DETERMINATION OF IN VITRO ANTIOXIDANT ACTIVITY OF HYDROCOTYLE JAVANICA THUMB WHOLE PLANT EXTRACTS

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ABSTRACT
Antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of Hydrocotyle javanica whole plant have tested using various antioxidant model systems viz., DPPH, hydroxyl, superoxide and ABTS. Methanol extract of H. javanica is found to possess highest DPPH, hydroxyl and ABTS radical cation scavenging activities. The superoxide radical scavenging activity of H. javanica, ethanol extracts records the highest. Like the antioxidant activity, reducing power of the extract increases with increase in concentration. This study indicates significant free radical scavenging potential of H. javanica whole plant which can be exploited for the treatment of various free radical mediated ailments.

KEYWORDS: Hydroxyl, Flavonoid, DPPH, Methanol.

INTRODUCTION
Reactive Oxygen Species (ROS) are constantly formed in the human body by normal metabolic action and these are exert oxidative damaging effects by reacting with nearly every molecule found in living cells including nucleic acids, proteins, lipids or DNA and may involve in several chronic and degenerative diseases including gastritis, reperfusion injury of many tissues, atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others. If excess ROS and free radicals are not eliminated by endogenous antioxidant system.
An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Currently available synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone (TBHQ) and gallic acid esters, have been suspected to cause or prompt negative health effects. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Moreover, these synthetic antioxidants also show low solubility and moderate antioxidant activity.[3,4] Recently, interest has increased considerably in funding naturally occurring antioxidant for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity.[5,6]

The whole plant of the Hydrocotyle species (Family – Apiaceae) are often used in Taiwan folk medicine for treating common cold, tonsillitis, cephalitis, enteritis, dysentery, zoster, eczema, period pain, hepatitis and jaundice. Hydrocotyle javanica is a naturally growing perennial herb. Traditionally, the fresh plant parts of H. javanica are used to crushed and ingested orally to cure sores of throats and lungs. Leaf juice is often used as eye drops to cure eye infection and leaf paste was used in dressing of wounds to reduce swelling and juice of shoots can treat gastritis and constipation.[7] However, no systematic attempts have been made to establish scientific basis of beneficial effects of H. javanica whole plant extracts. To our knowledge, no reports on the in vitro antioxidant activity of H. javanica whole plant. This study was therefore undertaken to evaluate the effect of five solvent extracts of whole plant of H. javanica on in vitro antioxidant activity.

MATERIALS AND METHODS

Collection of plant samples
The whole plant of Hydrocotyle javanica Thumb was collected from Melpallam, Kodaikanal Block, Dinidigul District, Tamil Nadu. With the help of local flora, voucher specimen were identified and preserved in the P.G. Department of Botany, A.P.A. College of Arts and Culture, Palani for further research.

Preparation of extracts for phytochemical analysis
Freshly collected whole plant sample of H. javanica were dried in shade and then coarsely powdered separately in a Wiley mill. The coarse powder (100g) was extracted successively with petroleum ether, benzene, ethyl acetate, methanol and ethanol, each 250ml in a Soxhlet apparatus for 24hrs. All the extracts were filtered through Whatman No. 41 filter paper and
all the extracts were concentrated in a rotary evaporator and used for in vitro antioxidant activity. The concentrated methanol extract was used for estimation of total phenolic and flavonoid content.

**Estimation of total phenolic content**

Total phenolic contents were estimated using Folin-Ciocalteau reagent based assay as previously described\(^8\) with little modification. To 1ml of each extract (100µg/ml) in methanol, 5ml of Folin-Ciocalteau reagent (diluted ten-fold) and 4ml (75g/L) of Na\(_2\)CO\(_3\) were added. The mixture was allowed to stand at 20°C for 30 min and the absorbance of the developed colour was recorded at 765 nm using UV-VIS spectrophotometer. 1 ml aliquots of 20, 40, 60, 80, 100µg/ml methanolic gallic acid solutions were used as standard for calibration curve. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE g/100g dry weight of extract).

**Estimation of flavonoids**

The flavonoids content was determined according to Eom et al.\(^9\) An aliquot of 0.5ml of sample (1mg/ml) was mixed with 0.1ml of 10% aluminium chloride and 0.1 ml of potassium acetate (1 M). In this mixture, 4.3ml of 80% methanol was added to make 5 ml volume. This mixture was vortexed and the absorbance was measured spectrophotometrically at 415 nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

**DPPH radical scavenging activity**

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H.\(^10\)

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method. Briefly, an 0.1 mM solution of DPPH in methanol was prepared, and 1ml of this solution was added to 3 ml of the solution of all extracts at different concentration (50, 100, 200, 400 & 800µg/ml). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517nm using a UV-VIS spectrophotometer (Genesys
10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

\[
% \text{ inhibition} = \left\{ \frac{(A_0 - A_1)}{A_0} \right\} \times 100
\]

Where, \(A_0\) is the absorbance of the control reaction, and \(A_1\) is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

**Hydroxyl radical scavenging activity**

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell *et al.*\(^{[11]}\) Stock solutions of EDTA (1 mM), FeCl\(_3\) (10 mM), Ascorbic Acid (1 mM), H\(_2\)O\(_2\) (10 mM) and Deoxyribose (10 mM) were prepared in distilled deionized water.

The assay was performed by adding 0.1ml EDTA, 0.01ml of FeCl\(_3\), 0.1ml H\(_2\)O\(_2\), 0.36ml of deoxyribose, 1.0ml of the extract of different concentration (50,100,200,400 & 800\(\mu\)g/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50mM, pH 7.9), 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37\(^{0}\)C for 1 hour. 1.0ml portion of the incubated mixture was mixed with 1.0ml of 10\%TCA and 1.0ml of 0.5\% TBA (in 0.025 M NaOH containing 0.025\% BHA) to develop the pink chromogen measured at 532 nm. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

**Superoxide radical scavenging activity**

The superoxide anion scavenging activity was measured as described by Srinivasan *et al.*\(^{[12]}\) The superoxide anion radicals were generated in 3.0ml of Tris – HCL buffer (16mM, pH 8.0), containing 0.5mL of NBT (0.3mM), 0.5mL NADH (0.936 mM) solution, 1.0 ml extract of different concentration (50,100,200,400 & 800\(\mu\)g/ml), and 0.5ml Tris – HCl buffer (16mM, pH 8.0). The reaction was started by adding 0.5 ml PMS solution (0.12mM) to the mixture, incubated at 25\(^{0}\)C for 5min and the absorbance was measured at 560nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.
Antioxidant activity by radical cation (ABTS +)

ABTS assay was based on the slightly modified method of Huang et al.\textsuperscript{[13]} ABTS radical cation (ABTS+) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS + solution were diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. After addition of sample or trolox standard to 3.9 ml of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC). The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Reducing power

The reducing power of the extract was determined by the method of Kumar and Hemalatha.\textsuperscript{[14]} 1.0ml of solution containing 50, 100, 200, 400 & 800 μg/ml of extract was mixed with sodium phosphate buffer (5.0ml, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 ml, 1.0%): The mixture was incubated at 50°C for 20 minutes. Then 5ml of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5°C) in a refrigerator centrifuge. The upper layer of the solution (5.0 ml) was diluted with 5.0 ml of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

RESULTS

Total phenolic and total flavonoid content

The total phenolic and flavonoid content of the methanol extract of \textit{H. javanica} whole plant was found to be 1.02g 100g\textsuperscript{-1} and 1.14g 100g\textsuperscript{-1} respectively.

DPPH radical scavenging activity

DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of \textit{H. javanica} whole plant was shown in figure 1. The scavenging effect increases with the concentration of standard and samples. Among the solvent tested, methanol extract exhibited highest DPPH radical scavenging activity. At 800µg/ml concentration methanol extract of \textit{H. javanica} possessed 131.14% scavenging activity on DPPH. The concentration of \textit{H. javanica} methanol extract needed for 50% inhibition (IC\textsubscript{50}) was found to be 39.60µg/ml, whereas 29.15µg/ml was needed for ascorbic acid (Table 1).
Hydroxyl radical scavenging activity
Hydroxyl radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *H. javanica* whole plant was shown in Fig. 2. Methanol extract showed very potent activity. At 800µg/ml concentration, *H. javanica* possessed 113.16% scavenging activity on hydroxyl radical. The IC$_{50}$ value of methanol extract of *H. javanica* on hydroxyl radical were found to be 29.15µg/ml and 28.16µg/ml for ascorbic acid, respectively (Table 1).

Superoxide radical scavenging activity
The different solvent extracts of *H. javanica* whole plant were subjected to be superoxide scavenging assay and the results were shown in figure 3. It indicates that ethanol extract of *H. javanica* whole plant (800µg/ml) exhibited the maximum superoxide radical scavenging activity of 149.36% which is higher than the standard ascorbic acid whose scavenging effect is 103.11%. The quantity of *H. javanica* ethanol extract required to produce 50% inhibition of superoxide radical was 40.18µg/ml and 29.88µg/ml for ascorbic acid (Table 1).

ABTs radical cation scavenging activity
The different solvent extracts of *H. javanica* whole plant were subjected to be ABTs radical cation scavenging activity and the results were shown in figure 4. The methanol extract exhibited potent ABTs radical cation scavenging activity in concentration dependent manner. At 800 µg/ml concentration, *H. javanica* whole plant possessed 116.92% scavenging activity on ABTs which is higher than the standard trolox whose scavenging activity is 103.16%. The IC$_{50}$ value of methanol extract of *H. javanica* on ABTs radical were found to be 32.16µg/ml and 29.16µg/ml for trolox, respectively (Table 1).

Reducing Power
Figure 5, showed the reducing ability of different solvent extracts of *H. javanica* whole plant compared to ascorbic acid. Absorbance of the solution was increased when the concentration increased. A higher absorbance indicates a higher reducing power. Among the solvent tested, methanol extract exhibited higher reducing activity.
Table 1: IC₅₀ values of different solvent extracts of *Hydrocotyle javanica* whole plant.

<table>
<thead>
<tr>
<th>Different Solvent extract</th>
<th>IC₅₀ (µg/ml)</th>
<th>DPPH assay</th>
<th>Hydroxyl assay</th>
<th>Superoxide assay</th>
<th>ABTS assay</th>
</tr>
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<tbody>
<tr>
<td>Petroleum ether</td>
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<td>21.50</td>
<td>27.93</td>
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<td>31.88</td>
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<td>Ethanol</td>
<td>31.60</td>
<td>26.96</td>
<td>34.16</td>
<td>28.15</td>
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<tr>
<td>Standard (Ascorbic acid)</td>
<td>29.15</td>
<td>28.16</td>
<td>29.88</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Standard (Trolox)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>29.16</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1: DPPH radical scavenging activity of different solvent extracts of *Hydrocotyle javanica*.

Fig. 2: Hydroxyl radical scavenging activity of different solvent extracts of *Hydrocotyle javanica*. 
Fig. 3: Superoxide radical scavenging activity of different solvent extracts of *Hydrocotyle javanica*.

Fig. 4: ABTs radical scavenging activity of different solvent extracts of *Hydrocotyle javanica*.

Fig. 5: Reducing power assay of different solvent extracts of *Hydrocotyle javanica*. 
DISCUSSION

Phenolics have attracted a great attention in relation to their potential for beneficial effects on health. Over the last few years, several experimental studies have revealed biological and pharmacological properties of phenolic compounds, especially their antimicrobial activity, antiviral, antiinflammatory and cytotoxic activity. It is a well documented fact that most medicinal plants are enriched with phenolic compounds and bioflavonoids that have excellent antioxidant properties. Phenolics are active incurring kidney and stomach problems as well as helpful as antiinflammatory in action.

Flavonoids are a group of polyphenolic compounds which influence the radical scavenging, inhibition of hydrolytic and oxidative enzymes and also act as antiinflammatory agent. The flavonoids show antioxidant activity and their effects on human nutrition and health is considerate. The mechanism of action of flavonoids are through scavenging or chelating process, they also inhibit microbes which are resistant to antibiotics. Flavonoids are free radical scavengers, super antioxidants and potent water soluble which prevent oxidative cell damage and have strong anticancer activity. The presence of these compounds such as total phenolics and flavonoids in H. javanica whole plant may give credence to its local usage for the management of oxidative stress induced ailments.

In vitro antioxidant activity of the petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of H. javanica whole plant were investigated in the present study by DPPH, hydroxyl, superoxide and ABTS radical cation scavenging activities. These methods have proven the effectiveness of the extracts in comparison to that of the reference standard antioxidants, ascorbic acid and trolox.

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Hence DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants. In the present study, the results revealed that the ethanol extract of H. javanica whole plant exhibited the highest DPPH radical scavenging activity with 131.60% followed by its benzene extract with 106.84%. These results indicated that the extracts have a noticeable effect on scavenging free radicals.
Hydroxyl radicals are major active oxygen species causing lipid peroxidation and enormous biological damage. Hydroxyl radical scavenging capacity of *H. javanica* whole plant extract is directly related to its antioxidant activity. This method involved *in vitro* generation of hydroxyl radicals using Fe$^{3+}$/ascorbate/EDTA/H$_2$O$_2$ system using Fenton reaction. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe$^{2+}$) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogan with thiobarbituric acid$^{[26]}$. When *H. javanica* extract was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction. Among the solvent tested, methanol extract possessed more hydroxyl radical scavenging activity when compared with standard ascorbic acid.

In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture.$^{[27]}$ In the process study, methanol extract of *H. javanica* showed potent superoxide radical scavenging activity.

ABTs radical cation scavenging activity is relatively recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTs for the estimation of antioxidant activity.$^{[13]}$ The present study, methanol extract of whole plant of *H. javanica* was fast and effective scavenging of ABTs radical and this activity was higher than that of trolox standard. Proton radical scavenging is an important radical has characteristic absorbance maxima at 734nm which decreases with the scavenging of the proton radicals.$^{[28]}$

Several reports indicated that the reducing power of bioactive compounds was associated with antioxidant activity.$^{[29]}$ Therefore, it is necessary to determine the reducing power of phenolic constituents contained in the plant extracts to elucidate the relationship between their antioxidant effect and their reducing power. In the present study, increase in absorbance of the reaction mixture indicates the reductive capabilities of *H. javanica* extract in concentration dependent manner when compared to the standard ascorbic acid.
As a conclusion, the methanol extract of *H. javanica* whole plant showed strong antioxidant activity, reducing power, DPPH radical, hydroxyl, superoxide and ABTs radical cation scavenging activities when compared to standards such as ascorbic acid and trolox. The results of this study show that the methanol extract of *H. javanica* can be used as easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. However, the phenolic compounds or other compounds responsible for the antioxidant activity of methanol extract of *H. javanica* are already unknown. Therefore, it is suggested that further work could be perfomed on the isolation and identification of the antioxidant compounds in *H. javanica* whole plant.

REFERENCES


