

## Screening and quantification of phytochemicals in the leaves and flowers of *Tabernaemontana heyneana* Wall. - a near threatened medicinal plant

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The present investigation is aimed to screen and quantify the phytochemicals present in the leaves and flowers of *Tabernaemontana heyneana* Wall. (Family-Apocynaceae). Various phytochemicals distributed in the leaves and flowers of this plant were extracted by shake flask method, screened and quantified by standard protocols. The phytochemical analysis of different solvent extracts of the leaves and flowers revealed the presence of alkaloids, flavonoids, tannins, phytosterols, cardiac glycosides, terpenoids, reducing sugars and saponins. Phlobatannins was absent in both the parts. The chloroform extract of leaf and flower contained  $58.5 \pm 1.5$  mg/g tissue and  $1.5 \pm 0.5$  mg/g tissue of phytosterols, respectively. Alkaloid content was found to be  $14.6 \pm 1.7$  mg/g tissue and  $7.9 \pm 0.85$  mg/g tissue, in leaf and flower, respectively. Flavonoid content was observed to be  $4.3 \pm 0.17$  mg/g tissue and  $1.2 \pm 0.13$  mg/g tissue, in both the parts, respectively. Most of the phytochemicals were found in the leaves and flowers. High content of sterols, moderate distribution of alkaloids and low amount of flavonoids were observed in both the parts. Moreover, studies proved that the methanol and ethanol were the best solvents for the extraction of the phytochemicals.

**Keywords:** Alkaloid, Flavonoid, Phytochemical, Phytosterol, *Tabernaemontana heyneana* Wall., Apocynaceae.

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### Introduction

The vast majority of people on this planet still rely on traditional *materia medica* (medicinal plants and other materials) for everyday health care needs<sup>1</sup>. According to World Health Organization (WHO), traditional medicine is defined as inclusion of diverse health practices, approaches, knowledge and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness. The practice of traditional medicine is widespread in various countries such as China, India, Japan, Pakistan, Sri Lanka and Thailand<sup>2</sup>. The practice of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed. It is also a fact that one quarter of all medical prescriptions are formulations based on substances derived from plants or plant-derived synthetic analogs, and according to the WHO, 80% of the world's population, primarily those of developing

countries, rely on plant-derived medicines for their healthcare<sup>3</sup>. Phytochemicals are defined as bioactive non-nutrient plant compounds present in fruits, vegetables, grains and other plant foods, whose ingestion has been linked to reductions in the risk of major chronic diseases. The different compounds included in this group can be classified according to common structural features as carotenoids, phenolics, alkaloids and nitrogen containing and organosulfur compounds. Phenolics, flavonoids and phytoestrogens have raised particular interest because of their potential effects as antioxidant, antiestrogenic, anti-inflammatory, immunomodulatory, cardioprotective and anticarcinogenic compounds<sup>4</sup>. Plant secondary metabolites are unique resources for pharmaceuticals, food additives, and fine chemicals. They also provide original materials used in other areas.

*T. heyneana* Wall. (Tamil- *Kundalam Paalai*), belonging to Apocynaceae family is a small shrub that can grow up to two meters, considered as a near threatened medicinal plant and ethnobotanically have been known to possess antimicrobial, anthelmintic, antioxidant, curative properties against nervous disorders, skin problems, respiratory and eye

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ailments, venereal diseases, diabetes, chronic bronchitis, snake bite and cardiotoxic ailments<sup>5-11</sup>. Previous reports<sup>12-16</sup> have revealed the presence of flavonoids in the leaves and flowers and alkaloids, sterols, triterpenoids, flavonoids and resins in the roots and fruits. In this context, our laboratory has focused on the screening and quantification of different phytochemicals present in the leaves and flowers of this plant.

## Materials and Methods

### Plant material and extraction process

The fresh leaves and flowers of *T. heyneana*, were collected during July 2006 from the medicinal garden of Kumaraguru College of Technology, Coimbatore, India (latitude 11°08; longitude 76°99). The species was identified and confirmed by Botanical Survey of India (BSI), Southern Circle, Coimbatore, India (BSI/SC/ 5/ 23/ 06-07/ Tech. 478). The flowering season of the species is between April to June and October to November, and the fruiting season is between January to February. About 5 g of air dried leaves and fresh flowers were dissolved in 50 mL of the solvent (methanol, ethanol, distilled water, chloroform, heptane and acetone) and kept in an orbital shaker for overnight. The residue was re-extracted under the same conditions. The obtained extracts were filtered with Whatman No.1 filter paper and the filtrate was used for the experimental analysis. All the chemicals and solvents used for experimental analysis were of analytical grade.

### Phytochemical screening

Phytochemical analysis of different solvent extracts was carried out using standard procedures proposed by Sofowara<sup>17</sup>, Trease & Evans<sup>18</sup> and Harborne<sup>19</sup>.

### Quantitative determination of phytochemicals

#### Sample preparation

Different solvents ranging from highly non-polar to polar (Heptane > Chloroform > Ethanol > Methanol > Aqueous) were used to extract various phytochemicals in leaves and flower samples. In a clean dry conical flask, weighed 0.5 g of dried leaves (fresh leaves were air dried in an incubator at 37°C for two days) and fresh flowers and added 15 mL of the solvent. Homogenized and centrifuged the homogenate at 10000 rpm for 20 minutes, saved the supernatant and evaporated the content to dryness. The residue was dissolved with distilled water and used for experimental analysis.

#### Estimation of total phenolic content (TPC) by Folin-Ciocalteu method

A method proposed by Singleton & Rossi<sup>20</sup> was adopted to determine the total phenolic content. To 0.1 mL of the extract, added 3.9 mL of distilled water and 0.5 mL of Folin's reagent. The tube was incubated at room temperature for 3 minute. To this added 2 mL of 20 % sodium carbonate and kept in a boiling water bath for 1 minute. The blue color formed was read at 650 nm. Gallic acid was used as a standard for constructing a calibration curve.

#### Estimation of total flavonoid content (TFC) by aluminium chloride method

TFC was estimated spectrophotometrically as proposed by Zhishen *et al*<sup>21</sup> with slight modifications. To 0.1 mL of the extract, distilled water was added to make the volume to 5 mL. To this added 0.3 mL 5% NaNO<sub>2</sub> and 3 mL of 10% AlCl<sub>3</sub>, 5 minutes later. After 6 minutes, 2 mL of 1 M NaOH was added and the absorbance was measured at 510 nm. Rutin was used as a standard for constructing a calibration curve.

#### Estimation of tannins by modified Prussian blue method

A method proposed by Graham<sup>22</sup> was espoused to quantify the tannins. To about 0.1 mL of the extract added 6.9 ml of distilled water, 1mL of 0.008M potassium ferric cyanide, 1 mL of 0.2M ferric chloride in 0.1M HCl and mixed well. The blue color formed was read at 700 nm. Tannic acid was used as a standard for constructing a calibration curve.

#### Estimation of alkaloids

Alkaloids were estimated spectrophotometrically by the method proposed by Singh & Sah<sup>23</sup>. To 1.5 mL of the extract, distilled water was added to make the volume to 10 mL in a 25 mL standard flask. To this added 1 mL of 0.01 M sodium meta periodate (SPI), 0.5 mL of 0.1M acetic acid and kept in a boiling water bath for 10 minutes. Added 2 mL of 0.01M 3-methyl 2-benzo thiazolinone hydrazone hydrochloride (MBTH) into all the flasks and boiled in a water bath for 2 minutes. Cooled the flasks and made up to the mark with double distilled water. The blue color formed was spectrophotometrically measured at 630 nm. Caffeine was used as a standard for constructing a calibration curve.

#### Estimation of Sterols by Liberman – Burchard method

Total sterol content was measured spectrophotometrically by Liberman – Burchard method<sup>24</sup>. To 1.0 mL of the extract, chloroform was added to make the volume up to 5 mL in a test tube.

To this added 2 mL of Liberman – Burchard reagent (0.5 mL of conc. sulphuric acid in 10 mL of acetic anhydride) and mixed well. The tubes were covered with black paper and kept under dark for 15 minutes. The green color complex formed was spectrophotometrically measured at 640 nm. Cholesterol was used as a standard for constructing a calibration curve.

#### Statistical Analysis

All determinations were carried out at least in three separate experiments (triplicates). The results were expressed as means  $\pm$  SD and the mean values were plotted in all figures and Pearson correlation coefficients ( $r^2$ ) were calculated using data of each triplicate. The level of significance was expressed using Students't-Test. All the analysis was carried out using GraphPad Prism 5 software (Trial version).

#### Results

Preliminary phytochemical analysis of leaves and flower extracts of *T. heyneana* Wall. using different solvents revealed the presence of various phytochemicals as summarized in Table 1 & 2. As per the tables, alkaloids, flavonoids, steroids, terpenoids and reducing sugars were observed to be highly extracted by ethanol, methanol and acetone in both leaves and flowers, whereas, studies proved that aqueous system was potent in the extraction of

tannins and cardiac glycosides. Chloroform was found to be a significant solvent in the extraction of sterols and heptane was the poor solvent in extracting most of the phytochemicals. Saponins were moderately extracted by all the solvents in the leaves. Phlobatanins were found to be absent in both the parts. Overall results suggested that methanol and ethanol were the best solvents, distilled water to be a moderate one and chloroform and heptane were poor solvents in the extraction of the phytochemicals.

The quantitative analysis of total phenolic content in leaves and flowers were found to be  $11.4 \pm 0.17$  mg/g tissue and  $6.9 \pm 0.13$  mg/g tissue, respectively. A positive correlation has been observed between standard gallic acid concentration and the absorbance values ( $r^2 = 0.999$ ) and the phenolic content of leaves and flowers ( $r^2 = 0.970$ ). The analysis of Students't-Test was proved to be significantly different at 5% level ( $p < 0.05$ ) between leaves and flowers which revealed that leaves possessed higher phenolic content than flowers (Fig. 1).

The amount of total flavonoids present in leaves and flowers was found to be  $4.3 \pm 0.17$  mg/g tissue and  $1.2 \pm 0.13$  mg/g tissue, respectively as depicted in Fig. 2. A significant positive correlation was observed between standard rutin concentration and the absorbance values ( $r^2 = 0.995$ ) and the flavonoid concentration of leaves and flowers ( $r^2 = 0.865$ ). The

Table 1—Phytochemical analysis in the leaves of *Tabernaemontana heyneana* Wall. using various solvents

Solvent Type/ phytochemicals present	Alkaloids	Flavonoids	Tannins	Phloba Tannins	Saponins	Steroids	Terpenoids	Cardiac glycosides	Reducing sugars
Aqueous	++	++	+++	-	++	+	+	+++	-
Methanolic	+++	+++	++			+++	+++	++	+++
Ethanolic	+++	+++	++			+++	++	++	+++
Heptane	+	+	+			+	-	+	-
Chloroform	+	+	+			+++	+	+	+
Acetone	+++	+++	++			++	+++	++	+++

+ = trace amount, ++ = moderately present, +++ = highly present, - = absent

Table 2—Phytochemical analysis in the flowers of *Tabernaemontana heyneana* Wall. using various solvents

Solvent type/ phytochemicals present	Alkaloids	Flavonoids	Tannins	Phloba Tannins	Saponins	Steroids	Terpenoids	Cardiac glycosides	Reducing sugars
Aqueous	++	++	++	-	+	+	+	+++	-
Methanolic	+++	+++	++			+++	+++	+++	++
Ethanolic	+	+	++			+++	+++	++	+++
Heptane	+	-	+		-	+	-	+	-
Chloroform	+	-	+		-	+++	-	+	+
Acetone	+++	++	+++		+	+++	++	+++	+++

+ = Trace amount, ++ = moderately present, +++ = highly present, - = absent

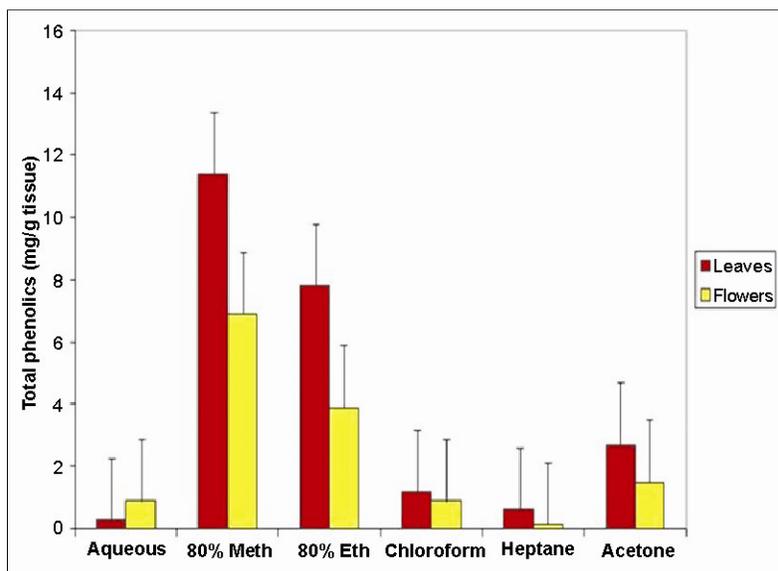


Fig. 1—Effect of various solvents in the extraction of total phenolics from the leaves and flowers of *Tabernaemontana heyneana* Wall.

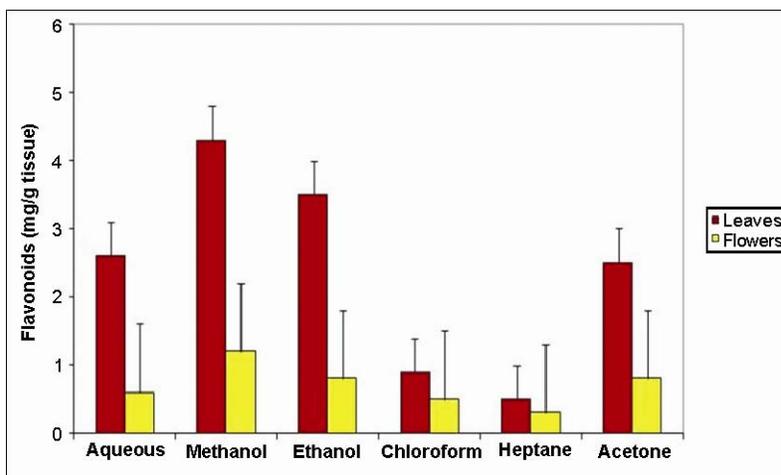


Fig. 2—Effect of various solvents in the extraction of flavonoids from the leaves and flowers of *Tabernaemontana heyneana* Wall.

Student's t-Test analysis performed was confirmed to be significantly different at 5% level ( $p < 0.05$ ), which showed that leaves possess higher flavonoid content than flowers.

The concentration of tannins present in leaves and flowers was found to be  $12 \pm 0.13$  mg/g tissue and  $6 \pm 0.17$  mg/g tissue, respectively. A remarkable positive correlation was observed between the standard tannic acid level and the absorbance values ( $r^2 = 0.999$ ), and the tannin concentration of leaves and flowers ( $r^2 = 0.962$ ). The Student's t-Test analysis carried out was established to be significantly different at 5% level ( $p < 0.05$ ) between leaves and flowers substantiate that the leaves had high tannin content than flowers.

The amount of alkaloids present in leaves and flowers was found to be  $14.6 \pm 1.7$  mg/g tissue and  $7.9 \pm 0.85$  mg/g tissue, respectively as shown in Fig. 3. A noteworthy positive correlation has been observed between standard caffeine concentration and the absorbance values ( $r^2 = 0.994$ ) and the caffeine concentration of leaves and flowers ( $r^2 = 0.827$ ). The Student's t-Test analysis carried out was established to be significantly different at 5% level ( $p < 0.05$ ) between leaves and flowers substantiate that leaves had high tannin content than flowers.

The amount of sterols present in leaves and flowers was found to be  $58.5 \pm 1.5$  mg/g tissue and  $1.5 \pm 0.15$  mg/g tissue, respectively. A significant positive correlation was observed between standard

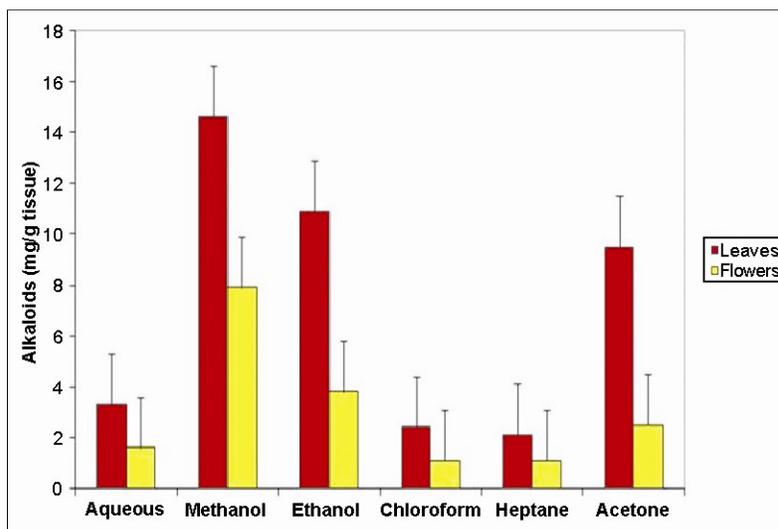


Fig. 3—Effect of various solvents in the extraction of alkaloids from the leaves and flowers of *Tabernaemontana heyneana* Wall.

cholesterol concentration and the absorbance values ( $r^2 = 0.963$ ) and the tannin concentration of leaves and flowers ( $r^2 = 0.761$ ). The Student's t-Test analysis examined has confirmed to be significantly different at 5% level ( $p < 0.05$ ) between leaves and flowers proved that leaves possessed high amount of sterols than flowers.

### Discussion

Phenolics are secondary metabolites synthesized by plants during normal development. In plants, phenolics may act as phytoalexins, antifeedants and attractants for pollinators, contributors to plant pigmentation, antioxidants and protective agents against UV light<sup>25</sup>. Simple phenolics such as hydroxycinnamic acid conjugates and flavonoids are important constituents of fruits, vegetables and beverages. These compounds show a wide range of antioxidant activities *in vitro* and are thought to exert protective effects against major diseases such as cancer and cardiovascular diseases. The Apocynaceae family plants were known to contain appreciable amounts of phenolic compounds. The order of secondary metabolites with respect to percentage of latex bearing plants are phenolics > alkaloids > cynogenic glycosides > tannins > flavonoids and saponins > terpenoids. Some authors have reported the presence of phenolics in *Tabernaemontana* species<sup>26-28</sup>. About five different phenolic acids like vanillic, gentisic, syringic, 4-hydroxybenzoic and salicylic acid have been identified and isolated from the stems of *T. coronaria*<sup>29</sup>. All these documents revealed appreciable distribution of

phenolics in the genus *Tabernaemontana* and the present study also endorsed an appreciable phenolic content in *T. heyneana*.

Flavonoids exert a broad range of biochemical and pharmacological properties, including cancer preventive activities. This effect has been endorsed by a wide variety of mechanisms, like free radical scavenging, inhibitors of lipid peroxidation, modifying enzymes that detoxify carcinogens and inhibiting the induction of the transcription factor activator protein-1 (AP-1) activity by tumor promoters<sup>30</sup>. The best described property of almost every group of flavonoids is their capacity to acts as antioxidants. Quercetin, kaempferol, morin, myricetin and rutin, by acting as antioxidants, exhibited beneficial effects such as anti-inflammatory, anti-allergic, antiviral as well as anticancer activity. They have also been suggested to play a protective role in liver diseases, cataracts and cardiovascular diseases<sup>31</sup>. Earlier investigation on the phytochemical screening of leaves, stem and root of *T. coronaria* revealed the presence of flavonoids<sup>26</sup>. Similar phytochemical screening done in the fresh methanolic leaf extract of *T. coronaria* by Mathivanan<sup>28</sup> also proved the presence of flavonoids. The detection of flavonoids in the leaves and flowers of *T. heyneana* that revealed in the present investigation were good in the agreement of the earlier observation of phytochemical screening in the leaves of *T. coronaria* and *T. citrifolia*<sup>27</sup>.

Tannins are a diverse group of polyphenols having molecular weights between 500 and 3000, and are formed as secondary metabolites in plants that include a wide range of oligomeric and polymeric

polyphenols. They have special properties such as the ability to precipitate alkaloids, gelatin and other proteins. Condensed tannins (syn. proanthocyanidins), gallotannins and ellagitannins are the most widely occurring tannins. Procyanidins are particularly abundant in the human diet and are responsible for the sensation of astringency (drying and puckering of the oral mucosa) by interacting with salivary proteins and found potent to act as  $\alpha$ -amylase inhibitors<sup>32</sup>. Condensed tannins have been demonstrated to exhibit numerous biological and pharmacological activities that are of interest in human and veterinary medicine, such as inhibition of lipid oxidation, antioxidant<sup>33</sup>, mutagenicity of carcinogens and tumor promotion. Tannins detected in the methanolic leaf extract of *T. coronaria*<sup>28</sup> are similar to the results observed in the leaves and flowers of *T. heyneana*, in contrast to an earlier report<sup>27</sup>.

Alkaloids are a diverse group of low-molecular-weight, nitrogen-containing compounds and are mostly derived from amino acids, and found in about 20% of plant species. As secondary metabolites, alkaloids are thought to play a defensive role in protecting the plant against herbivores and pathogens. Owing to their potent biological activity, approximately 12,000 known alkaloids have been exploited as pharmaceuticals, stimulants, narcotics and poisons. Alkaloids from *Tabernaemontana* species have shown hypotensive and muscle relaxant activity, antimicrobial activity against Gram-positive bacteria<sup>34</sup>, and effects of sedation, decreased respiration, decreased skeletal muscle tone, anti-leishmanial and antibacterial activities. At least 66 alkaloids were extracted from *T. divaricata* by several methods such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography-mass spectrophotometry (GC-MS)<sup>35</sup>. Alkaloids like coronaridine, voacangine, ibogamine and 19-oxocoronaridine has been isolated from the roots of *T. heyneana*. The methanolic leaf extract of *T. coronaria* has been reported to contain alkaloids<sup>28</sup>. Isolation of several unusual alkaloids like 15- $\beta$ -stemmadenine, tabernoxidine, coronaridine, voacangine and iboganine with antimicrobial activities has been reported previously<sup>13</sup>. All these review supported the distribution of alkaloids in the leaves and flowers of *T. heyneana* extracted by different solvents (ethanol, methanol, aqueous, acetone, chloroform and heptane).

Plant sterols (phytosterols), which chemically resemble cholesterol have been shown to block the

absorption of dietary and endogenously derived cholesterol from the gut. They are not synthesized by the human body and are minimally absorbed by the human intestine. Phytosterols are bioactive components of all vegetable foods. They are 28- or 29-carbon alcohols and resemble cholesterol in vertebrates in terms of both function (stabilization of phospholipid bilayers in plant cell membranes) and structure (steroid nucleus, 3 $\beta$ -hydroxyl group, 5, 6 double bond). In plants, more than 200 different types of phytosterols have been reported the most abundant being  $\beta$ -sitosterol (24- $\alpha$ -ethylcholesterol), campesterol (24- $\alpha$ -methylcholesterol) and stigmasterol ( $\Delta^{22}$ , 24- $\alpha$ -ethylcholesterol)<sup>36</sup>. The main function of phytosterols and phytostanols is to inhibit the uptake of dietary and endogenously produced cholesterol from the gut. Previous reports are there for the presence of phytosterols like campesterol, stigmasterol and sitosterol in the root bark of *T. hilariana* Müell.-Arg.<sup>37</sup>. A report made by Mathivanan *et al*<sup>28</sup> has revealed the presence of phytosterols in the ethanolic leaf extract of *T. coronaria* (Jacq.) Willd. All these scientific documentation are well correlated with the present observation regarding the presence of sterols in the leaves and flowers of *T. heyneana*.

### Conclusion

From the above results it is concluded that the leaves and flowers of *T. heyneana* contain diversified phytochemicals, except phlobatannins. Quantification has proved higher content of phytochemicals in leaves than flowers.

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