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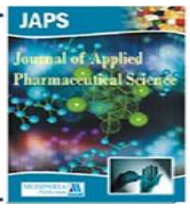

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In vitro antibacterial and antifungal activities of *Tabernaemontana heyneana* Wall. leaves

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ABSTRACT

Medicinal plants are the oldest source of pharmacologically active compounds and provided virtually the only source of medicinally useful compounds for centuries. They contain physiologically active principles that over the years have been exploited in traditional medicine for the treatment of various ailments. The increased likelihood of acute infections and inflammatory conditions in early humans could have set the stage for the natural selection of the use of medicinal herbs high in antimicrobial and anti-inflammatory components. In the present study, the *in vitro* antibacterial and antifungal activities of different solvent leaf extracts of *T. heyneana* Wall. was investigated. *K. pneumoniae* (26±1.0 mm) and *S. typhii* (9.0±2.0 mm) were proved to be inhibited maximally and minimally by the leaves of *T. heyneana*. Similarly, the maximum and minimum antifungal effect was observed against *Rhizopus mucor* (25±0.0 mm) and *Trichoderma viridins* (6.0±1.0 mm), respectively. Among the solvents methanol was proved to be the best one in the extraction of antimicrobial compounds. Ethanol and aqueous systems were proved to be moderate and chloroform as poor solvent in extracting antimicrobial components. Overall results has proved that *T. heyneana* leaves possess significant antibacterial and poor antifungal activities.

Keywords: Antibacterial, Antifungal, Medicinal plants, *Tabernaemontana heyneana*.

INTRODUCTION

Medicinal plants, since times immemorial, have been used in virtually all cultures as a source of medicine. The widespread use of herbal remedies and healthcare preparations, as those described in ancient texts such as the Vedas and the Bible has been traced to the occurrence of natural products with medicinal properties (DaSilva and Hoareau, 1999). Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. The development of microbial resistance to the available antibiotics has led researchers to investigate the antimicrobial activity of medicinal plants (Hammer *et al*., 1999). Several compounds like flavonoid, tannins, coumarins, quinones, terpenoids, essential oils, alkaloids, lectins and peptides have been reported to possess antimicrobial properties (Cowan, 1999).

The genus *Tabernaemonana* consists of shrubs or small trees. The latex is white and stems are repeatedly dichotomously branched. The leaves are opposite. Several of its species has been widespread in various countries like North America, South America, Africa and Asia (Ying and Ping-tao, 1977).

The plant *Tabernaemontana heyneana* Wall. (Apocynaceae, syn. *Ervatamia heyneana*) is included in the oldest script *Amarakosam or Namalingkanusasanum* written by Amarasshimhan in between somewhere in 1-6 century AD (Nambiar and Raveendran, 2008). It is generally found in south – western India in open forests of the western ghats from Konkan (Maharashtra) to southwards through Kerala up to 900 meter elevation. It is a small deciduous tree or shrub that grows up to 9 meter tall with rough grey bark. In south India, *Tabernaemontana heyneana* is used as a substitute for *Tabernaemontana coronaria,* which is a closely related and more widely distributed species. In Tamil, *T. heyneana* is known as *Kundaalam Paalai*, possess antioxidant (Sathishkumar *et al.,* 2012), curative properties against veneral, nervous and skin disorders (Ignacimuthu *et al.,* 2006). Several alkaloids like tabernoxidine, coronaridine and vocangine has been reported from *T. heyneana* (Roy *et al.,* 2002). Similarly, compounds like rutin, quercetin and few phenolic acids have been identified from the leaves of *T. heyneana* (Sathishkumar *et al.,* 2008).

Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for the development of novel drugs because of the great diversity in their chemical structure. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases (Rojas *et al.,* 2004). Therefore, researchers are increasingly turning their attention to ethno-medicine, looking for new leads to develop more effective drugs against microbial infections and this has led to the screening of several medicinal plants for potential antimicrobial activity. In this perspective, the present study has been focused to investigate the antibacterial and antifungal activities of *T. heyneana* Wall. leaves.

MATERIALS AND METHODS

Plant material and extraction process

The leaves were collected from the medicinal garden of Kumaraguru College of Technology, Coimbatore, India. The species was identified and confirmed at Botanical Survey of India (BSI), Southern Circle, Coimbatore, India (BSI/ SC/ 5/ 23/ 06-07/ Tech. 478). About 5g of air dried leaves were dissolved in 50ml of the solvent (Distilled water, methanol, ethanol, chloroform and acetone) and kept in an orbital shaker for overnight (Shake flask method). The residue was re-extracted under the same conditions. The obtained extracts were filtered with Whatman No.1 filter paper. The filtrate containing the solvent was dried, the residue was re-dissolved in distilled water and used for *in vitro* antibacterial and antifungal assays. All the chemicals and solvents used for experimental analysis were of analytical grade.

Preparation of inoculum

Pure cultures of both bacterial and fungal types of the following microorganisms were used for the investigation of antimicrobial analysis: *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhii, Klebsiella pneumoniae, Bacillus cereus, Bacillus subtilis, Aspergillus niger, Giberella fujikori, Pencillium chrysogenum, Aspergillus terreus, Candidda albicans, Rhizopus mucor and Trichoderma viridians.* The lyophilized pure bacterial cultures were thawed and inoculated on to nutrient broth and incubated overnight.

Then, the cultures were swabbed onto sterile nutrient agar in petri plates. The growth and colony morphology of the cultures was carefully observed. Then, all the microorganisms were subcultured in conical flasks containing sterile nutrient broth. The cultures were incubated in bacteriological shaker-incubators. The nutrient broth cultures were allowed to grow until the optical density reached 1.00 and were subcultured and preserved for further analysis. Similarly, Sabouraud and Potato dextrose agar were used for the analysis of antifungal activities. The fungal inoculum was prepared by the above mentioned protocol (instead of nutrient broth, potato dextrose and Sabouraud's dextrose broth was used).

In vitro antibacterial and antifungal activities by gel diffusion method

About 3g of nutrient agar was dissolved in 100ml of distilled water and autoclaved for 15 minutes at 121°C. 25 ml of sterile molten nutrient agar was poured into sterile petri plate and allowed to solidify. Then 0.1 ml of the bacterial cultures (containing 1 x 10^6 cfu /ml) were inoculated on the agar plates and the culture was uniformly spread using a sterile glass rod (Spread plate technique). The wells were bored with 8mm borer in the agar. About 200 µl of the herbal extract was added in each well. The plates were incubated at 37°C for 24 h. After incubation period was finished the zone of inhibition was measured and recorded. The inhibition diameters were measured in millimetres and averaged and the mean values were tabulated (Ignacimuthu *et al.,* 2006).

Likewise, 3.9g of potato dextrose agar and 5g of Sabouraud dextrose agar was dissolved separately in 100ml of distilled water and mixed well. Boiled the mixture for 5 minutes with frequent agitation for proper dissolution. Autoclaved the contents for 15 minutes at 121°C. Then 0.1 ml of the fungal cultures (containing 1 x 10^8 cfu /ml) were inoculated on the agar plates and the culture was uniformly spread using a sterile glass rod (Spread plate technique). The wells were bored with 8mm borer in the agar. About 200 µl of the herbal extract was added in each well. The plates were incubated at room temperature for 72 h. After incubation period was finished the zone of inhibition was measured and recorded. The microorganisms sensitivity to the reference antibiotics (controls) was checked at their respective recommended dosage for comparative antimicrobial efficacy. Ampicilin, gentamycin and cloxacilin were used for bacterial cultures; amphotericin B was used for fungal cultures.

RESULTS AND DISCUSSION

The antimicrobial activities of different solvent leaf extracts of *T.heyneana* and control antibiotics against various bacterial and fungal species have been depicted in Table 1, 2 and 3, respectively.

Maximum and minimum antibacterial activities of leaves were observed against *K. pneumoniae* (26±1.0) and *S. typhii* (9.0±2.0), respectively has proved the efficacy of the leaves in controlling the growth of bacterial species. Among the seven species tested, it was found that *K. pneumoniae , B. subtilis* and *B. cereus* were strongly controlled, and *E. coli* and *S. aureus* were moderately controlled by the extracts. Among the solvents methanol was proved to be the best one for the extraction of antimicrobial compounds which was followed by aqueous and ethanol systems (Plate 1 (a) and (b)). Chloroform was proved to be ineffective in the extraction of antimicrobial phytochemicals. The efficacy of aqueous extract (15±0.0) has documented high antimicrobial activity against *E. coli* than ampicillin (10±1.0). Similarly, the extract (26±1.0) possessed high antibacterial activity than cloxacilin (14±2) against *K. pneumoniae.* An equivalent effect of the extract (22±1.0) compared with ampicillin (22±0.0) against *B. subtilis* has confirmed the potent antibacterial effect of the plant, whereas, other bacterial species has shown to be sensitive against antibiotics.

Similar studies extended on fungal species has recorded a maximum and minimum antifungal effect against *Rhizopus* m*ucor* (25±0.0) and *Trichoderma viridins* (6.0±1.0). Among seven fungal species tested, the growth of *R. mucor, P. chrysogenum* and *A. niger* was found to be strongly inhibited and *A. terreus* and *C. albicans* were moderately inhibited. The inhibition zone values have proved that the solvent ethanol was very effective in the extraction of antimicrobial compounds than other solvents. Similarly, the analysis also revealed that *Trichoderma viridins* was too resistant than the other organisms. Appreciable antifungal effect of leaves has been observed against the skin pathogen *Candida albicans* (11±2.0). The antibiotic amphotericin B (17±1.0) was proved to be very effective in controlling the growth of all the fungal species except for *R. mucor, P. chrysogenum* and *A. niger*.

The antimicrobial properties of Indian medicinal plants were reported based on folklore information (Jeevan Ram *et al.,* 2004) and several plant species are used by many ethnic groups for the treatment of various ailments ranging from minor infections to dysentery, skin diseases, asthma, malaria and a horde of other indications (Perumal Samy and Ignacimuthu, 2000).

A large number of constitutive plant compounds have been reported to have antimicrobial activity. Well known examples include phenols, unsaturated lactones, saponins, cyanogenic glycosides, glucosinolates, tannins and phytosterols. Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds, which are effective against viruses, bacteria (Brantner *et al.,* 1996) and fungi. Similarly, hydroxylated phenols like catechol and pyrogallol are shown to be toxic to microorganisms. The position(s) and number of hydroxyl groups in the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. In addition, some authors have found that more highly oxidized phenols are more inhibitory. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Cowan, 1999). Flavonoids are hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection, it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. The mechanism of action of flavonoids against bacterial and fungal infections is: (1) to kill the bacterial or fungal cells and (2) to counteract the spread and the effects of the bacterial toxins. The bacteriocidal and fungicidal effect of the flavonoids may well be the result of a metabolic perturbation (osmotic imbalance, denaturation of enzymes and modification of ion channels), especially through complex formation with extracellular, soluble and cell wall proteins (Havsteen, 2002).

Previous literature studies have reported the *in vitro* antimicrobial activity of alkaloids like apparicine and voacamine isolated from *T. coronaria* (van Beek *et al.,* 1984). Guida *et al.,* (2003) has reported the potent antibacterial activity (standardized Kirby-Bauer proposed disc technique) of methanolic root bark of *T. catharinensis* against bacterial species like *B. subtilis* (25.5mm), methicillin resistant *S. aureus* (19.5mm), *S. aureus* (15.5mm), E. coli (10.5mm) and *K. pneumoniae* (7mm), and the present results of antibacterial activities in the leaves has well correlated with the above reported document. Not only the phytochemicals, even cysteine proteases isolated from latex of *Tabernaemontana coronaria* has proved to possess antibacterial activities (Kundu *et al.,* 2000). The present investigation has confirmed that the phytochemicals like alkaloids, flavonoids, tannins and sterols distributed in the leaves of *T. heyneana* may play a role in the inhibition of bacterial and fungal species.

Table. 1: Antibacterial activities of the crude *T. heyneana* leaf extracts against different bacterial strains

Test organism	Zone of inhibition (mm)				
	Aqueous	Methanol	Ethanol	Chloroform	Acetone
E. coli	15±0.0	8.0±1.0	9.0±1.0	8.0±0.0	6.0±0.0
S. aureus	10±1.0	14±1.0	14±1.0	11±0.0	9.0±0.0
S. typhii	6.0±0.0	6.0±0.0	7.0±2.0	5.0±0.0	9.0±2.0
K. pneumoniae	10±1.0	26±1.0	19±0.0	8.0±0.0	14±1.0
P. aeruginosa	13±0.0	7.0±1.0	9.0±1.0	6.0±0.0	9.0±0.0
B. cereus	11±0.0	17±0.0	15±0.0	9.0±0.0	10±1.0
B. subtilis	15±1.0	22±1.0	14±1.0	11±0.0	18±1.0

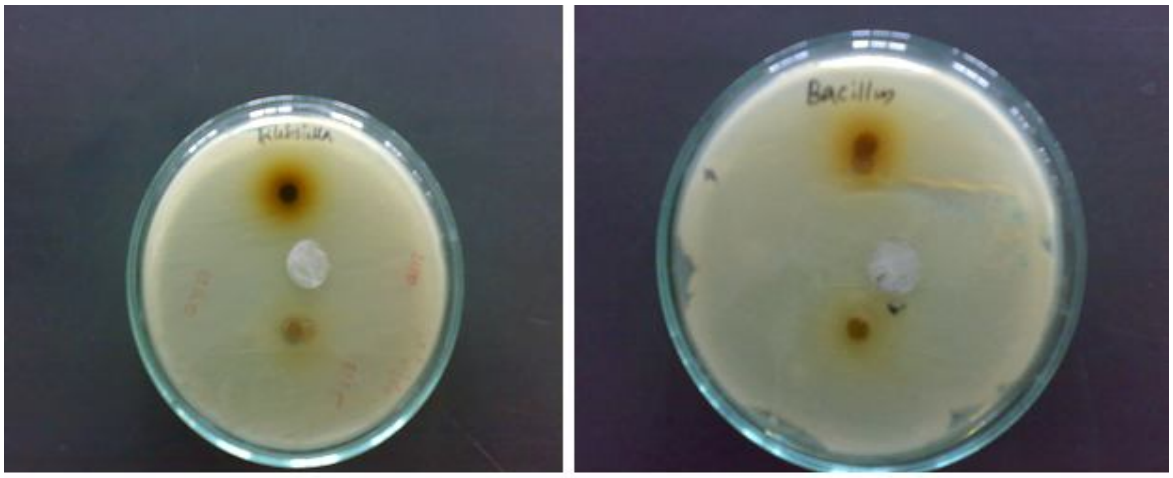
Table. 2: Antifungal activities of the crude *T. heyneana* leaf extracts against different fungal strains.

Test organism	Zone of inhibition (mm)				
	Aqueous	**Methanol**	**Ethanol**	**Chloroform**	**Acetone**
Aspergillus niger	9.0±1.0	8.0±0.0	18±0.0	7.0±1.0	11±2.0
Giberella fujikori	9.0±0.0	9.0±1.0	8.0±0.0	8.0±0.0	9.0±1.0
Pencillium chrysogenum	10±2.0	8.0±1.0	8.0±1.0	8.0±0.0	19±1.0
Aspergillus terreus	9.0±0.0	11±1.0	6.0±1.0	8.0±0.0	6.0±1.0
Candida albicans	6.0±0.0	9.0±1.0	11±2.0	8.0±0.0	7.0±1.0
Rhizopus mucor	11.0±0.0	19.0±1.0	25±0.0	6.0±0.1	20.0±1.0
Trichoderma viridians	ND	4.0±0.0	6.0±1.0	ND	ND

(ND=Not detected)

Table. 3: Antibacterial activities of different control antibiotics against different bacteria strains.

Test organism	Control Antibiotics (10mg/ml)	Zone of inhibition (mm)
E. coli	Ampicillin	10.0±1.0
S. aureus	Gentamycin	26±0.0
S. typhii	Gentamycin	22±2.0
K. pneumoniae	Cloxacilin	14±2.0
P. aeruginosa	Gentamycin	18±3.0
B. cereus	Ampicillin	20±2.0
B. subtilis	Ampicillin	22±0.0



A **B**

Plate. 1: Antibacterial affect of methanolic leaf extract against (a) *K. pneumonia* and (b) *B. subtulis.*

CONCLUSION

Scientists from divergent fields are investigating plants with an eye to their antimicrobial usefulness. Generally, antimicrobial properties of substances are desirable tools in the control of harmful microorganisms especially in the treatment of several infectious diseases. The active phytochemical components usually interfere with growth and metabolism of microorganisms. Laboratories of the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro*. In conclusion, it was found that the leaves of *T. heyneana* possess significant antibacterial and poor antifungal activities.

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