alithromycin [gram +ve bacteria] 250ug was dissolved in 250ml of distilled water which gave a concentration of 1000mcg/ml. From this stock solution 0.25ml was pipetted out and again diluted to 10ml with distilled water which gave a final concentration of 25ug/ml. Standared solution of Metronidazole[gram -ve bacteria] was also prepared in the same way in distilled water to get 25 ug/ml concentration.

[b] Preparation of test solution: Concentrations of test compounds is made to [100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ug/ml] by dissolving 30ug of the Tabernaemontana divaricata stem powder in 300ml of distilled water which gave a concentration of 1000ug/ml. From this stock solution is 1ml pipetted out and diluted to 100ml with distlled water to give a final concentration of 100ug/ml. Similarly the higher concentration i.e 1000ug/ml is prepared by taking 10ml of stock solution

and diluting it to 100ml. The test solutions of ethanol & aqueous extracts were prepared in the same manner.

PROCEDURE

Subcultured suspensions of
(1) Staphylococcus aureus [gram +ve] and
(2) Pseudomonas aerogenosa [gram -ve] were spread aspetically over the surface of an agar medium in three different petridishes and media was allowed to settle down. Then five wells were made in each petridish on the agar medium was allowed to settle down. In each petridish the wells was filled with standared (1000ug/ml concentration) and test solutions of 100, 200, 300, 400, 500, 600, 700, 800, 900 & 1000ug/ml concentration (Aqueous, Ethanol & Aqueous) were added to wells. Then the petridishes were incubated at 37^0c in an incubator for 24 hours. Following incubation, zones of inhibition are formed around the agar wells. Their diameter depends upon the concentration and potency of the drug present in each well. After 24 hours incubated plates are taken out and zones of inhibition of standard test samples are measured. The results are compiled in table.

Determination of diameter of zone of inhibition (inmm) produced by the various solvent[Ethanol and Aqueous] extracts of the stem of T. divaricate and its comparision with that of Alithromycin & Metronidazole against selected sensitive bacterial strains.

Table 4: Organism used: Staphylococcus aureus.

S. no.	Concentrations (ug/ml)	Alithromycin [+ ve]	Ethanol extract	Aqueous extract
1	100 ug/ml	10.26 mm	9.43 mm	-
2	200 ug/ml	12.7 mm	10.49 mm	-
3	300 ug/ml	12.9 mm	10.69 mm	-
4	400 ug/ml	12.48 mm	11.15 mm	-
5	500 ug/ml	12.88 mm	11.22 mm	-
6	600 ug/ml	13.6 mm	12.4 mm	-
7	700 ug/ml	13.73 mm	12.88 mm	-
8	800 ug/ml	14.17 mm	13.6 mm	-
9	900 ug/ml	14.58 mm	13.73 mm	-
10	1000 ug/ml	15.53 mm	14.58 mm	-

Table 5: Oraganism used: Pseudomonas aeruginosa.

S. no.	Concentrations (ug/ml)	Metronidazole (-ve)	Ethanol extract	Aqueous extract
1	100 ug/ml	11.1 mm	9.15 mm	-
2	200 ug/ml	11.6 mm	9.43 mm	-
3	300 ug/ml	11.97 mm	10.13 mm	-
4	400 ug/ml	12.06 mm	10.57 mm	-
5	500 ug/ml	12.25 mm	11.15 mm	-
6	600 ug/ml	13.1 mm	11.22 mm	-
7	700 ug/ml	13.6 mm	12.06 mm	-
8	800 ug/ml	13.73 mm	13.1 mm	-
9	900 ug/ml	14.0 mm	13.55 mm	-
10	1000 ug/ml	14.58 mm	13.73mm	-

RESULTS FOR ANTI BACTERIAL ACTIVITY

There are reports that, leaves are useful in treatment against infection by burns or wounds. In addition work in our laboratory has some constituents have been reported as Alkaloids, carbohydrates, flavonoids, glycosides, tannins, saponins, etc in the leaves also possess good antibacterial activity.Hence it was suspected that the stems may also posses antibacterial activity. Therefore the tabernaemontana divaricata and the extracts containing ethanol and aqueous were subjected to screening for antibacterial activity gram positive and gram negative bacteria. Alithromycin and Metronidazole were used as standared drugs for comparision. The extract i.e ethanol has got positive responses. But the aqueous failed to show any antibacterial activity.

ZONE OF INHIBITIONS FOR THE ANTIBACTERIAL ACTVITY

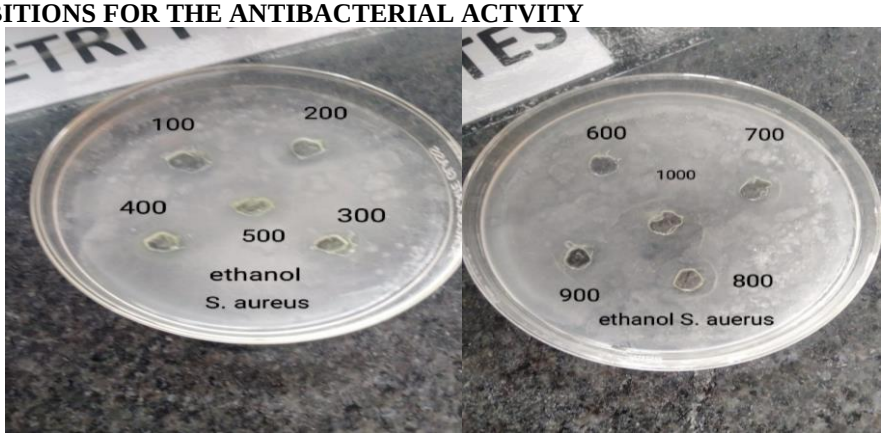

FIG. 3 &4 : Ethanol extract with Staphylococcous auerus.

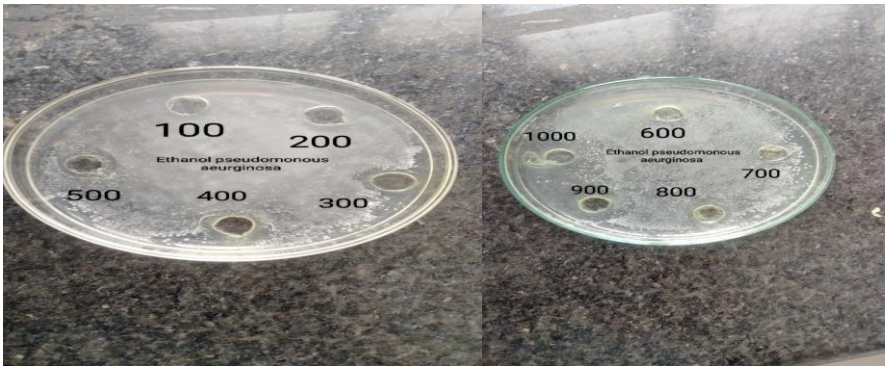

FIG. 5 & 6: Ethanol extract with Pseudomonas areuginosa.

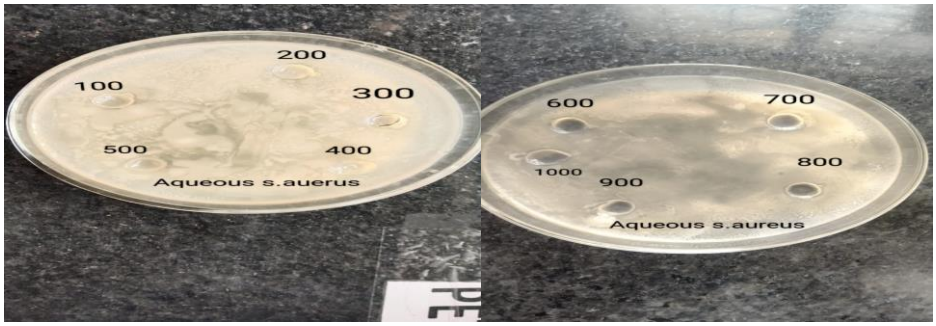

FIG. 7 & 8: Aqueous extract with Staphylococcus aureus.

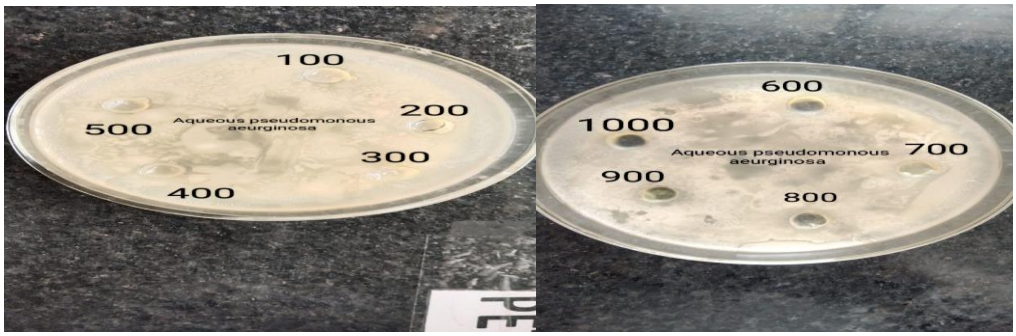

FIG. 9 & 10: Aqueous extract with Pseudomonas aeruginosa.

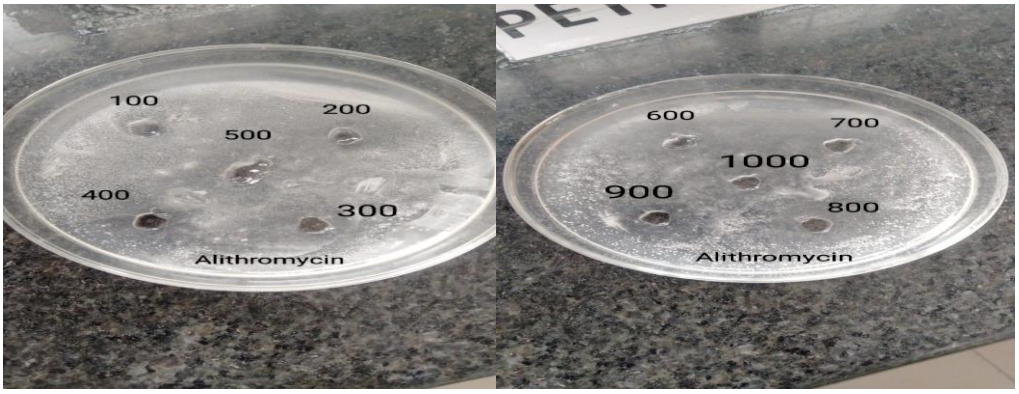

FIG. 11 & 12:Alithromycin [Standared drug (+ ve)].

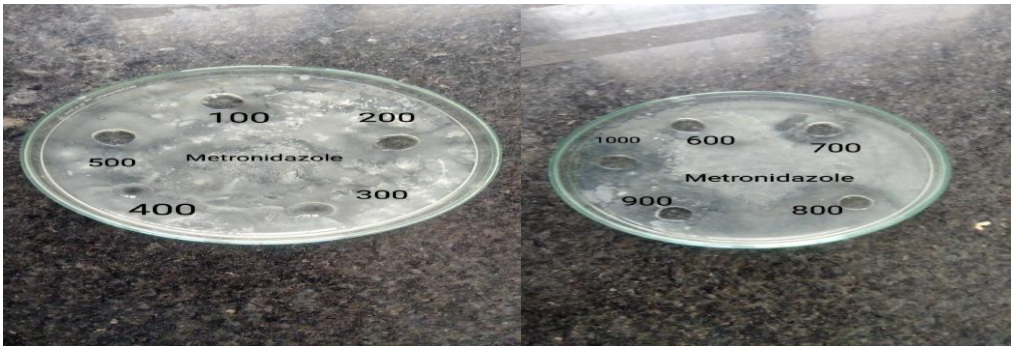

FIG. 13 & 14: Metronidazole [Standared drug (-ve)].

RESULTS AND DISCUSSION

Macroscopy

The following characteristics of stem were noted:
Color: dark green to brown
Odor:Characteristics
Taste: Acrid
Extra feature: Short and branched.

Microscopy

The transverse section of the stem shows the presence of undifferentiated cells known as the cortex in the outer region. They are followed by an extended part of the cortex, which consists of 7-8 layers of phellogen, followed by 6-7 layers pf phelloderm. The pericyclic fibres are in the circular motions which are collaged, followed by thin linning. The xylem are towards the upper surface followed by phloem in a concentric manner, they are known as open vascular bundle. The central portion consists of parenchyma cells called as pith with small number of mucilage cells. They are also composed of compactly packed cells with less space; these are called as collenchyma cells.

SAMPLE COLLECTION

- Tabernaemontana divaricata stems are collected in the month of October 2023 from the college herbal garden of Pulla Reddy Institute of pharmacy, Sanga Reddy Dist,. Hyderabad India. Later the samples was cleaned with water to remove the mud and others substances present on the stems.

AUTHENTICATION

- The plant specimen was identified and authenticated by L. Rasingam scientist E & HoO, Botanical survey of India, Deccan Regional Centre, Sultan Bazar, Kendriya Sadan Koti, Hyderabad, Telangana state. With reference No. BSI/DRC2023-24/ Identification/386. PRIP/PH. COG/ is the reference given to herbarium and it is stored in the department of pharmacognosy, Pulla Reddy Institute of pharmacy, Hyderabad, India.

DRYING AND PULVERIZING

- The collected stems were shade dried at temperature not exceeding 40^0c. After the removal of moisture content it was grounded into coarse powder with the help of mixers and then passed through the sieve number 16. Then the powder obtained was stored in well closed container and kept in dry place.

PREPARATION OF ETHANOL AND AQUEOUS EXTRACTS OF TABERNAEMONTANA DIVARICATA

- Freshly collected stems of tabernaemontana divaricate were washed with sterile and double distilled water and shade dried for 3 weeks at room temperature. Dired stems are grinded into coarse powder.
- Organic[Ethanol] and Non organic[Aqueous] extraction processes were carried out by maceration method, in the ratio of 1:10. The container with its contents were sealed and kept for minimum of 24hrs [1day] and maximum of 72hrs [3days] under dark condition.

- Obtained extracts are subjected to rota evaporator and later it is stored in refrigerator for further uses.
- We have proceeded with the dilution [100, 200, 300, 400, 500, 600, 700, 800, 900 & 1000ug/ml] of the antibacterial activity from that extract.

VISUALIZATION OF EXTRACTS

Dried residues were observed and color of the each extracts was noted.

SNO	NAME OF THE EXTRACTS	VISUALIZATION OF EXTRACTS
1	Aqueous [water]	Appearance of Brownish cream colour residue.
2	Alcoholic [Ethanol]	Appearance of Dark green colour residue.

CONCLUSION

According to research, leaves can help heal infections caused by burns or wounds. Furthermore, studies in our laboratory has revealed that various elements in the leaves, such as alkaloids, carotenoids, flavonoids, glycosides, lipids, triterpines, polyphenols, saponins, and so on, have high antibacterial activity.As a result, it was thought that the stems have antibacterial properties as well. As a result, the tabernaemontana divaricata and extracts including ethanol, chloroform, acetone, and water were tested for antibacterial activity against gram-positive and gram-negative bacteria. Alithromycin and Metronidazole were utilized as benchmark medications for comparison. All extracts, including ethanol, chloroform, and acetone, received positive reactions. However, the aqueous solution showed little antibacterial action. The investigation of the pharmacognostic properties of the stem of T. divaricate Linn.

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