Qualitative and quantitative screening of secondary metabolites in selected medicinal plants of Sri Lanka

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Abstract
Medicinal plants are valuable source of bioactive compounds such as secondary metabolites (SMs). Sri Lanka is a bio diversity hotspot of flora specially with untouched diverse nature of medicinal plants. The present study includes four endemics and one non endemic but a rare medicinal plant of the country. The methanolic crude extracts of these medicinal plants were qualitatively and quantitatively tested for alkaloids, phenols, flavonoids and tannins. The total phenolic compounds was found significantly higher amount in all tested plants specially in both leaves and flowers, followed by flavonoids, alkaloids and tannins. The highest amount of total phenolic compounds (80.28 ± 2.99 mg GAE/g dw) and tannin (30.81± 1.37 mg TAE/g dw) was resulted in O. octandra. Similarly, a significant high amount of total alkaloid content was recorded in B. ceylanica (3.55 ± 0.08 mg AE/g dw) and followed by in leaves (2.54±0.29) and flowers (0.07 ± 0.04) of W. antidysenterica which belongs to the family Apocynaceae. The total flavonoid content was highest in P. catarhendi var tomentosa (147.37± 29.38mg RE/g dw) compare to all others. The results of this study prove that these endemic and native medicinal plants are rich reservoirs of therapeutically important SMs and can be explored them for maximum utilization in traditional and alternative medicine.

Keywords: Secondary metabolites, medicinal plants, alkaloids, tannins, phenols, flavonoids

Introduction
Medicinal plants that contain one or many SMs that can used to heal diseases [1, 2]. Medicinal plants were used to treat human diseases from the beginning of human history. During that time, the active compounds of plants were not identified. But the discovery of morphine (widely used as a pain killer) from Opium poppy plant by Friedrich Serturner in 1804 was an eye opening of scientific community for exploring the varieties of SMs for using as therapeutic agents [3]. Scientists found out that some phytochemicals in medicinal plants such as phenols, alkaloids, flavonoids and tannins have an influence on the human physiology [2, 4, 5]. Such compounds are known as bioactive compounds. Since then, medicinal plants are predominantly analyzed for their bioactive compounds. In present, 74% of nature based drugs are produced from plant derived chemical [6]. In addition, the knowledge of SMs in medicinal plants are valuable to identify economic plant products i.e. oils, gums, dyes, aromatic compounds to produce perfumes, bio pesticides, precursors to produce complex chemicals etc. SMs are low molecular weight organic chemical compounds produced inside plant cells or organelles which are not growth essentials but they are found to be implicated with plant defense in instance with phytopathogens and herbivores [7]. Also, they support the plants to withstand unfavorable environmental conditions [8]. SMs are either by-products or intermediates of plant metabolic pathways. The quantity of SMs present in plants vary between the plants of the same species [9, 10]. There are around 100,000 SMs in plant kingdom. They are categorized into three major groups according to their biochemical synthetic pathways: Nitrogen and sulfur containing molecules (alkaloids, cyanogenic glycosides and glucosinolates), Phenolics (flavonoids and tannins), and terpenoids [8]. SMs are known to cause the distinct aroma, taste, dyes and colours in plants [3]. Most importantly they have the therapeutical properties on human and animals which were a revolutionized finding alternative medicines in the past few decades [11, 12]. 80% of world population and 70% of Sri Lankans still believe and use indigenous herbal medicine for their health requirements [13, 14]. Ayurveda (Sanskrit word meaning science of life) is the herbal medicine practice in India and Sri Lanka over 5000 years [2].
Ayurveda medicine strongly relies on the medicinal plants of the region. Sri Lanka as a tropical island with different topographic and climatic regions, has a rich endemism in medicinal plants. It has been highlighted that the Sri Lankan endemic medicinal plants should be investigated more for their medicinal values before they get extinct [14]. It is high time to study the Sri Lankan medicinal plants and identifying unique SMs that can be utilized to innovate new drugs to treat human diseases and to produce other economic products. Accordingly, five medicinal plants which are having many medicinal values for different types of human diseases were selected for the current experiments for analyzing the presence of SMs qualitatively and quantitatively (Table 1). The results of this study will be valuable for applying in fields such as pharmacognosy, pharmacology, phytochemistry, natural products, ethnobotany etc.

Table 1: Medicinal value of selected plants.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Medicinal Value</th>
<th>References</th>
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<tbody>
<tr>
<td><em>Berberis ceylanica</em></td>
<td>Schneider The stem extractions are prepared as tinctures (dissolving the drug in alcohol) and made it drink for the patients to treat fever, snake bites, syphilis, menorrhagia, jaundice and enlargement of liver and spleen.</td>
<td>[15, 16]</td>
</tr>
<tr>
<td><em>Walidda antidyssenterica</em></td>
<td>(L) M.Pichon Leaves and flowers are used for the treatment of ailments after delivery, abscess in genital organs, gonorrhea. The bark and leaves are used together to cure tonsillitis and bronchitis. The bark alone used for snake bites such as Russel's viper bite. The roots are used to treat epilepsy, chronic fever, cough, hemorrhoids and jaundice. The latex is used for toothy and gum ailments.</td>
<td>[17]</td>
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<tr>
<td><em>Osbeckia octandra</em></td>
<td>(L) DC Known to have Hepatoprotective, antidiabetic and antimicrobial properties. The leaf curvy of this herb is recommended as a treatment for jaundice. It is also used for hemorrhoids, ascites, hyperlipidemia and cancer</td>
<td>[18]</td>
</tr>
<tr>
<td><em>Valeriana moonii</em></td>
<td>(Am.ex Clarke) Whole plant is used to cure Alopecia, cancer, convulsion, cramp, debility, dysuria, epilepsy, Escherichia and snake bites</td>
<td>[19]</td>
</tr>
<tr>
<td><em>Plectranthus zatarhendi</em></td>
<td>(Frossk.) E.A.Bruce var tomentosa (Benth.) Codd Whole plant is used to cure fevers, dysentery, diarrhea, vomiting, excessive thirst, congestion of the liver, tarantula bites, abscess and heart diseases</td>
<td>[20]</td>
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Materials and Methodology
Preparation of crude extract
Fresh plant materials of selected plants were collected from different region of the country. All plants were identified and authenticated by Uva Provincial Department of Ayurveda, Ayurveda Commissioner’s office, Diyatalawa, Badulla, Sri Lanka. The collected plant parts were cleaned and shade dried for 2-3 weeks at room temperature (30°C) to obtain complete dryness. Dried plant materials were ground well using mechanical blender [21]. The resulting coarse powders were sieved through 212 micron sieve to get fine powders and stored properly in labeled air tight dark glass bottles at 4°C until use. A 10 g of each powdered plant material was soaked in 100 ml of 80% methanol and they were placed in mechanical shaker at 350 rpm for overnight. It was filtered using whatman filter paper No.1 and filtrate was placed in the rotary evaporator at 40°C to obtain a crude extract by evaporating the excess methanol [21, 22].

Qualitative screening
Alkaloids
Few drops of dilute HCl were added to 1ml of the plant extract and mixed well. One or two drops of freshly prepared Mayer’s reagent was added to it. Appearance of white precipitate indicated the presence of alkaloids [5, 11].

Phenolic compounds
FeCl₃ test- Three to four drops of 5% FeCl₃ solution were added to the test tube containing 2ml of plant extract. Appearance of blue-black or blue-green colour indicated the presence of phenols in the extract [5, 25].

Flavonoids
Alkaline reagent’s test- Two to three drops of NaOH (2N) were mixed with 1ml of plant extract. Appearance of intense yellow colour that disappears with the addition of diluted HCl and giving a clear solution was taken as a positive result for flavonoids [11, 25].

Lead acetate test: 1 ml of the plant extract was diluted with 2 ml of distilled water and 3 ml of 10% lead acetate was mixed with diluted plant extract. The presence of flavonoids was indicated as a bulky white precipitate [26].

Tannins
Wohler’s test- 1 ml plant extract was diluted by adding 9 ml of distilled water. The diluted extract (1.6 ml) was mixed with a 5 – 6 drops of 1% lead acetate. A white precipitate showed the presence of tannins [5]. Ferric chloride test- 10ml of distilled water was added to 1ml of plant extract and was boiled well after mixing. A few drops of filtrated solution was taken in a separate test tube and mixed with 1% ferric chloride. Appearance of bluish black or bluish green colour showed the presence of tannins [1].

Quantitative screening
Estimation of total tannin content
0.5ml plant extract was mixed with 0.25ml of Folin denis reagent, saturated Na₂CO₃ and 3.5ml of distilled water. The absorbance was taken within 30 minutes at 700 nm. Total tannin content in extracts were expressed as tannic acid equivalents (mg of TAE/g of extract) [34, 38].

Estimation of total Flavonoids
A 0.5ml of plant extract was mixed with 2ml of distilled water, 0.15ml of NaNO₂, 0.15ml of AlCl₃ and 2ml of NaOH. taken. After 15 minutes, the absorbance was recorded at 510 nm [34, 35]. Total flavonoid content in extracts were mentioned as rutin equivalents (mg of RE/g of extract) [21].

Estimation of total phenolic compounds
A 0.5ml of plant extract, 5ml of 10% Folin ciocalteau reagent and 4ml of 1M Na₂CO₃ were placed into a 50ml volumetric flask. The final volume was adjusted with distilled water. A 0.5 ml of mixture was withdrawn at the end of 30 minutes and its absorbance was measured at 760 nm using a blank solution without adding the plant extract [32]. Finally, the phenol concentration was determined by using the standard curve, then the total phenolic contents were stated as gallic acid equivalents (mg of GAE/g of extract) [29].
Estimation of total alkaloids
A 0.5 ml of plant extract, 5 ml of phosphate buffer (pH 4.7) and 5 ml of bromo cresol green were added in a separatory funnel and it was shaken vigorously with 5 ml of chloroform. The chloroform layer at bottom was carefully removed and its absorbance was measured at 470nm in the presence of blank without adding the plant extract [22, 29]. The alkaloid concentration was determined as atropine equivalents (mg of AE/g of extract) with reference to the standard curve of atropine.

Statistical Analysis
All experiments were carried out in three replicates and presented as mean± standard error (SE) using Excel. All graphs were plotted using Microsoft excel. One way analysis of variance (ANOVA) and statistically significant was considered at P = 0.05. Pearson correlation test was applied to determine the correlation coefficient value (R²).

Results and Discussion
The present study reveals the presence of SMs such as alkaloids, phenolic compounds, flavonoids and tannins in variable amount in the selected endemic and native medicinal plants of the country.

All extractions were carried out using methanol extractions. It has proven that organic solvents such as ethanol and methanol or a mixture of organic solvents with water would give the best yield of phenolic compounds [33]. Many literature of similar studies have demonstrated the use of methanol as an extracting solvent/ medium [21, 34]. Similarly, due to the fact that flavonoids and tannins are also coming under the phenolic group, methanol was proven as the suitable solvent [39]. In the case of alkaloids, they are present in plants as salts of organic and inorganic acids together with other water soluble compounds such as proteins, minerals etc. Therefore, it is more preferable to use water and alcohol mixtures to extract alkaloids [40]. Moreover, it has been mentioned that most alkaloids in plants are soluble in methanol or ethanol [44]. The details about the chemical constituents of new plants which have not been subjected to a similar study are generally investigated by conducting a qualitative screening. In this study, qualitative analysis showed some notable results for all the plant extractions as shown in Table 2. Two standard tests for each metabolite were used to ensure the accuracy of the results. At least a single test gave positive result for a particular metabolite hence subsequently, that extraction was subjected to the quantitative estimation. O. octandra gave no reaction for Mayer’s test but it gave moderate positive reaction for Dragendorff’s test. Similarly, V. moonii, leaves and flowers of W. antidysenterica gave no reaction to Dragendorff’s test but they gave weak positive reaction to Mayer’s test. Both these reagents test the presence of alkaloids and they react with different types of alkaloids i.e., Dragendorff’s reagent react mostly with tertiary amines and Mayer’s reagent react with many alkaloids including tertiary amines [41]. It was clearly seen in the results of qualitative screening, that the selected medicinal plants are having all four SMs of interest.

The quantitative estimation of the SMs showed that they all exist in plants in different quantities. In general, phenols and flavonoids are omnipresent in the plant kingdom [41]. The phenolic compounds were found in highest quantity in all tested plants, followed by flavonoids, alkaloids and tannins respectively as shown in Table 3. Spectrophotometric determination of total alkaloids using BCG is a simple and a sensitive method. BCG forms a yellow colour complex with alkaloids and it is completely extractable in chloroform at pH 4.7 [32]. BCG reacts with the alkaloids that contain nitrogen atom within its cyclic structure. But it cannot react with amine and amide alkaloids which causes a limitation in this method. Therefore, the obtained results are giving an overall idea about the total alkaloid contents in the plants [22, 42]. The colour was allowed to develop for a constant time and the absorbance were measured at 700nm.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Plant</th>
<th>B. ceylanica</th>
<th>Leaves of W. antidysenterica</th>
<th>Flowers of W. antidysenterica</th>
<th>O. octandra</th>
<th>V. moonii</th>
<th>P. zatarhendi var tomentosa</th>
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<tr>
<td>Mayer’s test</td>
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<td>Dragendorff’s test</td>
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<td>Ferric chloride test</td>
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<td>Alkaline reagent test</td>
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<td>Wohler’s test</td>
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<td>Tannin Ferric chloride test</td>
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(++++ strong positive reaction, ++ moderate positive reaction, + weak positive reaction, - no reaction).

Table 2: Qualitative test for alkaloids, phenolic compounds, flavonoids and tannin of crude extract

Table 3: Total contents of different SMs in the studied medicinal plants.

<table>
<thead>
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<th>Plant</th>
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<tr>
<td>B. ceylanica</td>
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<tr>
<td>3.56 ± 0.08</td>
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<tr>
<td>Leaves of W. antidysenterica</td>
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<tr>
<td>Flowers of W. antidysenterica</td>
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<tr>
<td>O. octandra</td>
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<tr>
<td>V. moonii</td>
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<tr>
<td>P. zatarhendi var tomentosa</td>
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The total tannin content in the medicinal plants extracts were expressed as mg tannic acid equivalent/g dry weight of the sample using tannic acid standard curve (γ=3.36x+0.04, R² = 0.96). Usually, tannins are abundant in parts which are more vulnerable for herbivores i.e., young leaves, flowers etc. because tannins are good defensive chemicals that act as toxins and feeding repellents for most herbivores [47]. But flowers of W. antidysenterica showed very low tannin content and it had no significant difference with total tannin contents of B. ceylanica, V. moonii, P. zatarhendi var tomentosa. Similarly, Tannins are largely found in the bark (specifically, in the layer between cortex and epidermis) protecting the
plants by forming a barrier for microorganisms. The only stem extraction in this study gave less total tannin content. It might be because the bark of the stems used for this study were removed prior to drying. In leaf tissues tannins are mostly present in the upper epidermis of young leaves. But in the leaves of evergreen plants they are equally distributed [44]. As shown in figure 1(a), O. octandra was having the richest tannin content (30.81± 1.37). When compared to all other metabolites, tannin content was relatively low in selected parts of every selected medicinal plant.

To determine total flavonoid content Aluminum chloride spectrophotometric method was used. It is a simple, precise and a widely used method. Aluminum chloride undergoes a complexation reaction with flavonoids. For that NaNO₂ should be present in alkaline medium. At first, the aromatic ring having a catechol group (unsaturated six carbon ring with two hydroxyl groups attached to adjacent carbons) in its third or fourth position unsubstituted or not sterically blocked, undergoes nitration. AlCl₃ is added afterwards [4]. Then, Al³⁺ reacts with hydroxyl groups of the flavonoid. It forms the yellow coloured complex which immediately turned to reddish colour with addition of NaOH. The intensity of colours are proportional to the flavonoid concentration in the extraction [33].

The total flavonoid content in the medicinal plants extracts were expressed as mg rutin equivalent/g dry weight of the sample using rutin standard curve (y=0.84x+0.00, R² = 0.97). As shown in figure 1(b), P. zatarhendi var tomentosa was having the richest flavonoid content (147.37± 29.36). That may be the reason for its very pleasant aroma. Because flavonoids are responsible for the colour, aroma and flavor of plants [30]. O. octandra was having (114.35 ± 11.61) the second highest flavonoid content. It is recorded that the plants grow in tropical countries and in high altitudes are having higher flavonoid content than the plants growing in the temperate region. Because flavonoids accumulate in the plant leaves to protect them from UV-exposure [31]. Both these plants were collected from the mountain regions of the country, thus they get overexposure to the UV radiation. Based on the results of mean separation at 95% confidence level, there were no significant difference of flavonoid content in B. ceylanica, V. moonii, leaves and flowers of W. antidysenterica and they all contain low flavonoid content (Table 3).

UV-Vis spectroscopy has proven as one of the best and accurate procedures to quantify the phenolic content in plant extracts. Mainly because of the chemical structure of phenols i.e., phenol ring is capable to absorb UV light [44]. Folin-ciocalteu method was used to quantify the phenol content, ever since it was introduced by Otto Folin and Vintila ciocalteu in 1927 with little modifications [45]. Folin-ciocalteu reagent is a mixture of two acids; phosphotungstic (H₃PW₁₂O₄₀) and phosphomolybdic (H₃PMO₁₂O₄₀). They efficiently react with phenols in the plant extract and form phosphotungstic-phosphomolybdic complex which is blue in colour. It is quantified at 760nm [44]. In this method it is very important to follow the order of chemical addition to the extract. It helps to ensure that the reaction takes in alkaline medium (Na₂CO₃ is added after the addition of folin-ciocalteu reagent). The maximum absorption is depending on the alkaline nature of the medium and the phenol concentration in the extract. This reagent is decomposable in the alkaline solution. Therefore, excess amount of folin-ciocalteu reagent need to be added to allow the completion of the reaction. The reagent already contains lithium salts to prevent the buildup of turbidity or precipitates due to excess addition [46].

Fig 1: Total content of different secondary metabolites; tannin (1a), flavonoid (1b), phenol (1c) and alkaloid (1d) in tested medicinal plants.
The total phenol content in the medicinal plant extracts were expressed as mg gallic acid equivalent/g dry weight of the sample using the gallic acid standard curve \((y=6.48x+0.01, \quad R^2=0.99)\). Phenols are extensively found in higher plants [47]. They protect the plants from UV radiation through their radical scavenging ability and from herbivores by acting as enzyme inhibitors and feeding deterrents [46]. The highest phenol content was measured in O. octandra (80.28 ± 3.00).

Whereas, the lowest phenolic content was measured in B. ceylanica (7.75±2.30) as illustrated in figure 1(c). According to the results of mean separation at 95% confidence interval, there was no significant difference between the phenolic content in leaves of W. antidysenterica and V. moonii. Similarly, it showed that flowers of W. antidysenterica and P. zatarhendi var tomentosa had no significant difference in their phenolic content at 95% confidence interval.

The alkaloid content in plant extracts were expressed as mg atropine equivalent/g dry weight of the sample using atropine standard curve \((y=1.37x+0.03, \quad R^2=0.98)\). As shown in the Figure 1(d), the highest alkaloid content was measured in the extraction obtained from the stems of B. ceylanica (3.56 ± 0.08). In angiosperms some plant families such as Paparvaceae, Fabaceae, Boraginaceae, Asclepiadaceae, Asteracea, Lilaceae, Gentaceae, Ranunculaceae, Rubiaceae, Solanaceae, Rutaceae, Apocynaceae and Berberidaceae are known to have rich content of alkaloids [43]. Genus Berberis of family Berberidaceae is well known in the world for its high content of isoquinoline alkaloid berberin [16]. B. ceylanica is one among the three species of that genus that grow in Sri Lanka. The second highest amount of alkaloid was recorded from the leaves of W. antidysenterica (2.535±0.297) which belongs to the family Apocynaceae. The lowest was in the extraction of Flowers of W. antidysenterica (0.070 ± 0.039) (Table 3).

The distribution of SMs are vary among the parts of plants tested. Phenol is the major secondary metabolite in flowers of medicinal plants that used for this experiment and followed by the presence of flavonoid (fig. 2). However, phenol and flavonoid are dominantly prevailing substances in leaves compared to alkaloid and tannin. The figure 2 reveals that more alkaloid can be extracted from leaves.

Different SMs in plants have provided an outstanding contribution to the pharmaceutical industry [4, 45]. It shows the possible relationship of quantified SMs with the medicinal values of these plants proving that these endemic and native medicinal plants are rich reservoirs of therapeutically important SMs.

**Conclusions**

The present study revealed that the stems of B. ceylanica, leaves of W. antidysenterica, flowers of W. antidysenterica, O. octandra, V. moonii and P. zatarhendi var tomentosa contains significant quantities of alkaloids, phenols, flavonoids and tannins. The highest alkaloid content was recorded in B. ceylanica stem extraction. O. octandra was having the highest phenol and tannin content. The highest flavonoid content was recorded in P. zatarhendi var tomentosa plant extraction. The SMs found in these valuable medicinal plants should be further studied to isolate, identify, and elucidate the structures and to produce them chemically or biotechnologically to encourage their utilization in pharmaceutical and other possible industries. Due to the fact that this study contains four endemics and one native but a rare plant, scientists should urge to study them more as well as to distribute and conserve them for the future generations.

**5. References**


