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Original Article

ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *TABERNAEMONTANA HEYNEANA* WALL. AN ENDEMIC PLANT OF WESTERN GHATS

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ABSTRACT

Objective: The aim of this study was to investigate the antioxidant and antimicrobial properties of different crude extracts of leaves of *T. heyneana*

Methods: Crude extracts of methanol, chloroform, dichloromethane and dichloroethane of leaf were evaluated for antimicrobial activity by disc diffusion method and antioxidant activity by DPPH (diphenyl-2-picrylhydrazyl) and reducing power assay. Quantitative analysis of total phenolics was done by Folin-Ciocalteau method and total flavonoids by aluminium chloride method.

Results: Methanolic extract of *T. heyneana* exhibited the presence of all the phytochemicals tested except triterpenoids and saponins. The highest phenolic content of 14.0±0.45 mg GAE/g and flavonoid content of 81.62±0.47 mg QE/g were found in methanol extract. The highest DPPH scavenging activity (IC_{50} 20.3±0.56 µg/ml) and reducing power was exhibited by methanolic extract. The methanolic extract showed maximum antibacterial activity of 12.66±0.57 mm zone of inhibition against *Staphylococcus aureus* and least of 9.23±0.25 mm against *Proteus vulgaris.*

Conclusion: These findings provide scientific evidence to support the traditional use of *Tabernaemontana heyneana* Wall*.* and also indicate that the leaves of this species are a promising potential for the development of antioxidant and antimicrobial agents.

Keywords: *Tabernaemontana heyneana* Wall, Antioxidant activity, Antibacterial activity.

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years because of their traditional medicinal practice [1]. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. They also serve in plant defense mechanisms to prevent damage by microorganisms, insects, and herbivores [2]. Recently plants have been exploited as powerful and potential medicinal drugs [3]. Herbal drugs are mainly focused as an alternative source against manifestations caused by various microorganisms due to the increasing resistance of existing antimicrobial agents [4]. This could lead to its successful use as a potential drug, which confers to its therapeutic use.

The genus *Tabernaemontana* consists of shrubs or small trees with white latex, repeatedly dichotomously branched stem with leaves opposite. Several of its species have been widespread in various countries like North America, South America, Africa and Asia [5]. *Tabernaemontana heyneana* Wall. (Apocynaceae) known as Maddarassa gida or Madle mara or Nagarkuda in Kannada 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 m tall with rough grey bark. The plant is known to possess antimicrobial activity against skin and venereal diseases, respiratory problems, nervous disorders and various other diseases [6, 7]. Several alkaloids like tabernoxidine, coronaridine and vocangine have been reported from *T. heyneana* [8]. Compounds like rutin, quercetin and few phenolic acids have also been identified from the leaves of *T. heyneana* [9].

Among natural antioxidants, phenolic substances have been of special interest because they are widely distributed in the plant kingdom, constituting a large group of secondary metabolites, with more than 8000 phenolic structures currently known, ranging from simple molecules such as phenolic acids, to highly polymerized substances such as tannins [10]. Phenolics possess a wide spectrum of biochemical activities such as antioxidant, antimutagenic, anti carcinogenic as well as the ability to modify the gene expression [11].

Free radicals are chemical species possessing an unpaired electron that can be considered as fragments of molecules, which are generally very reactive. Reactive free radicals formed within the cells can oxidize bio-molecules and lead to cell death and tissue injury [12]. Overproduction of reactive free radicals results in oxidative stress that can cause damage to cell structures, including lipids and membranes, proteins and DNA. An antioxidant is a molecule capable of inhibiting the oxidation of free radical intermediates by being oxidized themselves as reducing agents such as thiols, ascorbic acid or polyphenols [13]. Antioxidants help in preventing degenerative diseases such as cancer, coronary heart disease and even altitude sickness [14].

Infectious diseases cause a large proportion of the health problems in developing countries. Medicinal plants constitute a rich source of antimicrobial agents. Plants are used medicinally as a source of potent and powerful drugs [15]. Considering the vast potentiality of plants as sources for antimicrobial drugs with reference to antibacterial and antifungal agents, a systematic investigation was undertaken to screen the local flora for antibacterial and antifungal activity.

Previously, no work has been carried out on the antimicrobial and antioxidant activities of leaf extracts of *T. heyneana*, the present study dealing with the preliminary evaluation of phytochemicals, antioxidant and antimicrobial activities of leaves of *T. heyneana* was undertaken.

MATERIALS AND METHODS

Collection of plant material

The leaves of *T. heyneana* were collected from plants located in and around Mangalore university campus, (12.8158 ° N and 74.9241 ° E) Karnataka, India and the voucher specimens have been deposited in the herbarium of Department of Applied Botany, Mangalore University. The collected samples were shade dried, ground into fine powder using domestic grinder and stored in sterile polythene bags at 4 °C until used.

Preparation of the extract

The coarsely powdered leaves of *T. heyneana.* were extracted with methanol, chloroform, dichloromethane and dichloroethane by

Soxhlet extraction for 36 h. The extracts obtained were evaporated to dryness and were stored in bottles until further use.

Preliminary phytochemical screening

The freshly prepared crude solvent extracts of *T. heyneana* were qualitatively tested for the presence of phytochemical constituents such as alkaloids, flavonoids, terpenoids, phenols, tannins etc., by standard methods [16-18].

Estimation of total phenols

The total phenolic content was determined by Folin-Ciocalteau colorimetric method [16] with slight modifications. A small quantity (3 ml) of extracted solution (100 µg/ml) and 0.1 ml of Folin-Ciocalteau reagent (50 % v/v) were mixed thoroughly. After 4 min, 2 ml of Na2CO3 (2%) was added and the volume was made up to 10 ml by water. The mixture was incubated in dark for 30 min at room temperature. The absorbance was measured at 760 nm using Shimadzu UV/Vis 2401 spectrophotometer. The phenolic content was calculated using calibration curve for Gallic acid (20-100 µg/ml) and the results were expressed as mg Gallic acid equivalents/g dry extract.

Estimation of flavonoids

Total flavonoid content was determined following the Aluminium chloride method [19]. A small quantity (4 ml) of extracted solution (100 µg/ml) and 0.3 ml of $NaNO_2$ (5 %) were mixed and incubated At 5th min, 0.3 ml of 10% $AlCl_3$. H_2O solution was added followed by the addition of 2 ml of 1 M NaOH solution at 6th min and the total volume was made up to 10 ml using distilled water. The absorbance against blank was determined at 510 nm. Results were expressed as mg Quercetin equivalents (QE)/g of extract.

Evaluation of DPPH scavenging activity

Varying concentrations of 50 to 500 µg/ml of the extract were mixed with 0.3 ml of 0.5 mM DPPH in ethanol [20], the reaction mixtures were kept in dark for 30 minutes at room temperature and the decrease in absorbance was measured at 517 nm using Ascorbic acid as standard. The experiment was carried out in triplicate. The ability to scavenge DPPH radical was calculated as:

$$\%\ \text{DPPH radical scavenging activity} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{(\text{Absorbance of control})}$$

The activity was expressed as 50% inhibitory concentration (IC_{50}) based on the percentage of DPPH radicals scavenged. Lower the IC_{50} value, the higher is the antioxidant activity.

Reducing power assay

The reducing power of the plant extract was determined by the method of Oyaizu *et al.* [21]. Varying concentrations of 50 to 500 µg/ml of plant extracts were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 7.4) and 2.5 ml of 1% potassium ferricyanide and the mixture was incubated at 50 °C for 20 minutes. After incubation, 2.5 ml of trichloroacetic acid was added to the mixture and centrifuged at 3000 RPM for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.1% ferric chloride (0.5 ml) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Anti bacterial activity

The antibacterial activity of different plant extracts was evaluated by using the disc diffusion technique. Two Gram positive bacteria, *Staphylococcus aureus* (NCIM 2079) and *Bacillus subtilis* (ATCC 6633) and three Gram negative bacteria, *Escherichia coli* (NCIM 2931), *Pseudomonas aeruginosa* (NCIM 2200) and *Proteus vulgaris* (NCIM 2813) obtained from National chemical laboratory, Pune, India were used in the study. These bacteria were maintained on nutrient broth (NB) at 37 °C. The bacterial inoculum was prepared by subculturing the micro organism on the nutrient broth and incubated at 37 °C overnight. Two hundred µl of overnight grown culture of each organism was inoculated into sterile nutrient broth and incubated for 4-5 h at 37 °C.

Twenty ml of sterilized nutrient agar was poured into sterile petriplate and after solidification of the media, 100 µl of microbial inoculums (containing 1 x 10^6 cfu/ml) was swabbed on the respective plates. The sterile antimicrobial susceptibility filter paper discs (5 mm in diameter) obtained from Himedia Lab. was impregnated with 20 µl of the extract were placed on test organism swabbed plates. Streptomycin sulfate (10 µg/ml) was used as positive control. The activity was obtained by measuring the zone of inhibition around the discs. The experiment was repeated thrice and the mean values were presented.

Statistical analysis

All the experiments were performed in triplicates (n=3). Statistical analysis was carried out using SPSS Software version 20. Statistical differences between extract activities were determined using one way ANOVA with Duncan's multiple range test grouping. Differences were considered statistically significant at $p<0.05$.

RESULTS

Preliminary phytochemical screening

The preliminary phytochemical screening of *T. heyneana* extracts showed the presence of bioactive secondary metabolites such as alkaloids, flavonoids, saponins, tannins, glycosides, resins, steroids and triterpenoids (table 1). Methanol extract of *T. heyneana* revealed the presence of all the phytochemicals tested except for triterpenoids and saponins.

Total phenolics and flavonoid content

The phenolic content in different extracts ranged from 3.8±0.26 to 14.0±0.45 mg GAE/g (Table. 2). The highest phenolic content was exhibited in methanol extract of *T. heyneana* (14.0±0.45 mg GAE/g) followed by dicholoromethane (5.7±0.15 mg GAE/g), dicholoroethane (4.4±0.4 mg GAE/g) and chloroform extract (3.8±0.26 mg GAE/g). The flavonoid content ranged between 51.12±0.85 to 81.62±0.47 mg QE/g, the highest being 81.62±0.47 mg QE/g in methanol extract followed by dichloromethane, chloroform and dichloroethane extracts (table 2).

Table 1: Results of Preliminary phytochemical analysis of *T. heyneana*

Phytochemicals	Chloroform	Dichloroethane	Methanol	Dichloromethane
Phenols	+	-	+	-
Flavonoids	+	+	+	+
Tannins	+	+	+	-
Saponins	-	-	-	-
Alkaloids	+	+	+	+
Glycosides	+	-	+	+
Triterpenoids	-	-	-	-
Resins	+	+	+	+
Steroids	+	+	+	-

(+): Present (-): Absent

Table 2: Total phenolic and flavonoid contents in different extracts of *T. heyneana*

Extract	mg of GA/g of extract	mg of quercitin/g of extract
Chloroform	3.8±0.26[c]	72.75±0.95[c]
Dichloroethane	4.4±0.4[c]	51.12±0.85[d]
Methanol	14.0±0.45[a]	81.62±0.47[a]
Dichloromethane	5.7±0.15[b]	75.37±0.94[b]
F value	6050.897	94472.102

Values are mean±SD for three experiments. (n = 3). Results with different alphabets indicate significant difference at p<0.05.

Antioxidant activity

Scavenging effects of samples on DPPH radicals were in the following order: methanol>dichloroethane>dichloromethane>chloroform (table 3). The IC50 values of scavenging DPPH radicals were 20.3±0.56, 169±3.61, 199.66±4.04, 217.66±2.52 μg/ml of extract respectively in *T. heyneana*.

Table 3: Antioxidant activity by DPPH method

Extracts	DPPH IC_{50} (μg/ml)
Chloroform	217.66±2.51[d]
Dichloroethane	169±3.60[b]
Methanol	20.33±0.57[a]
Dichloromethane	199.66±4.04[c]
Std Ascorbic acid	230
F–Value	29931.325

Values are mean±SD for three experiments. (n = 3). Results with different alphabets indicate significant difference at p<0.05.

The reducing power increased with an increase in the concentration of the sample. Among all the extracts, methanol extract exhibited potent activity, which showed higher activities than the standard ascorbic acid. The activity was in the order of methanol>dichloroethane>chloroform>dichloromethane (fig. 1).

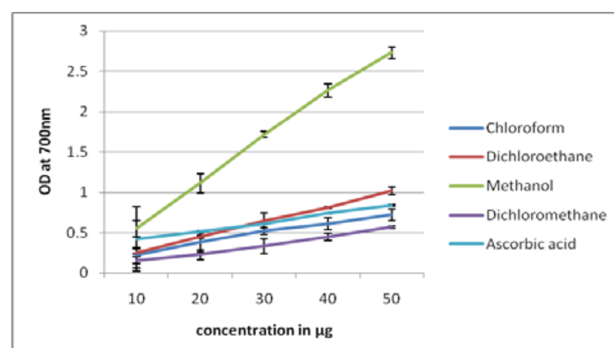

Fig. 1: Reducing power assay of different extracts of *T. heyneana* Values are mean±SD for three experiments (n = 3)

Antibacterial activity

All the extracts of *T heyneana* were found to posses antibacterial activity but methanolic extract showed maximum inhibition against all the tested bacteria and proved to be the best solvent for the extraction of antimicrobial compounds compared to all other extracts (table 4) Methanolic extract possess highest inhibition of 12.66±0.57 mm against *Staphylococcus aureus* and least of 9.23±0.25 mm against *Proteus vulgaris.* However, the antibacterial activity was lower in plant extracts compared to the standard Streptomycin.

Table 4: Antibacterial activity of *T. heyneana*

Extract	Zone of inhibition(diameter in mm)				
	PA	PV	SA	EC	BS
Chloroform	10±1[bc]	8.33±1.79[abc]	11.33±0.76[c]	8±0[c]	10.33±0.75[cd]
Dichloroethane	8.66±2.57[b]	8±1b[c]	11.66±2[c]	11.3±1.56[b]	9.66±0.857[d]
Methanol	11±1[b]	9.23±0.25[ab]	12.66±1.2[b]	11.66±2.17[b]	12.66±1.247[b]
Dichloromethane	10.16±0.288[b]	8.66±0.457[abc]	11.66±2.5[c]	8.66±1.47[c]	10.83±0.288[c]
Streptomycin	17.6±2.37[a]	9.66±0.57[a]	17.66±0.98[a]	14.66±2.7[a]	18.33±2.47[a]
F-Value	3778.571	3217.976	11700.00	6642.250	10195.630

BS-*Bacillus subtilis*, SA-*Staphylococcus aureus*, EC-*Escherichia coli*, PA-*Pseudomonas aeruginosa*, PV-*Proteus vulgaris.* Values are mean±SD for three experiments. (n = 3). Results with different alphabets indicate significant difference at p<0.05.

DISCUSSION

The plants in general posses primary and secondary metabolites such as phenolic compounds, flavonoids, alkaloids, tannins, saponins and steroids. The preliminary phytochemical screening of leaf extracts of *T. heyneana* revealed the presence of majority of the secondary metabolites in the methanolic leaf extracts. The results of the preliminary phytochemical screening of *Tecoma stans* flower also revealed the presence of terpenoids, alkaloids, cardiac glycosides, tannins, phenols, flavonoids and steroids [22]. Phytochemical screening of selected plants belonging to apocyanaceae members namely *Catharanthus roseus, Rauvolfia tetraphylla, Nerium oleander, Tabernaemontana divaricata, Allamanda cathartica, Thevetia peruviana* and *Plumeria alba* revealed the presence of reducing sugars and Phenolic compounds in all the seven plants while triterpenoids, sugars and anthroquinones were present only in *C. roseus* [23]. Catechin and tannins were present in all the plants except *N. oleander* and *T. peruviana* respectively. Steroids were present in *C. roseus, R. tetraphylla, T. peruviana* and *P. alba*. Flavonoids were present in *A. cathartica* and *P. alba* whereas aminoacids were absent in both the plants. Saponins were present in *C. roseus* and *N. oleander*. Alkaloids were present in *C. roseus, R. tetraphylla, N. oleander* and *P. alba*. An investigation was carried out to determine the possible phytochemicals from leaf and fruit rind of *Thevetia peruviana* Schum [24]*.* The results revealed the presence of carbohydrates, saponins, oils and fats, alkaloids, flavonoids, cardiac glycosides, coumarins and anthraquinone glycoside in plant leaf as well as fruit extracts.

A total phenolic content of 30.17±2.36 mg/g and a total flavonoid content of 21.33±0.94 mg/g (quercetin equivalents/g dry extract) were reported in methanolic extract of *Wrightia tinctoria* (Roxb) R. Br. when estimated through Folin Ciocalteu reagent method and aluminum chloride colorimetric method respectively [25]. Total phenol and flavonoid contents in *Thespesia populnea* flower extracts were found to be 31.2±4 mg/g and 25.05±0.18 mg/g of extract respectively [26]. Ghosh *et al.* [27] also reported higher phenolics of 511.472±22.304 mg gallic acid equivalent (GAE) and flavonoids of 230.785±5.439 mg/g quercetin equivalent in methanol extracts of *Litsea polyantha* Juss. The present study revealed the higher flavonoids of 81.62±0.47 mg/g quercetin equivalent and phenolics of 14.0±0.45 mg gallic acid equivalent (GAE) in the methanolic extract.

Phenolic compounds exhibit a wide spectrum of biological effects including antioxidant and free radical scavenging [28]. Antioxidant activity was evaluated in various fractions of methanolic extracts of

Quercus robur L by DPPH radical scavenging assay, which showed moderate activity against the standard drug querceitin [29]. In the present study methanolic extract of *T. heyneana* showed a good activity against quercitin while chloroform extract showed the least. The results obtained here revealed that there might be a positive correlation between total phenolic and antioxidant capacity of different extracts of *T. heyneana* which corroborates the results of Sathishkumar *et al.* [30]. Negative correlation between DPPH activity and flavonoids was also reported earlier by Mohammed *et al.* [31].

Different species of *Tabernamontana* have been reported to contain a large number of Indole alkaloids like vincosan, corynanthean, vallesiachotaman, strychnan, aspidospermatan, plumeran, eburnan, ibogan, tacaman, bis-indole and miscellaneous which are known to posses antimicrobial activity [32, 33]. The antimicrobial activities of different solvent extracts of leaves of *T. heyneana* collected from medicinal garden of Kumaraguru College of Technology, Coimbatore, India revealed a maximum activity against *K. pneumoniae* (26±1.0 mm) and the minimum against *S. typhii* (9.0±2.0 mm) [34]. In the present study also methanolic extract exhibited highest inhibition of 12.66±0.57 mm against *Staphylococcus aureus* and least of 9.23±0.25 mm against *Proteus vulgaris.* A number of studies have been conducted on the antimicrobial properties on herbs, spices and their derivates such as essential oils, extracts and decoctions [35-37].

CONCLUSION

T. heyneana leaves possesses active pharmacological principles that contribute to free radicals scavenging and antimicrobial properties. Thus, these plants have great potential as antimicrobial agents and can be used in the treatment of infectious diseases caused by resistant microorganisms. Such screening and identification of various natural organic compounds present would possibly lead to the successful prediction of the active agent present and could further lead to drug development. Further studies are needed to isolate, characterize and elucidate the structure of the bioactive compounds present in this plant.

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CONFLICT OF INTERESTS

Declared None

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