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Phytochemical and Pharmacological Screening of *Tabernaemontana divaricata* Leaves Extracts for Anti-Microbial and Wound Healing Activity

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HUMAN

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ABSTRACT

Plants are traditionally used as medicine. The term medicinal plant include various types of plants used in herbalism and some of these plants consider as a rich resource of ingredients that can be used in development and synthesis, TD is a common garden plant having attractive appearance. Belonging to the family Apocynaceae and subfamily Plumerideae.TD commonly used for various curative purposes. The study is revealed that TD is used for various diseases like anti-cancer, anti-diuretic anti-depressant, anti-hypertensive anti-microbial, anti-anxiety, antiulcer, anti-fertility, and so on. Morphological and microscopical characteristics of the drug was done. Extraction was done by continuous hot extraction. Preliminary phytochemical screening of powdered drug was done such as ash value, acid and water-soluble ash, loss on drying. *The In-Vitro* antioxidant study activity, phenolic content, and flavonoid content was determined of both the extracts The antioxidant study was carried out by DPPH radical scavenging activity, phenolic content by gallic acid equivalent method, and flavonoid content by Filin- Ciocalteu method. The results were observed and microscopical characteristics of a thin section of leaf and the powdered drug characteristics was observed that shows the presence of stomata. xylem vessels, parenchyma, xylem, phloem, cuticle, trichomes. The DPPH radical activity shows good results, all three extracts possesses good radical scavenging activity. The present study revealed that *In-Vivo* wound healing activity of methanolic as well as ethanolic extracts by excision wound model on the species of Wistar Albino rats eighter sex shows the significant result of both the extracts ($P<0.05$ to $P<0.001$). *In-Vitro* anti-microbial activity was done by using the agar cup and plate method and anti-fungal Activity shows significant results of both the extracts.

INTRODUCTION:

This plant is traditionally used by people in many parts of the world to treat various disorders, like abdominal tumor athralgia, asthma, diarrhea, epilepsy, eye infection, fever, fractures, leprosy, mania, oedema, paralysis, piles, rabbis, anti-hypertensive, aphrodisiac, diuretics, immense, headache, inflammation, eye-infections, fever, fracture pain, skin disease, ulceration, and vomiting. The various *In-Vitro* or *In-Vivo* pharmacologica screening has done on *Tabernaemontana divaricata* like anti-cancer [(1)], Anti-Convulsant [(2)] , Anti-microbial [(3)], anti-diarrheal activity [(4)], Anti-ulcer activity [(5)], Anti-diabetic [(6)] , Anti-inflammatory [(7)]. (Synonyms: *Ervatamia coronaria, E.divaricata, T. coronaria*) belongs to the family Apocynaceae and subfamily plumiredeae. The common vernacular names are crape Jasmine, Carnation of India, East Indian Rosebay and Pinwheel Flower (English), Nandivrksha (Sanskrit), Chandani (Hindi), Nandivardhanam (Telugu), Nandiarvattai and Nantiyavattam (Tamil), Sagar(Gujrati), Ananta and Tagar(Marathi), Nantyarvattam(Malayalam), Kottuble(Kanada) and Nanta and Phool(Konkan). TD an evergreen shrub 1.8-2.4 meter in height with silvery grey bark and milky latex; leaves are simple, opposite, elliptic or elliptic-lanceolate smooth, glossy green, acuminate and wavy margins; flowers are white sweety fragrant in 1-8 flowered cymes at the bifurcation of the branches, lobe of corolla overlapping to the right in the bud; fruits follicles are 2.5-7.5 cm long, ribbed and curved, orange or bright red within narrowed into a slender curved beak; seeds are dull brown, minutely pitted, irregular enclosed in a red plop aril [(8,9,10)]. Alkaloids, flavonoids, terpenoids, and tannins are the main secondary metabolite that possess many physiological and pharmacological properties on living cells [(9,10)].

The anti-oxidant study of different leaves extracts of *T. divaricata*(TD) ware done by DPPH (2,2 Diphenyl-1-Picrylhydrazyl) radical scavenging activity [(14,15,16).] Total phenolic content ware determined by Folin-Ciocalteu Method [(11,12,)] and Total flavonoid content ware determined by Aluminium Chloride Colorimetric method. [(11,12,13,14,15)] The wound healing activity was screened by using the Excision Wound Healing Model [(17,18,19)] of two different extracts methanolic extract and ethanolic extract using four different concentrations of extract ointment, *T. divaricata* 2% Mathanolic Ointment (TDM2), and *T. divaricata* 5% Methanolic Ointment (TDM5), same concentration was prepared for ethanolic extracts like *T. divaricata 2%* ethanolic ointment (TDE2) *T. divaricata*5% (TDE5) Same concentration was screened out for Anti-microbial [(3,31,32,)] and Anti-fungal activity(3,31,32,). The wound healing activity was done on ethanolic extracts TD [(31)] , and anti-microbial activity was

done [3] but in this study, we have tried to attempt the comparative study of two different extracts of TD leaves along with pharmacognostic evaluation, phenolic content, flavonoid content, *in-vitro* antioxidant study, In-vitro antimicrobial and anti-fungal activity and In-vivo wound healing activity. the wound healing through several cellular mechanisms, chelating of the free radicals and reactive species of oxygen, promoting contraction of the wound, and increasing the formation of capillary vessels and fibroblasts. Flavonoids are strong scavengers of reactive oxygen species that provide enabling support to the healing process initiated by the moderation of superoxide anions and later by enhancing the expression of vascular endothelial growth factors [32,33].

MATERIAL AND METHOD:

Collection and Authentication:

T. divaricata leaves were procured in the month of August 2017, from the local region of Nanded, Maharashtra state India. The plant ware authenticated by Taxonomist Dr. Shrirang Bodake, HOD, Dept. of Botany, Yashwant Mahavidyalaya, Nanded. The specimen no was H-4.

Processing of crude drug:

Fresh leaves of plant *T. divaricata* were subjected to shade drying and it was pulverized to a moderately coarse powder and then the powder is passed through mesh 14 and stored in airtight container for further study [9].

Pharmacognostic Evaluation [9]

Organoleptic properties:

Organoleptic properties of *T.divaricata* leaves was assessed for color, odour, taste, shape, and size.

Microscopic Evaluation:

Study of Transverse section of leaf: The microscopical study is the anatomical study which is done by taking an appropriate section of the plants under study. Free-handed sections of the leaf were cut into thin sections manually with the sharp cutting edge of the blade. Then transferred on the slide, cleared by warming with chloral hydrate, stained with phloroglucinol and Conc. HCl and mounted in glycerin[9].

Powder microscopy [9]

The powder microscopy was performed according to the method mentioned in Khandelwal [9].

Physicochemical Analysis:

Ash Value: Accurately weighed 2gm of the powdered drug was taken in a tared silica crucible. The crucible was supported on the pipe on a ring of retort stand. Heating was done with burner using a flame about 2cm, high and supported the dish about 7cm above cases to be evolved. This dish was cooled in desiccators. The total ash weighed and calculated the percentage of total ash [9].

Determination of Acid Insoluble Ash: Total ash was taken and boil for 5 min in 25 ml 1M HCL into a 100 ml beaker. Then filtered through an ashless filter paper, the residue was washed twice with hot water. The crucible ignited in the cool and weighed. The residue was kept in filter paper and put into a crucible. Heat gently until vapours to be evolved and then more strongly until all carbon was removed. The crucible was cooled in desiccators the residue was weighed and acid insoluble ash was calculated [9].

Determination of Water-Soluble Ash: Total ash was taken and boil for 5min in 25 ml water into a 100ml beaker. Then filtered through an ashless filter paper, the residue was wash twisely with hot water. The crucible ignited in the cool and weighed. The residue was kept in filter paper and put into a crucible. Heat gently until vapours to be evolved and then more strongly until all carbon was removed. The crucible was cooled in desiccators the residue was weighed and acid insoluble ash was calculated [9].

Loss on Drying: Loss on drying is a widely used test method to determine the moisture content of a drug, although occasionally it may refer to the loss of any volatile matter from the sample. About 1gm of the powdered drug was taken in a tared silica3crucible. The powder was heated at 105°C till the constant weight of the powder was obtain Crucible was kept in desiccators for cooling. The loss in weight is usually recorded as moisture [9].

Extractive Value: Weigh about 4g of the coarsely powdered drug in a weighing bottle and transfer it to a dry 250ml conical flask. Fill a 100 ml graduated flask to the delivery mark with a solvent (90 % alcohol). Wash out a bottle and pour the washings together with the remainder of the solvent into the conical flask. Cork the flask shakes and it well and to stand

for one hour. Shake well and filter rapidly through a dry filter. Filter into a 50ml cylinder. When sufficient filtrate has been collected, transfer 25ml of the filtrate to a weighed, thin porcelain dish. Evaporate to dryness for 12 hours. Cool in desiccators for 30 minute and weigh immediately. Calculate the percentage w/w of extractive value with reference to the air-dried drug [(9)].

Extraction of plant material:

Extraction of TD leaves was done by Soxhlet extraction method. Soxhlet apparatus was used for the solvent extraction and ethanol, as well as methanol, was selected as a solvent for extraction while petroleum ether was used for defatting of the waxy materials. 300 g of coarsely dried powder of leaves was first defatted separately with petroleum ether (50-60 ºC) for 48 hours to remove fatty materials and then extracted with ethanol using soxhlet apparatus for 72 hr at 60-80 ºC. After complete extraction, the extract was collected and dried at room temperature for 5 days, the percentage yield of extract was calculated and the dried crude extract was stored in airtight container at 2-8ºC for further study [(31)].

Preliminary Phytochemical Screening:

Preliminary phytochemical screening of all three extracts that is Petroleum Ether, Ethyl Acetate, and Methanol Extract was done. Preliminary phytochemical screening was done for the detection of phytoconstituents such as carbohydrates, glycosides, tannins, terpenoids, phenols, flavonoid, steroids, proteins, amino acids, etc. [(9)].

Chromatographic Fingerprinting:

A slurry of silica gel G was prepared in *distilled* water and poured over a glass plate to form a thin layer. The prepared plates were air-dried for setting and then kept in an oven at 100-120°C (30min) for activation. The extracts were dissolved in respective solvents and spotted over an activated plate (1cm above from the bottom). The spotted plates were kept in a previously saturated developing chamber containing mobile phase, and allowed to run 3/4th of the height of the prepared plate 20. The plates were air-dried number of spots were noted and Rf values were calculated. Spots were visualized by respective spraying agents. Numbers of solvents systems were tried but the maximum resolution was shown in benzene: chloroform: Ethyl acetate and benzene: chloroform and spraying agent was used H2SO4 for ethyl acetate and methanol extracts. Rf values calculated [(35)].

Formula:

$$Rf = \frac{\text{Distance Traveled by Solute}}{\text{Distance Traveled by Solvent}}$$

Total Phenolic Content:

Folin-Ciocalteu Method

Total phenolic content was analyze by Folin –Ciacaltue Colorimetric method. A standard gallic acid (5,10,15,20 and 20 µg/ml) solution was prepared in methanol and a 100 µg/ml stock solution of test sample (plant extract) also prepared. The test sample was prepared by adding 0.3ml of distilled water, 0.4ml of Folin – Ciacaltue reagent was added and the sample allowed to stand for 6min before adding 4ml of 7 % sodium carbonate (Na2CO3). The water was used to adjust the volume up to 10ml. After incubated for t 90min, the absorbance was recorded at 765nm. Reference curve were prepared using (5-25 µg/ml) of gallic acid and results were prepared are presented at the amount of phenolic content (Gallic acid Equivalent) per dry weight. The blank solution was prepared using 0.4ml of Folin-Ciacaltue reagent, 4ml of 7 % of sodium carbonate, and water was used to adjust the volume up to10ml [11,12,13,14,15] .

Total Flavonoid content:

Aluminium Chloride Colorimetric Method.

Flavonoid determination was done using the aluminium chloride colorimetric method. 100 µg/ml of quercetin stock solution was prepared aliquots of 5,10,15,20 & 25 µg/ml using distilled water. Test sample (plant extract) were prepared by mixed with 4ml distilled water,0.30ml of 5 % NaNO2 was added and allowed to stand for 5min, then added 2ml of 1M NaOH, and water was used to adjust the volume up to 10ml. Absorbance was taken at 330nm against blank. The development of yellow colour indicates the presence of flavonoid content and results are expressed as the amount of (Quercetin Equivalent QE) per dry weight. Blank solution was prepared using 0.30ml of 5 % (NaNO2) and water were used to adjust the volume up to 10ml [11,12,15].

Anti-oxidant Study:

The free radical scavenging activity of plant extract was estimated according to DPPH radical scavenging activity.1000µg/ml of stock solution of DPPH was prepared in methanol. The four standards was taken as BHT, ascorbic acid, gallic acid, and ellagic acid. Prepared the aliquots of 5,10,15,20 & 25µg/ml of ascorbic acid in methanol. And plot the calibration curve. The test solution (plant extract) was prepared by using 0.5ml freshly prepared DPPH reagent was added into 2.5ml (5,10,15,20 & 25ml) of the test sample. The control solution was prepared by using 0.5ml DPPH and 2.5ml methanol. The decrease in absorbance at 514nm was recorded after 30min at room temperature and the percentage of DPPH radical scavenging ability was calculated from absorbance value at 30min duration by using formula [14,15,16].

Formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance (0min)} - \text{Absorbance (30min)}}{\text{Absorbance (0min)}} \times 100$$

Preparation of Ointments by Fusion Method

Preparation of 2 % Ethyl acetate (TD) extract (100g)

2g of ethyl acetate leaves extract of *T. divaricate* was added slowly to the 98g of simple ointment bases and stirred thoroughly until the mass cools down and a homogenous product is formed. The ointment was packed in wide mouth container.

Preparation of 5 % Ethyl acetate (TD) Extract (100g)

5 g of ethyl acetate leaves extract of *T. divaricate* was added slowly to the 95 g of simple ointment bases and stirred thoroughly until the mass cools down and a homogenous product is formed. The ointment was packed in a wide mouth container.

Preparation of 2 % methanolic extract (TD) ointment (100g)

2g of methanolic leaves extract of *T. divaricate* was added slowly to the 98 g of simple ointment bases and stirred thoroughly until the mass cools down and a homogenous product is formed. The ointment was packed in a wide mouth container.

Preparation of 5 % methanolic extract (TD) ointment (100g)

5g of methanolic leaves extract of *T. divaricate* was added slowly to the 95 g of simple ointment bases and stirred thoroughly until the mass cools down and a homogenous product is formed. The ointment was packed in wide mouth container.

Pharmacological Screening of Plant extract:

1) *In- Vitro* antimicrobial activity

Agar and Cup Plate Method [36]:

The antimicrobial activity was measured by the agar cup method. Nutrient agar (Himedia) was prepared and sterilized at psi 15 minutes in the autoclave. It was allowed to cool below 45°C and seeded turbid suspension of test bacteria separately, prepared from 24 hours old slant culture. 3 % inoculum was use every time. The bacterial culture selected were, two gram-positive and two gram-negative cultures *viz.Echerichia coli, Salmonella typhi* and two gram positive culure *viz. Staphylococcus aureus, Bacillus subtilis*. This seeded preparation was then procured in a sterile Petri plate under aseptic condition and allowed to solidify.

Cup of 10mm diameter bordered in the agar plate with a sterile cork borer. 100µl of a compound solution prepared in Dimethyl Sulphoxide (1%) was added to cup under the aseptic condition with the help of a micropipette. 100µl of DMSO was placed in the cup as blank (Negative Control). A standard antibiotic disk impregnated with 10units of Penicillin was also placed on the seeded nutrient agar surface as a standard reference antibiotic (Positive control).

The plates were kept in refrigerator for 15minute o allow diffusion of the compound from the agar cup into the medium. Then the plates were shifted to the incubator at 37^0C and incubated for 24hours.

After incubation plates were observed for the zone of inhibition of bacterial growth around the agar cup. Results were recorded by measuring the zone of Inhibition in millimeters (mm) using zone reader [3,36].

2) Anti-Fungal Activity

Poison Plate Method [36]:

Antifungal activity was performed by Poison Plate Method. The medium used was Potato Dextrose Agar (Himedia). The medium was prepared and sterilized at 10Psi in autoclave for 15minutes. Then the compound to be tested is added to the sterile medium in aseptic condition get the final concentration 1 %. A plate with DMSO was prepared as blank (negative control) similarly a plate with 1 % Gresiofulvin was prepared as a standard reference plate (positive control). Aspergillus niger, Penicillin chrysogenum, Fusiriummoneliforme, and Aspergillus were selected as test fungal culture. They were allowed to grow on a slant for 48 hours to get perfuse sporulation. 5mi of 1:100 aqueous solution of Tween 80 was added to the slant and spores were scraped with the help of nichrome wire loop form suspension. The fungal suspension was spot inoculated on the prepared using compound with the help of the nichrome wire loop. The plates were incubated at room temperature for 48hours. After incubation plates were observed for the growth of inoculated fungi. Results were recorded as the growth of fungi (no antifungal activity), reduced growth of fungi (moderate antifungal activity), and no growth of inoculated fungi (antifungal activity)[36].

In-vivo Wound Healing Study:

Excision Wound Healing Model[17,18,19]

In vivo wound, healing activity study of *T. divaricate* leaves extracts was carried out using Excision wound healing model.

IACE Approval:

Male and female rats weighing 150-220g were used in the present study. The experimental animals were maintained under standard laboratory conditions in an animal house approved by the committee for the purpose of control and supervision on experiments on animals (CPCSEA) under light/dark cycle and controlled temperature (24 ± 2^0C) and feed with commercial diet and water *ad libitum*. The experimental protocol was approved but the Institutional Animal Ethics Committee, Nanded Pharmacy College, Nanded, And Maharashtra India.

Animals used: Albino Rats

Weight: 150-220

Routes of administration: Topical

Housing Condition: Animals were housed in a group of seven in separate cages under controlled conditions of temperature (22 ± 2^0C). All the animals were given a standard diet and water regularly.

The wound is defined as disruption of the cellular and anatomic continuity of tissue, with or without microbial infection, and is produced due to any accidents or cut with sharp-edged things. Some diseases like diabetes, ischemia, and conditions like malnourishment, local infection, and local tissue damage due to burn o delay in wound healing.

Excision wound model

The excision wound model was the 21days study and the animals were anesthetized by using ketamine (50mg/kg i.e.) and with the help of Veet (Hair removing cream) hairs on the dorsal thoracic region of rats were removed. The excision wound was create 5cm away from the ear, a circular wound with approximately 500mm^2 area and 2mm depth was excised on depilated dorsal thoracic region of excised rats. The entire wound was left open. And the treatment was done topically in all the cases. The wound was measured by scale wound contraction measured by day-to-day progress for all groups observed for completely healing of the wound.

Grouping of Animals

Animals was divided in seven groups, each group consisting of six rats.

Experimental Design:

The following were considered for the study, each group containing six animals.

Table No. 1: Animal grouping for the study *In-vivo* wound healing activity

Group No.	Group	Treatment(Topical application)
I	Standard	Soframycin Ointment
II	Positive control	Plane ointment base
III	Negative control	Non treated
IV	Ethyl acetate extract (TD)	2 % E.A ointment
V	Ethyl acetate extract (TD)	5 % E.A ointment
VI	Methanol extract (TD)	2 % Me. Ointment
VII	Methanol extract (TD)	5 % Me. Ointment

Wound healing activity:

***In vivo* Method**

In vivo wound, healing activity study of *T. divaricate* leaves was carried out using Excision wound healing model.

Forty-two male Wistar rats (200-220g) of approximately two months of age were used as experimental animal. The animal was divid in 7 groups of rats viz Positive control, Negative control, Standard drug-treated, and four extract-treated groups. The animal was housed in standard environmental conditions of temperature, humidity,12hrs light/dark cycle. During the experiment, the rats were administered a standard pellet diet and water *adlibitum.* All the animals were anesthetized with ketamine (50mg/kg i.e.). The back of the rat was depilated by careful shaving. The excision wound was created 5cm away from the ear, a circular wound of approximately 500mm2 area and 2mm in depth. The wound will be left open. The extract and the vehicle ointment were applied topically. The wound will be completely healed. The progressive changes in the wound area were monitored by a camera every other day. At the end of the treatment wound contraction was calculated as a percentage of the reduction in the wounded area.

Statistical analysis: Presentation of results was done in tabular form. All results expressed as mean ± Standard Error. The results were expressed as mean ±S.E.M. Data was analyze by one-way ANOVA test.

RESULTS AND DISCUSSION:

Morphological Characters: Leaves are simple, colour is dark green, odour characteristic.

Morphological Characters: Leaves are simple, color is dark green, odor characteristic, taste acrid, and shape is lanceolate**.** Size varies from 4-6 inches wide narrowed towards the base.

Microscopical Evaluation: The T.S. of the leaf was shown lower epidermis, upper epidermis, vascular bundles (xylem and phloem), calcium oxalate crystal, palisade, stomata, and cuticle.

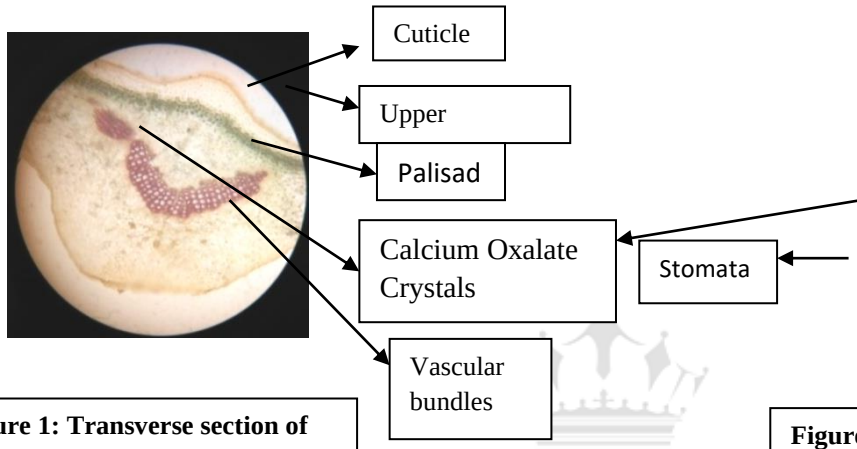


Figure 1: Transverse section of TD leaf

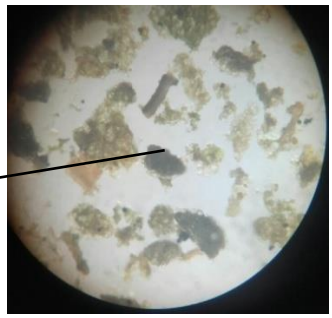


Figure 2: Powdered microscopy of TD leaf

Table No.2: Phytochemical Screening of Extracts

Sr.No	Test For	Petroleum Ether	Ethyl Acetate	Methanol
1.	Alkaloids	+	+	+
2.	Carbohydrate	-	+	+
3.	Glycosides	-	+	+
4.	Flavonoids	+	+	+
5.	Proteins	-	+	+
6	Fats and Oils	+	+	+
7	Tannins & Phenols	-	+	-
8	Saponin Glycosides	+	+	+

Table no. 2 shows the phytochemical screening of TD extracts it showed the presence of alkaloids, carbohydrate, glycosides, flavonoids, protiens, falts and oils, tannins, phenols and saponin glycosides**.**

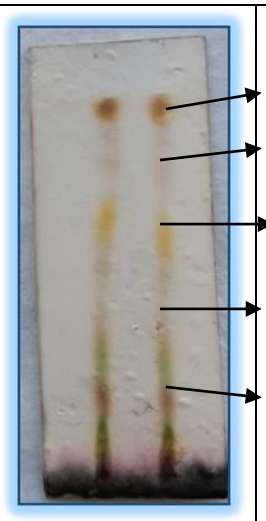
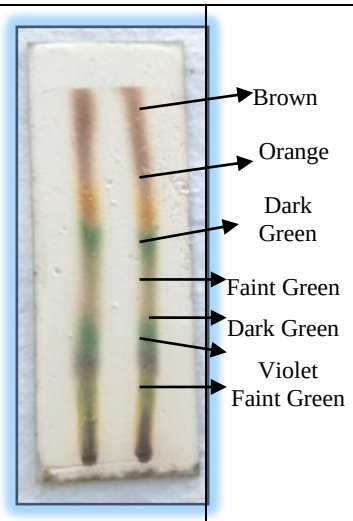
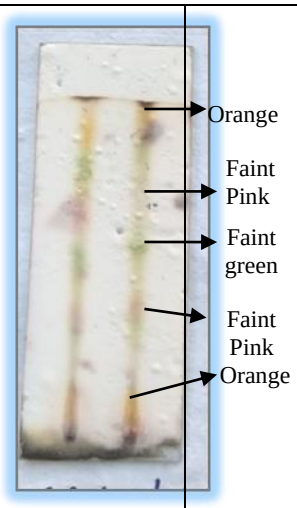
Table No.3: Physicochemical Evaluation

Sr. No	**Parameters**	**Values**
1	**Ash Value**	
a.	Total Ash	**7.1%**
b.	Water-soluble ash	**4%**
c.	Acid insoluble ash	**5%**
2	**Extractive Value**	
a.	Chloroform	**16.5%**
b.	Methanol	**38.5%**
c.	Ethanol	**41.5%**
d.	Ethyl Acetate	**40.5%**
E	Water	**10%**
3	**Loss on drying**	**8%**

Table no. 3 shows the physicochemical evaluation of TD leaves showed the significant results. Physical parameters like ash value, total ash, water-soluble ash and acid insoluble ash was determine. Extractive values was determine using various solvents like chloroform, methanol, ethanol, ethyl acetate and water. And loss on drying also determined.

Chromatographic Fingerprinting: Table no. 4 shows confirmation of phytoconstituents with the help of TLC fingerprinting of all three extracts was done by using various concentration of mobile phase and Rf values was alculated**.** The various phytoconstituents was found by TLC fingerprinting like flavonoid glycosides,6 Hydroxy flavons, tannins, steroids, etc.*T. divaricata* leaf contain a largeamount of phytoconstituents.

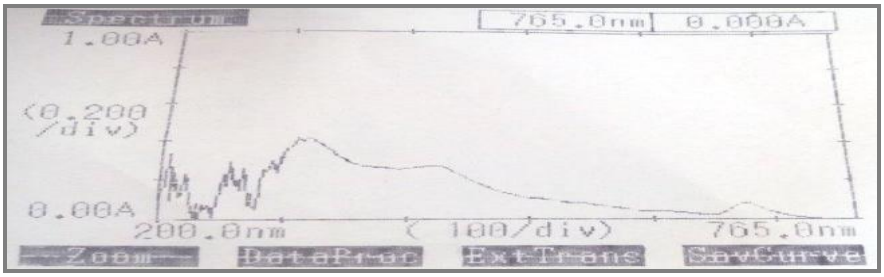
Table No.4: Chromatographic Fingerprinting of extracts

TLC for Petroleum Ether	**TLC for Ethyl Acetate**	**TLC for Methanol**
		
Solvent System: Benzene+ Chloroform + Ethyl Acetate :4:0.5:0.5	Benzene+ Chloroform+ Ethyl Acetate :4:0.5:0.5	Benzene + Chloroform+ Ethyl Acetate :4:0.5:0.5
Spraying Agent: H_2SO_4		
Rf Values: 0.78, 0.65, 0.54, 0.3, 0.28,0.24, 0.10	**:**0.87, 0.8, 0.7, 0.6, 0.5, 0.3,0. 18, 0.10	0.9, 0.8, 0.7, 0.650.5,0.3,0.1,0

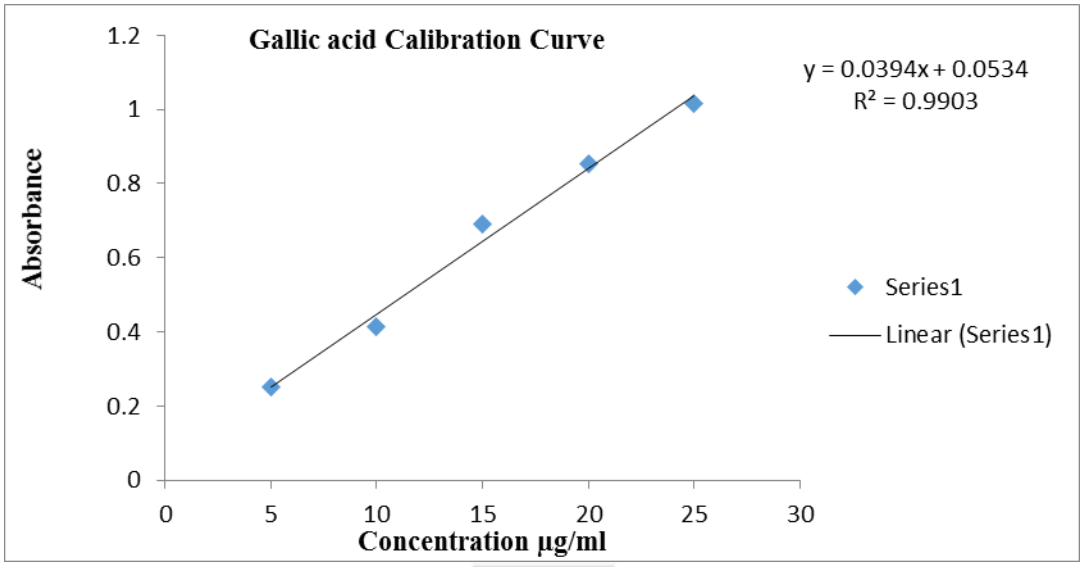
Phenolic Content:

Folin-Ciocalteu Method

λmax refer to the wavelength in the absorption spectrum where the absorbance is maximum. Generally, the molecule absorbs in a wavelength range centered on the λmax. It acts as a single quantitative parameter to compare the absorption range of different molecules. The colored solution of the sample shows the maximum absorbance at 765nm. And calibration curve was determined of standard solution. A calibration curve is a standard curve, it is a general method for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of a standard sample of known concentration.



λ max Determination for Gallic acid



Graph No.1. Calibration Curve for Gallic Acid

Table No.5: Phenolic Content of Standard

Sr.No	Conc. (µg/ml)	Absorbance	Phenolic Content of Standard(µg/ml)
1.	**5**	**0.250**	**6.7872**
2.	**10**	**0.414**	**9.1440**
3.	**15**	**0.690**	**15.236**
4.	**20**	**0.852**	**18.825**
5.	**25**	**1.016**	**22.435**

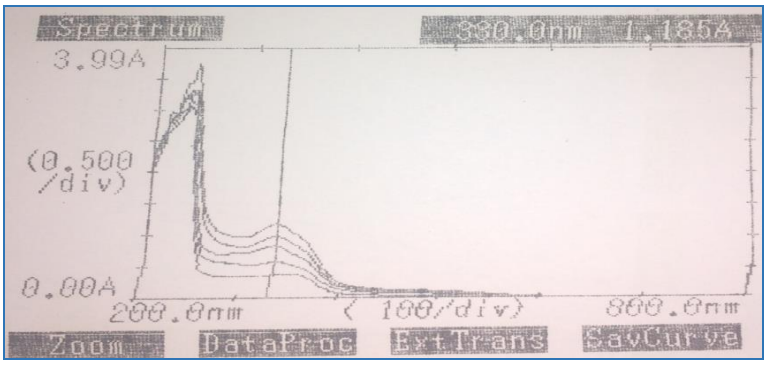
Table No. 6: Note: Data expressed as (±) SD (n=3). (µg GAE/ µg DW denote Gallic acid equivalent per dry weight of extract

SrNo	Conc. µg/ml	λmax(nm)	Extract	Absorbance	Total phenolic content
1	100	765	Petroleum Ether	1.653	39.07 ± 0.008
2	100	765	Ethyl Acetate	1.838	43.42± 1.45
3	100	765	Methanol	2.051	48.46 ±0.01

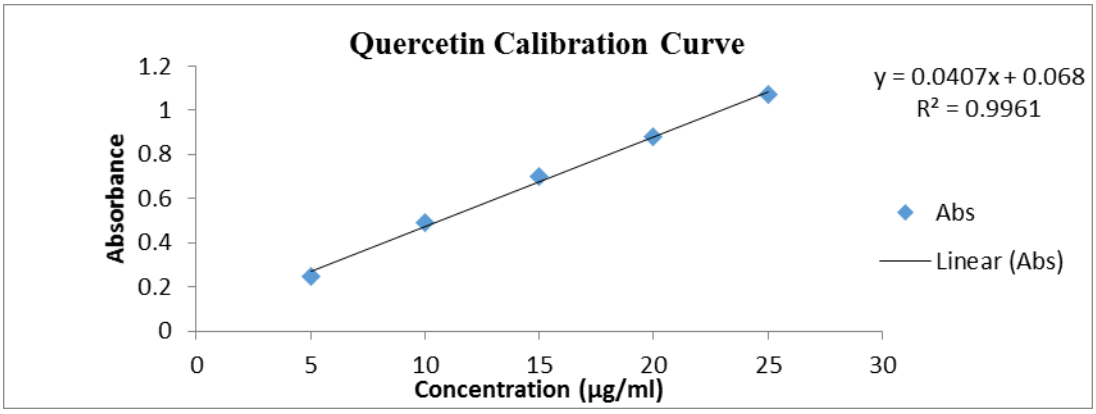
Above table no.5 shows the phenolic content of the standard and table no.6 shows the test sample. The standard drug shows the maximum phenolic content at low concentration. In the case of the test sample, Methanolic extracts showed the maximum phenolic concentration**48.461 ±0.015** as compared to the ethyl acetate extract and petroleum ether extract. The concentration of the test sample was taken 100µg/ml because herbal drugs contain maximum adulterant substances. At low concentration, it was not shows the proper results.

Flavonoid Content:

Aluminium Chloride Colorimetric Method.



λ max Determination for querecetin



Graph No.2: Calibration Curve for Querecetin

Table No.7: Flavonoid Content for Quercetin (µg/ml)

Sr. No	Conc. (µg/ml)	Flavonoide Content For Quircetin (µg/ml)
1.	5 µg/ml	0.370
2.	10 µg/ml	0.552
3.	15 µg/ml	0.729
4.	20 µg/ml	0.871
5.	25 µg/ml	1.039

Table No.8: Note: Data expressed as (±) SD (n=3). (µg GAE/ µg) denote Galic acid equivalent per dry weight of querecetin

Sr. No	Conc. (µg/ml)	Extract	Absorbance	Flavonoide Content (µg GAE/ µgDW)
1.	100µg/ml	Flavonoide Content Of P.E	0.625	14.062±0.01
2.	100µg/ml	Flavonoide Content Of E.A	0.698	43.427±0.00
3.	100µg/ml	Flavonoid Content Of Methanol	0.816	48.461±0.00

Table no. 8 shows the total flavonoid content of T.D extract varied from43.427±0.00 to 48.461±0.00 Methanolic extract shows the maximum flavonoid content as compared to petroleum ether and ethyl acetate extract.

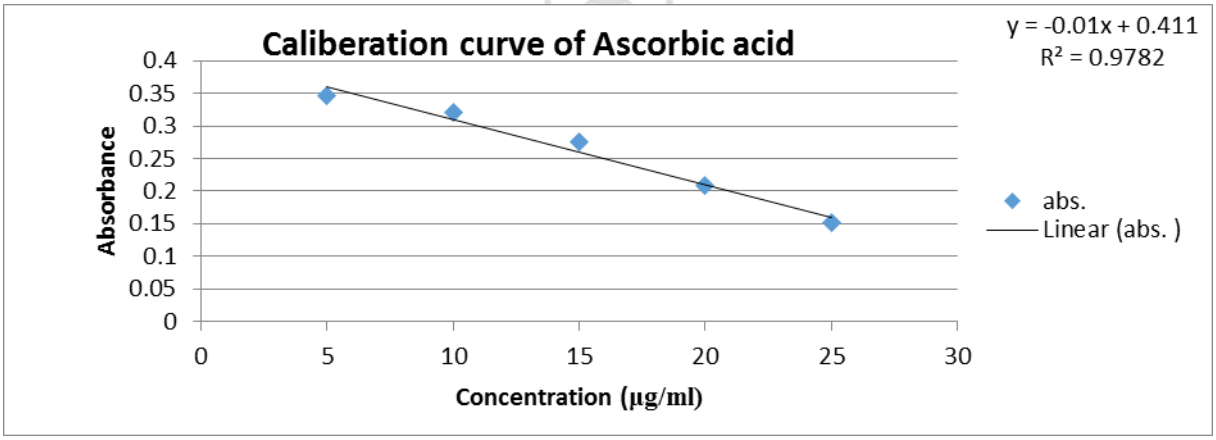
Antioxidant Activity:

DPPH Radical scavenging activity of different standards ware used.

Table No.9: Data expressed as mean ± SD (n=3). µg GAE,EA,AA and BHT/ µg DW per dry weight

Sr. No	Conc.	DPPH	λ max	Ellagic acid	Ascorbic acid	BHT	Gallic acid
1	5 µg	0.5	514	65.99±0.02	69.50±0.00	88.13±0.01	93.67±0.01
2	10 µg	0.5	514	68.36±0.01	71.88±0.01	89.89±0.01	93.93±0.01
3	15 µg	0.5	514	69.15±0.02	75.83±0.01	93.67±0.01	94.11±0.01
4	20 µg	0.5	514	71.96±0.01	81.71±0.01	94.63±0.01	95.25±0.03
5	25 µg	0.5	514	76.88±0.01	86.64±0.01	94.99±0.01	95.34±0.02

Calibration Curve for Ascorbic Acid



DPPH Radical scavenging activity of *Taernaemontana divaricate* Extracts

Table No: 10: Note: Data expressed as mean ± SD (n=3). µg GAE/ µg DW denote the gallic acid equivalent to microgram per dry weight of the sample

Sr.No	Extract	Conc.(µg/ml)	λmax(nm)	% Inhibition (µg GAE/ µg DW)
1	**Pet .Ether**	100	514	74.87±0.00
		50	514	54.77±0.01
2	**Ethyl Acetate**	100	514	62±002
		50	514	59±0.03
3	**Methanol**	100	514	81±0.02
			514	75.5±O.3

Table no.10 shows the antioxidant activity of *T. divaricate* leaves extracts of Petroleum ether, Ethyl acetate, and methanolic extracts by DPPH assay gives essentially significant results. This assay found the concentration-dependent. The reducing power assay revealed a promising amount of compound with reducing power. This may be due to the biologically active compounds present in the extracts indicating that they are electron donors which act as primary and secondary antioxidants. The above table shows the methanolic extracts showed the maximum reducing power, Petroleum ether, and ethyl acetate extracts also show satisfying results. Ascorbic acid is a very good oxidizing agent so ascorbic acid ware taken as a standard. The test sample shows the result in the range of 78.87±0.00 to 75.5±0.3.

***In-Vitro* Antimicrobial Activity**

Agar Cup and Plate Method:

The abbreviations used during the study are

TDEA = Ethyl acetate of TD at concentration of 100mg/ml

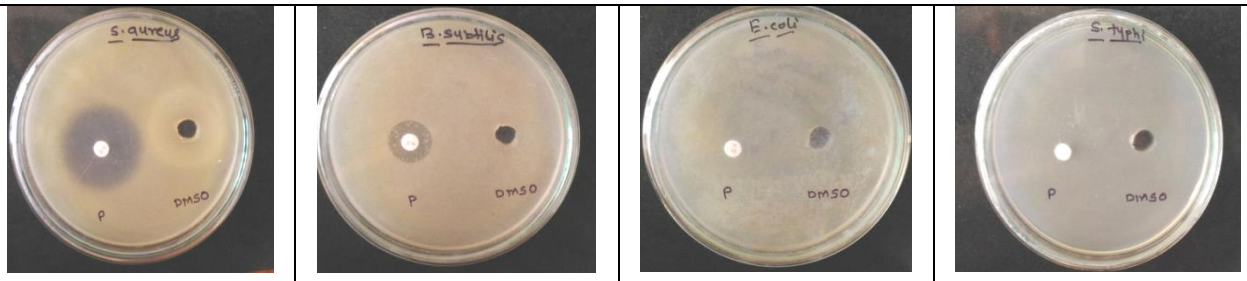
TDEA = Ethyl acetate of TD at concentration of 50mg/ml

TDME = Methanolic extract of TD at concentration of 100mg/ml

TDME = Methanolic extract of TD at concentration of 50mg/ml

Table No.11: Antimicrobial Activity of Standard Drug(Penicillin) and different concentrations of *Tabernaemontana divaricata* leaves extracts

Zone of inhibition of reference standard (*Penicillin*)



Zone of inhibition of *Tabernaemontana divaricata* leaves extract at different concentration

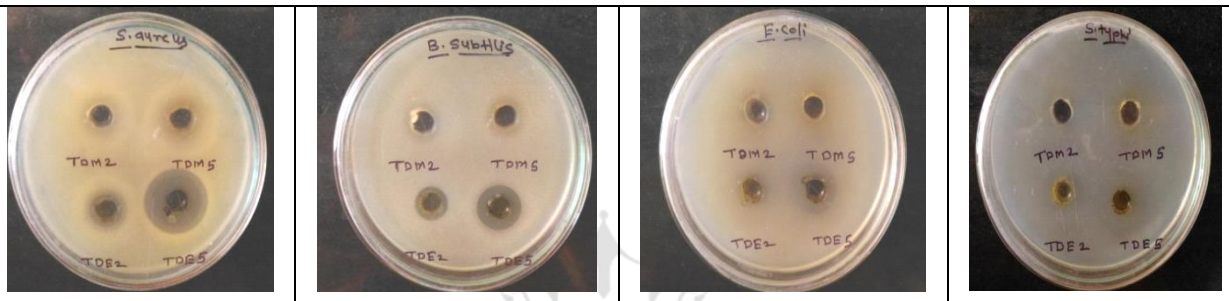


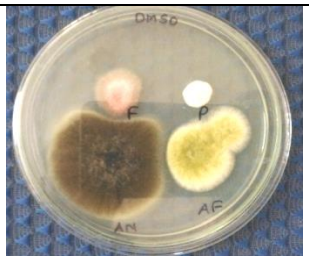
Table No: 12 Legends: - -ve no Antibacterial activity Zone of inhibition:- in mm

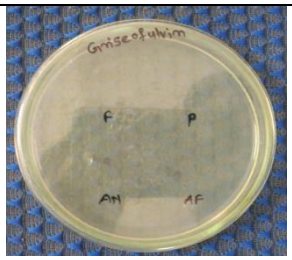
Sr.No	Dose (mg/ml)	Compound	*Escheriaa coli*	*Salmonella typhi*	*Staphylococus aureus*	*Bacillus subtilis*
1	50	TDM 2	12mm	-ve	-ve	12mm
2	100	TDM 5	12mm	12mm	-ve	12mm
3	50	TDE 2	12mm	16mm	-ve	14mm
4	100	TDE5	12mm	**21mm**	-ve	**19mm**
5	100	DMSO	-ve	-ve	-ve	-ve
6	100	Penicillin	11mm	24mm	-ve	30mm

Table no. 12 shows the results show both the extracts have moderate to good antibacterial activity when tests against Gram –ve strain. The maximum zone of inhibition was shown in TDE5 (ethyl acetate extract at concentration 100g/ml) i.e. 21mm against Salmonella typhi bacterial strain.

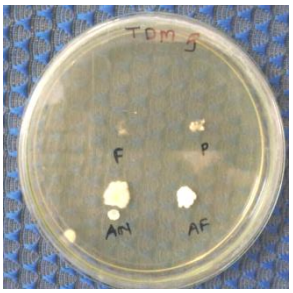
Antifungal Activity

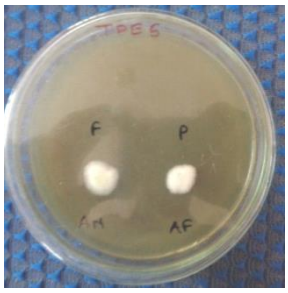
Effect of reference standard drug (*Griseofulvin*)

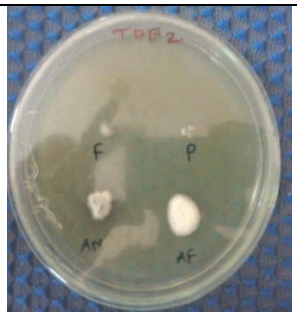




Zone of inhibition of *Tabernaemontana divaricata* leaves extract at different concentration Of *Tabernaemontana divaricata* leaves extracts







Anti-fungal activity of reference standard (*Griseofulvin)* and different concentrations

Table No: 13 Legends: +ve – Growth (Antifungal activity absent)-ve - No Growth (More than90 % reduction in growth Antifungal activity present)RG - Reduced growth (More than 50 % and less than 90 % reduction in growth observed)

Sr.No	**Dose**	**Compound**	***Penicillin chrysogenum***	***Fusarium moneliforme***	***Aspergillus flavus***	***Aspergllus sniger***
1	50	TDM 2	-ve	-ve	+ve	+ve
2	100	TDM 5	-ve	-ve	-ve	RG
3	50	TDE 2	-ve	-ve	-ve	RG
4	100	TDE 5	-ve	-ve	RG	RG
5	50	DMSO	+ve	+ve	+ve	+ve
6	100	Greiseofulvin	+ve	-ve	-ve	-ve

Table no. 13 shows the in an antifungal activity as well, the methanolic extract showed reduced antimicrobial activity potential ant of fungal strain. Therefore, we conclude that

results the methanolic extract (TD 5) of *T. divaricate* **leaves extract possess potential antimicrobial activity against the bacterial and fungal strain.**

Pharmacological evaluation of *Tabernanaemontana divaricate* leaves extract

Acute oral toxicity study:

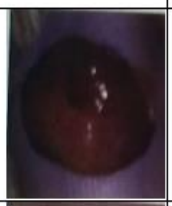
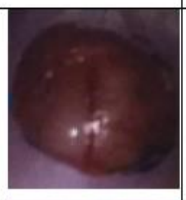
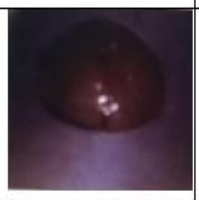
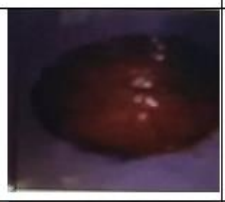
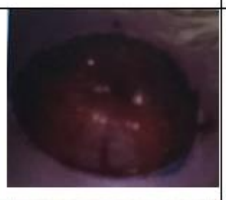
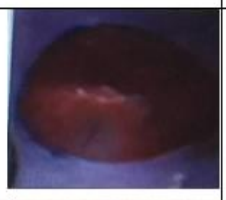
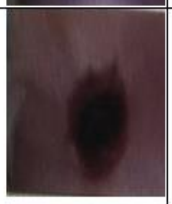
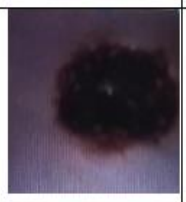
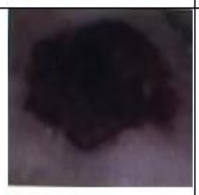
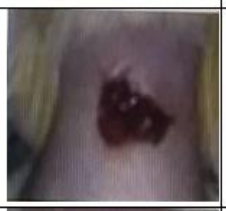
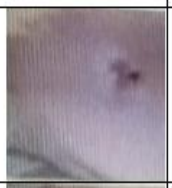
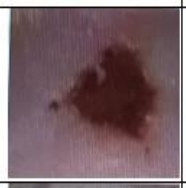
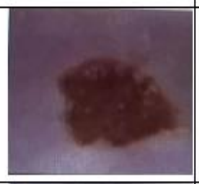
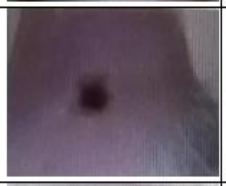
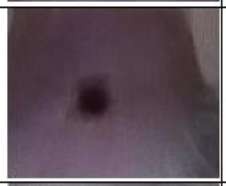
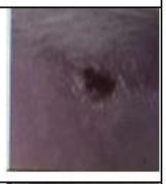
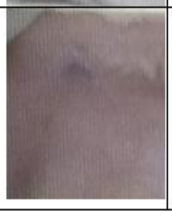
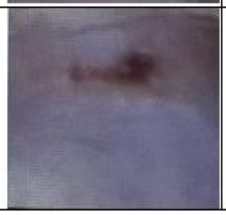
Acute oral toxicity studies conducted that the administration of graded doses extract (up to a dose of 2000mg/kg) of extract did not produce significant changes in behaviours such as alertness, motor activity, breathing, restlessness, diarrhoea, convulsion, coma, and appearance of the animals.

No death was observed up to a dose of 2000mg/kg body weight. The rat was physically active. These effects were observed during the experimental period (72hrs).

The results indicate that acute single oral treatment of extract at a dose of 2000 mg/kg did not produce any sign of toxicity or death in the rat during observation.

The results showed that in a single dose; the plant extracts had no adverse effect, indicating that the median lethal dose (LD_{50}) could be greater than 200mg/kg body weight in the rat.

Table No 14: Evaluation of wound healing activity of *Tabernaemontana divaricate* leaves extract

Day	Standerd	Positive Control	Negative Control	2% TDM	5% TDM	2% EA	5 % EA
0 Day							
7 Day							
14 Day							
21 Day							

Excision wound Model

Excision wound Model

Table No: 15 Evaluation of wound healing activity of TD leaves extract Data expressed as mean ± SD (n=3). µg GAE, EA, AA and BHT/ µg DW per dry weight N=**6; Significant difference when standard and test compared with the positive control (P<0.05); ** when test compare with the positive control (P< 0.001); # No significant difference when test compared with the standard; $ Significant difference when compare standard but activity more than standard**

Groups **Days**	**Wound area (cm) post wounding days**			
	0 Day	**7 Day**	**14 Day**	**21 Day**
Group –I(Standard)	2±0.00	1.51±0.07	1.31±0.007	0.03±0.08
Group – II(Positive control)	2±0.00	1.65±0.009	1.31±0.009	1.07±0.007
Group - II(Negative control)	2±0.00	1.83±0.009*#	1.62±0.007*	1.25±0.007
Group - IV(TDM 2 %)	2±0.00	1.76±0.009*$	1.10±0.01*#	0.19±0.007**
Group – V (TDM 5 %)	2±0.00	1.66±0.01**$	0.55±0.009**	0.09±0.16**$
Group –VII(TDEA 2%)	2±0.00	1.71±0.01*$	1.18±0.11**	0.29±0.01**#
Group – VIII (TDEA 5 %)	2±0.00	1.60±0.01*$	0.66±0.01**$	0.39±0.001**$

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Table no 15. shows data obtained was presented as mean ± standard error of the mean (S.E.M) for the number of animals in each group (n=6). The groups were compared using one-way analysis of variance (ANOVA), P<0.05 was considered significant difference when standard and test compared with positive control and negative control in excision wound model and P<0.001 was considered highly significant difference when test compared with positive control in excision wound model.

On 7 days, the group of animals treated with standard ointment i.e.Soframycin showed no significant decrease in wound area when compared with the positive control group. The test group i.e., TDM 2%, TDM 5 %, TDEA 2 %, and TDEA 5 % showed a significant decrease

(P<0.05) in the wound area when compared with the positive control group. The test group showed a significant decrease in wound area as compared with the standard.

On 14 days, the group of animals treated with standard ointment i.e., Soframycin showed a highly significant decrease in wound area when compared with the positive control group. The test group i.e., TDM 2%, TDM 5 %, TDEA 2 %, and TDEA 5 % showed a highly significant decrease (P<0.001) in wound area except TDM 5 % showed a significant decrease (P<0.05) in wound area when compared with the positive control group. The test group showed no significant change in wound area as compared with the standard.

On 21 days, the group of animals treated with standard ointment i.e., Soframycin showed a highly significant decrease in wound area when compared with the positive control group. The test groups. TDM 2%, TDM 5 %, TDEA 2 %, and TDEA 5 % showed a highly significant decrease (0.001) in wound area when area compared with the positive control group. The test group showed no significant change in wound area when compared with the standard.

From the above observations, it was found that the *T. divaricata* leaf extract possess a significant wound healing activity, and TDM 2% and TDM 5 % showed a highly significant decrease in wound area when compared with positive control and significant difference when compared with standard. TDME 5 % extract showed a significant difference in wound contraction.

CONCLUSION:

Standardization is an important tool for the assessment of herbal drugs for establish their identity, purity, safety, and quality. In order to standardize a drug, various macroscopic, microscopic, ash value extractive value, acid insoluble ash, water-insoluble ash. The microscopic method is one of the cheapest and simplest methods to start with establishing the correct identification of the source material. Morphological and microscopical studies of the leaf will enable us to identify the crude drug. Values help in the evaluation of the purity of drugs. The information obtained from the preliminary phytochemical screening will be useful in finding out the quality of the drug. Morphological and microscopic studies was helpful for detecting adulteration. These studies will help for a selection of manufacturing drugs. The present study showed the *T. divaricata*. Phenolic content of T.D shows in the range of 39.077±0.08 to 48.46±0.01. And flavonoid content shows the result in the range

between 39.07±0.01 to 48.46±0.00 and anti-oxidant results showed the results in the range of 78.87±0.00 to 75.5±0.3. Phenolic, flavonoid, and antioxidant show satisfactory results showed the maximum amount of phenols, flavonoid, and radical scavenging activity.

Acknowledgement:

We provide our sincere thanks to the entire faculty of Department of pharmacy and my friends.

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