EXPLORATION OF IN VITRO ANTIOXIDANT, THROMBOLYTIC ACTIVITY, NEUROPHARMACOLOGICAL AND ANTI-PYRETIC ACTIVITY OF LEAF EXTRACTS OF HOYA PARASITICA (WALL.)

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A B S T R A C T

The objective of the present study was to investigate the in vitro phytochemical screenings, thrombolytic, antioxidant, in vivo neuropharmacological and anti-pyretic activity of leaf extracts of Hoya parasitica Wall. Preliminary phytochemical screening was done for determining the nature of phytoconstituents or bioactive constitutes, which were assessed for their possible clot lysis, antioxidant, neuropharmacological and anti-pyretic activities as compared with the known drugs. Different classes of phytochemicals were present in the leaf extract. The methanol extract exhibited highest percentage of clot lysis (14.67%) as compared to 28.26% clot lysis produced by standard streptokinase. Among the three extracts, methanol and ethanol extracts showed relatively better cupric reducing antioxidant capacity with IC₅₀ value (61.15 µg/ml) and satisfactory antioxidant potential in hydrogen peroxide scavenging assay with IC₅₀ value (61.48 µg/ml) in comparison with L-ascorbic acid. Chloroform extract of H. parasitica was found to possess the highest total antioxidant capacity (1.86 mg/gm). Ethanol and chloroform extracts (100 and 200 mg/kg body weight) and methanol extracts (200 mg/kg body weight) shortened the immobility period significantly (**p<0.01; ***p<0.001) in comparison with standard. In open field test, ethanol extracts (100 & 200 mg/kg body weight) significantly (**p<0.01) decreased the rate of movement with time in a dose dependent manner. Significant (*p<0.05) antipyretic activity was observed by methanol and chloroform extracts in a dose dependent manner compared to standard. It can be concluded from the plant extracts of H. parasitica have significant thrombolytic, antioxidant, neuropharmacological and anti-pyretic activity which justifies its use as traditional medicine.

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INTRODUCTION

Hoya parasitica Wall is a climbing epiphyte of the family Asclepiadaceae is one of 300 species in the genus Hoya locally known as Chera pata, Pargacha, Fassya gaas (Rahman et al., 2007) in Bangladesh. It is an evergreen tropical perennial shrub native to tropical wet forests and humid climate of southern Asia, Singapore, the Andaman Island (Khatun et al., 2014) Australia, Polynesia (Reza et al., 2007), Bangladesh (Sadhu et al., 2008). It is a parasite creeper with a fragrant flower. They are evergreen perennial vines or rarely shrubs. Its stems are stout or slender, glabrous. Its leaves are ovate elliptic or lanceolate and acuminate penduncles are solitary or in pairs short or long slender or stout, pedicelsslender long glabrous, coronal processes longer than the corolla tube. The plant bears aesthetic flowers in May to June. Mukherjee et al., (1986) reported the plant to contain triterpenic 3,4-seco acid 3,4-secolup-20(29)-en-3-oic acid, along with lupeol and lupenone from stem and Sadhu et al., (2008) reported to contain an androstanoïd, a sesquiterpene, and a phenolic compound, together with a known triterpene, dihydrocanaric acid. According to Khatun et al., (2014) the steam extracts of H. parasitica was found to contain Reducing sugar, alkaloid, steroid, tannin, flavonoid, saponin. This plant has also been used in traditional medicine for the treatment of constipation (Hossan et al., 2009), bronchitis, diabetes, urinary tract disorders, frequent or infrequent urination, kidney disorders, bleeding, paralysis, rheumatic pain (Hanif et al., 2009; Biswas et al., 2010) fever, body pain (Khisha et al., 2012; Rahman et al., 2007), jaundice (Rahman et al., 2007), antirheumatic and in acute renal failure (Ahmed, 1997). H. parasitica has antibacterial, antinociceptive, cytotoxicity, activity and growth inhibitory effects of dihydrocanaric acid against both HeLa and SW480 cells (Ahmed et al., 2008;
Khatun et al., 2014; Reza et al., 2007; Mukherjee et al., 1986). The leave extracts of H. parasitica demonstrated to have in vitro antioxidant activity, membrane stabilizing activity and in vivo gastrointestinal motility with no potential acute toxicity (Tania et al., 2016). As a part of our continuing studies on H. parasitica, the organic soluble materials of the leaf extracts of H. parasitica were evaluated for Phytochemical screening, thrombolytic, antioxidant, neuropharmacological and antipyretic activity.

**MATERIALS AND METHODS**

**Collection, Identification and Processing of Plant Samples**

The leaves of H. parasitica were collected from Sylhet, Bangladesh and then plant sample was submitted to the National Herbarium of Bangladesh, Mirpur-1, Dhaka for its identification and the voucher specimen is DACB- 41159. Leaves were sun dried for seven days in order to remove the moisture contents and then ground into coarse powder using high capacity grinding machine (Jaipan designer mixer grinder, jaipan, India) which was then stored in air-tight container with necessary markings for identification and kept in cool, dark and dry place for the investigation.

**Extraction Procedure**

The powdered plant parts (30 gm) were successively extracted in a soxhlet extractor at elevated temperature using 500 ml of distilled methanol (40-60 °C which was followed by ethanol, and chloroform. After drying all extracts were labeled and kept in refrigerator at 4°C for future investigation.

**Preliminary Phytochemical Screening**

Different extracts of H. parasitica were subjected to preliminary phytochemical screenings for determining nature of phytoconstituents by using standard protocols (Tiwari et al., 2016).

**Streptokinase (SK)**

Commercially available lyophilized alteplase (Streptokinase) vial (Popular pharmaceutical Ltd.) of 15, 00,000 I.U, was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 μl (30,000 I.U) was used for in vitro thrombolytic activity evaluation.

**Blood Sample**

Blood (n=6) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1ml of blood was transferred to the previously weighed micro centrifuge tubes and was allowed to form clots.

**Thrombolytic Activity**

The thrombolytic activity of all extracts was evaluated by the method developed by Prasad et al., (2006) and slightly modified by Sharif et al., (2014) using streptokinase (SK) as the standard.

**Antioxidant Activity**

**Cupric reducing antioxidant capacity**

The Cupric Reducing Antioxidant Capacity of all extracts was conducted as described previously by Shahriar et al., (2015).

**Hydrogen peroxide scavenging activity**

The hydrogen peroxide scavenging activity of all extracts was conducted as described previously by Bakhtiar et al., (2015).

**Determination of total antioxidant capacity**

The total antioxidant capacity was evaluated by the phosphomolybdenum method (Jayaprakasha et al., 2004). 0.3ml of extract and sub-fraction in methanol, ascorbic acid used as standard (12.5-200μg/ml) and blank (methanol) were combined with 3ml of reagent mixture separately and incubated at 95°C for 90 minutes. After cooling to room temperature, the absorbance of each sample was measured at 695nm against the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid and was calculated by the following equation: A = (c x V)/m

Where, A = total content of antioxidant compounds, mg/gm plant extract, in ascorbic acid equivalent c = the concentration of ascorbic acid established from the calibration curve, mg/ml, V = the volume of extract in ml, m = the weight of crude plant extract, gm.

**Experimental Animal**

For the experiment Swiss albino mice of either sex, 4-5 weeks of age, weighing between 20-30 gm were collected from ICDDR, B, Mohakhali, Dhaka. Animals were maintained under standard environmental conditions [temperature: (27.0±1.0) °C, relative humidity: (55-65)% and 12 hour light/12 hour dark cycle] and free access to feed and water. The animals were acclimatized to laboratory condition for one week prior to experiments. All protocols for animal experiment were approved by the institutional animal ethical committee.

**Neuropharmacological Study**

To check the neuropharmacological effects or side-effects of drug, two types of experiment is carried out which are forced swimming test and open field test.

**Forced swimming test**

According to Porsolt et al., (1977) swimming test was performed with slight modification by Khandaker et al., (2016).

**Open field test**

According to previous work with slight modification open field test was performed to monitor behavioral responses in mice that were placed in a novel and bright arena (Gupta et al., 1971). Rodents tend to stay away from brightly illuminated areas. The experiment also assesses a range of anxiety induced, locomotor activity and exploratory behaviors. The animals were divided into 8 groups of 5 mice each. The first group was given 10ml/kg of 1% Tween 80 orally and served as control. Group 2 was served 2 mg of Clonazepam per kg of body weight and it served as standard. Groups 3, 4 received methanol extract of the leaves of H. parasitica at 200 and 400 mg/kg of body weight, and groups 5, 6 received ethanol extracts of the leaves of H. parasitica at 200 and 400 mg/kg of body weight. Group 7, 8 received chloroform extracts of the leaves of H. parasitica at 200 and 400 mg/kg of body weight. The open field apparatus is made of hardboard (60cm x 60cm; 40cm walls). Blue lines drawn on the floor divide the floor into thirty six squares 10cm x
10cm squares alternatively colored black and white and Central Square (10cm x 10cm) in the middle clearly marked. The number of squares visited by the animals was calculated for 2min at 0, 30, 60, 90 and 120min subsequent to oral administration of the experimental crude extracts.

**Antipyretic Activity**

Forty two Swiss Albino mice of both sexes (20-30 gm) were randomly divided into 7 groups and fasted overnight before the experiment with free access to water. The normal body temperature of each mouse was measured rectally at predetermined intervals and recorded. Fever was induced according to the method described by Hambourger and Smith, (1935). A lubricated thermometer probe was inserted 3-4 cm deep into the rectum and fastened to the tail by adhesive tape. Temperature was measured on digital thermometer. After measuring the basal rectal temperature, animals were injected subcutaneously with 10 ml/kg body weight of 20% w/v brewer’s yeast in NSS in the dorsum of the mice. Mice were then returned to their housing cages. Eighteen hours after brewer’s yeast injection, the animals were again restrained for rectal temperature recording, as described previously. Only mice that showed an increase in temperature of at least 0.5˚ to 1˚C were used for this study. The extracts at the doses of 100 & 200 mg/kg body weight were administered orally to four groups of animals. The control group received paracetamol (50 mg/kg body weight) orally. Rectal temperature was measured at 1 hr intervals for 4 hr after the extract/drug administration (Opo et al., 2016). The rectal temperature of normal mice (normothermic) was also measured at 1 hr. intervals for 7 hr. as stated by Chomchuen et al., (2010). The results are expressed as percentage of the pre-drug temperature recorded for the same animals using the formula of Makonnan et al., (2003).

**Statistical Analysis**

Data was expressed as Mean ± Standard deviation. IC50 values for antioxidant activities by the extracts were calculated from the dose - response curve by using Microsoft Excel 2010. The results were analyzed statistically by ANOVA followed by Dunnet’s test. Results below *p<0.05, **p<0.01 and ***p<0.001 are considered statistically significant.

**RESULTS**

**Phytochemical Screening**

The leaf of *H. parasitica* showed either presence or absence of different phytochemicals. The results are listed below in the Table 1.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Name of Extracts</th>
<th>Methanol</th>
<th>Ethanol</th>
<th>Chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

(+) Presence & (-) Absent

**Thrombolytic Activity**

The extractives of *Hoya parasitica* were assessed for thrombolytic activity and the results are presented in Table 2. Addition of 100 μl Streptokinase (30,000 I.U.) to the clots along with 90 minutes of incubation at 37˚C, showed 28.26% clot lysis. After treatment of clots with 100 μl methanol, ethanol, chloroform extract of *H. parasitica*, clot lysis 14.67%, 11.65%, 13.12% was obtained respectively.

**Table 2** % Clot lysis by different extracts of *H. parasitica* and standard

<table>
<thead>
<tr>
<th>Samples</th>
<th>% of RBC lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>14.67±1.75</td>
</tr>
<tr>
<td>Ethanol</td>
<td>11.65±0.92</td>
</tr>
<tr>
<td>Chloroform</td>
<td>13.12±1.48</td>
</tr>
<tr>
<td>Control</td>
<td>8.72±1.75</td>
</tr>
<tr>
<td>Streptokinase</td>
<td>28.26±1.81</td>
</tr>
</tbody>
</table>

(Values are expressed as mean ± S.D)

**Antioxidant Activity**

**Cupric reducing antioxidant capacity**

All the methanol, ethanol and chloroform extracts showed dose dependent reducing capacity. Among the three extracts of *H. parasitica*, methanol (61.15 μg/ml) and ethanol (55.57 μg/ml) extracts showed relatively better cupric reducing antioxidant capacity compared to the standard L-ascorbic acid (Table 3).

**Table 3** IC<sub>50</sub> Values of different extracts of leaves of *H. parasitica* in cupric reducing antioxidant capacity

<table>
<thead>
<tr>
<th>Samples</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Ascorbic Acid</td>
<td>48.09</td>
</tr>
<tr>
<td>Methanol</td>
<td>61.15</td>
</tr>
<tr>
<td>Ethanol</td>
<td>55.57</td>
</tr>
<tr>
<td>Chloroform</td>
<td>46.30</td>
</tr>
</tbody>
</table>

**Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging activity**

Scavenging of hydrogen peroxide of different leave extracts of *H. parasitica* is presented in Table 4. The standard, L-ascorbic acid showed 91.43% inhibition. Among the three extracts, ethanol and chloroform extracts showed highest inhibition 87.25% and 82.34% respectively, whereas the methanol extract showed significant result (77.88% inhibition).

**Table 4** IC<sub>30</sub> Values of different extracts of leaves of *H. parasitica* in H<sub>2</sub>O<sub>2</sub> scavenging assay

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC&lt;sub&gt;30&lt;/sub&gt; Value (μg/ml)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Ascorbic Acid</td>
<td>79.94</td>
<td>91.43%</td>
</tr>
<tr>
<td>Methanol</td>
<td>56.01</td>
<td>77.88%</td>
</tr>
<tr>
<td>Ethanol</td>
<td>61.48</td>
<td>87.25%</td>
</tr>
<tr>
<td>Chloroform</td>
<td>52.67</td>
<td>42.34%</td>
</tr>
</tbody>
</table>

**Total antioxidant capacity**

The crude methanol, ethanol and chloroform extracts of *H. parasitica*, were subjected to assay for total antioxidant capacity following standard protocol and the obtained results were represented in Table 5. Among all extracts, chloroform extract of *H. parasitica* leaves was found to possess the highest total antioxidant capacity (1.86 mg/gm) followed by ethanol (1.77 mg/gm) and methanol extracts (1.67 mg/gm).
the same time frequency of standing dose dependent manner when compared with corresponding (**).

Extracts of parasitica represented in Table 6 and Table 7. Following standard protocol and the obtained results were parasitica. The crude methanol, ethanol and chloroform extracts of parasitica exhibited a dose dependent antidepressant activity. For Open field test, ethanol and chloroform extracts at doses of 100 and 200 mg/kg body weight showed significant level (*p<0.05) of lowering pyrexia from elevated level, compared to standard drug Paracetamol.

**Neuropharmacological Study**

**Forced swimming test**

Forced swimming test was performed to evaluate the effect of anti-depressant effect of leaf extracts of H. parasitica on mice. After investigation of leave extracts of H. parasitica, following data were observed (Table 6). During the test ethanol and chloroform extracts at doses of 100 and 200 mg/kg body weight and methanol extract of 200mg/kg body weight shortened the immobility period significantly (**p<0.01; ***p<0.001) in comparison with standard and exhibited a dose dependent antidepressant activity.

Table 6 Effect of different extracts of leaves of H. parasitica in forced swimming test

<table>
<thead>
<tr>
<th>Group</th>
<th>Duration of immobility (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>116.3±3.29</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>88.17±9.29</td>
</tr>
<tr>
<td>Methanol 100 mg/kg</td>
<td>71.17±4.38</td>
</tr>
<tr>
<td>Methanol 200 mg/kg</td>
<td>44.17±2.45**</td>
</tr>
<tr>
<td>Ethanol 100 mg/kg</td>
<td>40.66±2.92**</td>
</tr>
<tr>
<td>Ethanol 200 mg/kg</td>
<td>28.66±1.76**</td>
</tr>
<tr>
<td>Chloroform 100 mg/kg</td>
<td>31.00±1.15**</td>
</tr>
<tr>
<td>Chloroform 200 mg/kg</td>
<td>21.33±1.83***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. (n=6), *p<0.05; **p<0.01; ***P<0.001 significant when compared with the corresponding value of control.

**Open field test**

The crude methanol, ethanol and chloroform extracts of H. parasitica were subjected to assay for open field test following standard protocol and the obtained results were represented in Table 6 and Table 7. This experiment was performed to assay general locomotor activity levels. In the present study it was observed that both the doses of ethanol extracts of 100 & 200 mg/kg body weight significantly (**p<0.01) decreased the rate of movement with time in a dose dependent manner when compared with corresponding value of standard (Table 7). These extracts also decreased the frequency of standing, entrance into center and stool count at the same time (Table 8). So it can be said that ethanol extract of H. parasitica have the ability to relieve stress and had an antioxilytic effect on the rodents.

Table 7 Effect of Different Extracts of H. parasitica in open field test (Movement)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Movement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-30 mins</td>
</tr>
<tr>
<td>Control (1% Tween 80)</td>
<td>35±4.01</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>34.4±3.75</td>
</tr>
<tr>
<td>Methanol Extract 100 mg/kg</td>
<td>60±4.08</td>
</tr>
<tr>
<td>Methanol Extract 200 mg/kg</td>
<td>52.5±2.04</td>
</tr>
<tr>
<td>Ethanol Extract 100 mg/kg</td>
<td>47.33±3.35**</td>
</tr>
<tr>
<td>Ethanol Extract 200 mg/kg</td>
<td>43.16±2.94**</td>
</tr>
<tr>
<td>Chloroform Extract 100 mg/kg</td>
<td>44.33±4.46</td>
</tr>
<tr>
<td>Chloroform Extract 100 mg/kg</td>
<td>46.83±2.83</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. (n=6), *p<0.05; **p<0.01; ***P<0.001 significant when compared with the corresponding value of control.
levels of H₂O₂ in biological systems may be important. Naturally occurring iron complexes inside the cell believed to react with H₂O₂ in vivo to generate highly reactive hydroxyl radicals and this may be the origin of many of its toxic effects. Methanol, ethanol and chloroform extracts showed noticeable radical scavenging activity with IC₅₀ values 56.01, 61.48 and 52.67 μg/ml respectively whereas the standard showed IC₅₀ value of 79.94μg/ml (Table 4).

Total antioxidant capacity of the different extracts of H. parasitica was evaluated by the phosphomolybdenum method and was expressed as ascorbic acid equivalents per gram of plant extract. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid (y = 1.025x – 0.961; R² = 0.878). The total antioxidant capacity was highest in chloroform extracts 1.86 mg/gm and lowest in methanol extracts 1.67 mg/gm. Presence of total phenol content and flavonoid in the plant extracts may be a reason for this activity (Hossain et al., 2016). Ethanol and chloroform extracts showed decreased immobility with dose dependently like the standard clonazepam which indicates its antidepressant effect. The CNS depressant effect of the extracts may be responsible for chemical constituents, as flavonoids are responsible for the decrease in immobile phase in the swim test and so does alkaloids as well which were also observed in the present study. The extract significantly decreased the locomotor activity as shown by the results of the open field. This activity is a measure of the level of excitability of the CNS and this decrease may be loosely related to sedation resulting from depression of the central nervous system. Ethanol and chloroform extracts decreased movement of rodents in a dose related manner and this may be the origin of many of its toxic effects. Methanol, ethanol and chloroform extracts showed noticeable radical scavenging activity with IC₅₀ values 56.01, 61.48 and 52.67 μg/ml respectively whereas the standard showed IC₅₀ value of 79.94μg/ml (Table 4).

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CONCLUSION

All the conducted experiments in the present study are based on crude extract and are considered to be preliminary and more sophisticated research is necessary to reach a concrete conclusion.
References


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