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Phytochemical and antibacterial properties of *Tabernaemontana divaricata* L. against ocular infection

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Abstract
In the present investigation suggested that the phytochemical and antibacterial properties of *Tabernaemontana divaricata* L. against ocular infection were performed The analysis of phytochemical constitutents such as alkaloids, carbohydrate, flavonoids, glycosides, phenol, saponin, steroid and tannin were estimated with four different solvents of aqueous, ethanol, hexane and acetone of *T.divaricata* flowers extract, Qualitative and Quantitative phytochemical concentration were determined with respective plant. Effect of antibacterial activities of *T.divaricata* flowers extract of different concentration of 25, 50, 75 and 100µl were used. The antibacterial activity of *T.divaricata* against used ocular infecting bacteria are *Bacillus* sp, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella* sp., *Pseudomonas* sp, *Streptococcus* sp and *Staphylococcus aureus.* Maximum antibacterial properties at 100 µl concentration of test plant flowers extract against ocular infecting bacteria.

Keywords: *Tabernaemontana divaricata* L, ocular infecting bacteria, traditional medicine

Introduction

Medicinal plants have been used for centuries as remedies for diseases. They are enriched with antimicrobial agents which are biologically active chemical molecules. Recently plant derived compounds have become a great interest because of their various applications (Baris 2006) [2]. Traditional medicinal system Ayurveda practices drug derived from plant parts. It has been estimated that 28% of higher plant species are used medicinally (Ncube 2008) [16] plants depends on the presence of some phytochemical constituents. Plants have endless ability to synthesize aromatic secondary metabolites, most of which are phenols or their oxygen substituted derivatives (Geissman 1963) [9]. These phytochemical substances in the medicinal plants can generate physiological consequences in the human body system (Rajendra Prasad 2013) [20]. There are two forms of phytochemicals. Primary metabolites are aminoacids, proteins, sugars and Chlorophyll, Secondary metabolites comprises of phenols, alkaloids, flavonoids, terpenoids, tannins, saponins and glycosides (Kiritikar 1987) [13]. *Tabernaemontana divaricata* known as crapejasmine (English), Nandivardhanam (Telugu). Chandani (Hindi), Nandiyavattai (Tamil), it has dichotomous branch containing herb, or small tree is extremely dispersed all over in India, Bangladesh and Elements of South East Asia. In India one of the ornamental as well as shrine plants is *Tabernaemontana divaricata* L. It belongs to Apocynaceae family. It gorgeous white – coloured aroma flowers and may become visible intermittently during the year. The flowers are sleek, hefty and deep green colour.

The bioactive compounds of phenols, phenolic acid, quinones, flavones, tannin and coumarine were most commonly presented in the extraction of plants. These group of compounds showed that antimicrobial effect and serve as as plant defense mechanism against pathogenic microorganisms. Flavones, flavonoids and flavonols are phenolic structure with one carboxyl group. They are synthesized by plants in response to microbial infection. (Dixon 1983) [6]. Studies reported the antimicrobial activities of flavones (Bennet 1994) [3]. Totally 66 alkaloids including the enzymes, pyrolytic oil, hydrocarbons, terpenoids and phenolic acids are also present (Ankita Kulshethra 2019). *T.divaricata* was first described by Linnaeus in 1753 *T.divaricata* has four typical charecteristics including ever green shrub forms shaped like symmetrical rounds 6-feet height, horizontal branches having the appearance of an attractive, appearance almost horizontal shrub, large shiny, deep green leaves, 6 or more inches length and 2 inches wide waxy blossoms with white, five petals pinwheels, gathered in small clusters on the stem tips. *T.divaricata* has been used in traditional medicine and for other purpose. (Kalaimagal 2015) [11]. The secondary metabolites within the genus have demonstrated huge medicinal potential for the treatment of infections, pain, injuries, and various diseases. (Clarissa Macella Naidoo *et al* 2021) [17].

Chemical constituents of *Tabernaemontana divarcata* are indole alkaloids. Alkaloids are the organic products of natural or synthetic origin which are basic in nature and contain one or more nitrogen atoms, normally of heterocyclic nature, and possess specific physiological actions on human or animal body, when used in small quantities. (Bindu Rathaur *et al* 2020) [4]. In traditional medicine *Tabernaemontana divaricata* is used to treat various diseases like epilepsy, abdominal tumours, eye infections, fractures, fever, headache, inflammation, mania, oedema, leprosy, diarrohea. Many primary and secondary

metabolites are present in the different parts of *Tabernaemontana divaricata*. (Ankita Kulshethra *et al* 2019).

Materials and Methods (Harborne (1973))

Collection of plant material

The fresh flowers of *Tabernaemontana divaricata* flower was collected, washed with running tap water to remove the surface dust particles and blotted with clean white muslin cloth. The flowers were shade dried completely and grind into a fine powder.

Extract preparation

Tabernaemontana divaricata flowers were collected from the plant at early morning then washed shade dried and powdered. For the preparation of extract by using different solvents such as aqueous, ethanol, hexane and acetone were taken for 200ml in each four conical flask separately and 30g of powdered material taken for extraction. After 24 hours the solvents were dispersed and dried extracts used for further analysis. Each extracts were mixed with suitable amount of respective solvents at time usage. This hot percolation method was done in Department of Microbiology A.V.V.M Sri Pushpam college (Autonomous), Poondi, Thanjavur.

Qualitative Phytochemical Analysis

The phytochemical analysis was carried out for the flowers of *T.divaricata* with standard methods. It was done to assess the qualitative chemical composition of crude extracts using commonly employed, precipitation and colourations reaction to identify the major natural chemical groups such as Alkaloids, Carbohydrate, Flavonoids, Glycosides, Phenol, Saponin, Steroid, Tannin. General reactions in these analyses revealed the presence or absence of these compounds in the plant extracts.

Qualitative analysis of phytochemical constituents

Test for Alkaloids

Mayer's reagent (2ml of 5%) was added to 1ml of flowers extract of *T.divaricata*. The formation of green precipitate indicates the presence of alkaloids.

Test for Carbohydrates

Treated the extract with Benedict's reagent (alkaline solution of cupric citrate complex) absence of red precipitates boiling on water bath indicates the absence of carbohydrates.

Test for Flavonoids

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Test for glycoside

4 ml of extract solution was dried till 2 ml. To it was added 1-2 ml of Ammonium Hydroxide and shaken. Appearance of cherish red color indicates the presence of glycosides.

Test for Phenols

Ferric chloride and few drops of ethanol was added to 1ml of flower extract of *T.divaricata* Formation of violet colour indicates the presence of phenols.

Test for Saponins

Few drops of water and two drops of coconut oil were added to flower extract of *T.divaricata* leaf formation of layer or foam, indicates the presence of saponins.

Test for Steroids

Acetic acid (2ml) was added to 2ml of flowers extract of *T.divaricata* leaf boiled then allowed to cool and added sulphuric acid the formation of upper green colour layers is positive and presence of steroids.

Test for tannins

5 ml of the *T.divaricata* extract was placed in a test tube and then 2 ml of 5 % of $FeCl_3$ solution was added. A greenish-black precipitate indicates the presence of tannins.

Quantitative Phytochemical analysis (Harborne 1973)

Estimation of Alkaloids

Alkaloid determination by using Harborne (1973) method. One gram of the *T.divaricata* sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and its covered and allowed to stand for 4 hrs. It was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated NH_4OH was added by drop wise to the extract until the precipitation was completed. The whole solution was allowed to settle and the precipitate was collected and washed with dilute NH_4OH and then filtered. The residue is the alkaloids which was dried and weighed.

Estimation of carbohydrate (Layne, 1975)

1. **Anthrone reagent:** Dissolved 2g of Anthrone in 1 litre of concentrated H_2SO_4. Used freshly prepared reagent for the assay.

2. **Glucose stock solution:** 200µg glucose per mL distilled water. Pipetted out into a series of test tubes different volumes of glucose solution from the supplied stock solution (200µg /ml) and made up the volume to 1 mL with distilled water. Consider tube 1 as blank and tubes 2 through 9 for construction of a standard curve. Tubes 10-15 are for the unknown samples. To each tube add 5 mL of the anthrone reagent (supplied) and mixed well by vortexing. Cooled the tubes. Covered the tubes with marbles/ Caps on top and incubated at 90^0C for 17 minutes or boiling water bath for 10 minutes. Cooled to room temperature and measured the optical density at 620 nm against a blank. Prepared a standard curve of absorbance vs. µg glucose.

Determination of total flavonoids: (Kumaran, *et al* 2003)

The method is based on the formation of the flavonoids - aluminium complex which has an absorbtivity maximum at 415nm. 100µl of the sample extracts in methanol (10 mg/ml) was mixed with 100 µl of 20 % aluminum trichloride in methanol and a drop of acetic acid, and then diluted with methanol to 5ml. The absorption at 415 nm was read after 40 minutes. Blank samples were prepared from 100 ml of sample extracts and a drop of acetic acid, and then diluted to 5ml with methanol. The absorption of standard rutin solution (0.5 mg/ml) in methanol was measured under the same conditions. All determinations were carried out in triplicates.

Determination gylcosides: [El-Olemy 1994].
Glycoside content in the sample was evaluated using Buljet's reagent as described by El-Olemy. 1g of the fine powder of *T.divaricata* flowers was soaked in 10ml of 70% alcohol for 2hrs and then filtered. The extract obtained was then purified using lead acetate and Na_2HPO_4 solution before the addition of freshly prepared Buljet's reagent (containing 95ml aqueous picric acid + 5ml 10% aqueous NaOH). The difference between the intensity of colours of the experimental and blank (distilled water and Buljet's reagent) samples gives the absorbance and is proportional to the concentration of the glycosides.

Estimation of Total Phenols (Harborne 1973)
The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. 5 ml of the extract was pipetted out into a 50 ml flask, then 10 ml of distilled water was added. 2 ml of NH_4OH solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark and left to react for 30 min for colour development. This was read at 550nm.

Estimation of total saponins (Harborne 1973)
The samples were ground. 20g of each plant samples were dispersed in 200 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separator funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight. The saponin content was calculated in percentage.

Estimation of total steroids (Harborne 1973)
The extract (1 g) was macerated with 20 ml of ethanol and filtered. To the filtrate (2 ml), 2 ml of chromagen solution was added and the solution left to stand for 30 min. The absorbance was read at 550 nm.

Estimation of tannins (Van – Burden and Robinson, 1981)
500mg of the *T.divaricata* flower sample was weighed into a 50 ml plastic bottle. 50ml of acetone solvent was added and shaken for 1 hr in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipette out into a test tube and mixed with 2 ml of 0.1M $FeCl_3$ in 0.1 N HCL and 0.008 M potassium ferriccyanide. The absorbance was measured at 120 nm with in 10 mm.

Determiantion of antimicrobial activity (Perez *et al.*, 1990)
Preparation of extract
The aqueous extracts of *T.divaricata* were prepared in similar concentration of 100 µg/ml and used for antibacterial activity by well diffusion method.

Test microorganisms
The following bacterial were used for the screening of antibacterial activity, *Bacillus cereus, B. subtilis, Enterococcus* sp, *Escherichia coli, Klebsiella* sp, *Pseudomonas* sp, *Streptococcus pyogenes and Staphylococcus aureus.* were selected for this study.

ii) Agar well – diffusion method: (Kirby and Bauer 2009)
Agar well-diffusion method was followed for determination of antibacterial activity. Nutrient agar (NA) plates were swabbed (sterile cotton swabs) with 24 hours culture and 48 hours old-broth culture of respective bacteria. Agar wells (5mm diameter) were made in each of these plates using sterile cork borer. About 25, 50, 75 and 100µL of aqueous and ethanol extracts were added using sterilized dropping pipettes into the wells and plates were left for 1 hour to allow a period of pre-incubation diffusion in order to minimize the effects of variation in time between the applications of different solutions. The plates were incubated in an upright position at 37˚C ± 2˚C for 24 h for bacteria. Results were recorded as the presence or absence of inhibition zone. Triplicates were maintained and the average values were recorded for antibacterial activity.

Results and Discussion
The phytochemical analysis of the flowers of *Tabernaemontana divaricata* reveals the presence of secondary metabolites were analysed. The phytochemical analysis of *Tabernaemontana divaricata* flowers of ethanolic extract such as alkaloid, carbohydrate, flavonoids, glycosides, phenol and tannin are presents. In the hexane extract the following phytochemical such as carbohydrate, flavonoids, glycosides, phenol, saponin, steroid and tannin were screened. Alkaloid and steroids are absent in the ethanol extract. In aqueous extract phytochemicals such as carbohydrate, flavonoids, glycosides, steroids and tannin are present.(Table :1, and 2)
Tabernaemontana divaricata flower extracts was used for this present study. flower was shade dried and prepared for extraction. Ethanol showed good antimicrobial activity than other solvents. In 100µl of ethanol extract of *Tabernaemontana divaricata* flower the maximum zone of inhibition was 30mm in *streptococcus* sp represented respectively. *Staphylococcus aureus* was 26.2mm zone of inhibition. *Pseudomonas* sp was 26mm zone inhibition, *E.coli*. was 26mm zone of inhibition are the maximum zone of inhibition from the isolated bacteria. *Bacillus* sp. was 16.2mm zone of inhibition *Bacillus subtilis* 17.1mm zone inhibition, *Klebsiella* sp was 11.5mm zone of inhibition was observed respectively.(Table : 3)
Plant produces various metabolic products for their growth and development. The components which are essential for the growth and survival for the producer plant are known as primary metabolites and secondary metabolites which are derived biosynthetically from primary metabolites. Alkaloid, carbohydrate, flavonoids, glycoside, phenol, saponin, steroid and tannin are belong to this class (Padmaja 2011).
The higher antibacterial activities present in the extracts of *T.divaricata* when compared with *M.oleifera* justified it uses in folk medicine. The antimicrobial activity of the extracts of petals of the flowers *M.oleifera* inhibited seven of the ten isolated bacteria tested *S.epidermis, G.vaginalis E.faecalis, C.macbinleys, B.cereus, B.subtilis* and *E.coli*. *T.divaricata*

flower extracts inhibited nine ocular pathogens are *S.aureus*, S. epidermis, *G.vaginalis, E. faecalis, S. agalactiae, P. acens, C. macbinleys, B.cereus* and *B. subtilis* (sumitha 2015).

The phytochemical components of the stem extracts of *T.divericata* has a high quality of phenolic compounds and glycoside compounds so this plant is a potential source for identifying novel phyto compounds which can have a good anti oxidant activity and can be used in pharma industries for producing potent drug (Sruthi S Nair 2021)

T.divaricata flowers have the presence of flavonoids, terpenoids, phenols, tannins, carbohydrates and proteins in all parts,but alkaloids and steroids were absent only in flowers (Kalaimagal *et al* 2015) [11].

Tabernaemontana extracts as natural antibiotics, monoterpenoid indole alkaloids, vocamine type and 3-hydroxy –iboga are biologically active compounds and are reportedly used as antimicrobial agents inhibiting the growth of bacteria fungi and parasites (Flavio Marinho *et al* 2016).

Table 1: Qualitative phytochemical test for *Tabernaemontana divaricata* with different solvents

Name of phytochemicals	Aqueous	Acetone	Ethanol	Hexane
Alkaloid	+	+	+	+
Carbohydrate	-	-	-	-
Flavonoids	+	+	+	+
Glycosides	+	+	+	+
Phenol	-	+	+	+
Saponin	+	+	-	+
Steriod	-	-	-	-
Tannin	+	+	+	+

(+) Present, (-) absent

Table 2: Quantitative phytochemical test for *Tabernaemontana divaricata* with different solvents

Phytoconstituents	Quantity (mg/g)			
	Aqueous	Acetone	Ethanol	Hexane
Alkaloid	12.0±0.13	11.0±0.36	10.0±1.0	10.2±0.12
Carbohydrate	-	-	-	-
Flavonoids	13.0±0.32	09.0±0.36	16.2±0.48	9.32±0.36
Glycosides	11.2±0.03	8.00±0.12	9.00±0.36	8.02±0.69
Phenol	-	6.35±0.23	15.2±0.45	9.63±02.3
Saponin	10.0±0.02	8.03±0.44	11.0±0.03	-
Steriod	-	-	-	-
Tannin	16.2±0.78	8.63±0.23	19.2±0.57	8.00±0.02

Values are expressed by mean ± S.D

Table 3: Antibacterial activity of *Tabernaemontana divaricata* against ocular pathogens

Ocular bacterial isolates	*Tabernaemontana divaricata* flower extracts and Zone of inhibition (mm)			
	25µl	50µl	75µl	100µl
Bacillus sp	11.2±2.20	13.0±0.20	15.2±12.0	16.2±1.80
Bacillus subtilis	10.0±0.50	12.2±1.42	14.0±1.00	17.1±3.40
E.coli	18.0±0.5	22.4±0.5	24.0±0.5	26.0±2.0
Klebsiella sp	8.2±1.2	9.0±2.1	10.2±1.5	11.5±0.5
Pseudomonas sp	16.0±2.0	22.3±3.5	24.2±2.0	26.0±1.5
Streptococcus sp	18.5±2.5	23.0±1.3	25.2±5.0	30.0±2.1
Staphylococcus aureus	12.0±0.2	18.2±2.2	23.2±2.5	26.2±2.5

Values are expressed by mean ± S.D

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