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Evaluation of *In vitro* Antioxidant and *In vivo* Pharmacological Activity of Leaf Extracts of *Hoya parasitica* (Wall.)

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ABSTRACT

In present study, leaf extract of *Hoya parasitica* Wall. was evaluated for *in vitro* antioxidant and membrane stabilizing activity along with *in vivo* gastro intestinal motility and acute toxicity. Five different assays were performed to evaluate antioxidant activity. In DPPH free radical scavenging activity, methanol, ethanol and chloroform extract exhibited IC_{50} value similar to standard ascorbic acid. The presence of flavonoid and phenolic contents was also similar in all the plant extracts. However, chloroform extract showed remarkable reducing power capacity (69.10% at 200µg/mL). In case of membrane stabilization, the chloroform extract showed maximum inhibition (32.62 %) of haemolysis, whereas the ethanol extract showed a significant (p<0.001) human RBC membrane stabilizing effect. *In vivo* gastrointestinal motility test indicates significant (p<0.001) increase in gastrointestinal motility by Methanol extract (100 and 200 mg/kg b.w.) and ethanol extract (200 mg/kg b.w.) compared to standard. Highest dose introduced as 1000, 2000 and 3000 mg/kg body weight of each extracts in acute toxicity study and did not shown any sign of toxicity in Swiss albino mice. The result obtained from this study, can be considered as preliminary and further sophisticated investigation is needed to isolate new bioactive compounds that might act as led compounds in future.

INTRODUCTION

As a natural source of medicine, plants have been used as medicine since ancient time. However, in recent years there has been growing interest in the therapeutic use of natural products, because of availability, less side effects and less abusive potential (Yalavarthi and Thiruvengadarajan, 2013; Rates, 2001). Among the 350000 plant species identified so far, around 35000 plants are being used as medicinal purpose worldwide. According to WHO estimation, herbal medicines are used to treat of about 80% of the world population, especially the people of the rural areas of the developing countries (Kong *et al.,* 2003). A large number of people in Bangladesh is solely dependent on Ayurvedic treatment for maintaining their health. It is estimated that about 250 species of medicinal plants are used for the preparation of traditional medicines which is the half of total species of plants grown in Bangladesh. However, most of these plants have not yet undergone chemical, pharmacological, and toxicological screening to identify their bioactive compound(s) (Khatun *et al.,* 2014). *Hoya parasitica* Wall. is a climbing epiphyte of the family Asclepiadaceae. The common name of Hoya genus is waxvine, waxflower or simple Hoya. Bengali name of the plant is Chera pata, Pargacha and tribal name is Fassya gaas (Rahman *et al.,* 2007). It is an evergreen tropical perennial shrub native to tropical wet forests and humid climate of southern Asia, Australia and Polynesia (Reza *et al.,* 2007). It is a parasite creeper with a fragrant flower.

In Bangladesh the plant is distributed in Sylhet, Moulvi Bazar, Chittagong and Chittagong Hill Tracts, Cox's Bazar, Sunderbans, Jessore, and Satkhira (Sadhu *et al.,* 2008). Among the species of *Hoya*, *Hoya parasitica* Wall. is selected for the current study because it is the species of Hoya genus on which a very few scientific investigations have been conducted although it is widely used as traditional medicine.

Among the Chakma tribe the leaf extract is used externally to alleviate body pain and for the treatment of fever. However local people of Rema-Kalenga use the leaf extract for the treatment of jaundice (Rahman *et al.,* 2007). However, the fruits of *Hoya parasitica* Wall. are used by the Tripura tribe for the treatment of constipation (Hossan *et al.,* 2009).

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MATERIALS AND METHODS

Collection and processing of plant samples

Fresh leaves of *Hoya parasitica* Wall. were collected from Sylhet, Bangladesh in December 2014 before its flowering stage and a plant sample was submitted to the Bangladesh National Herbarium for identification (Accession number- 41159). Cleaned leaves were shade dried for two days followed by oven dried for 72 hours more at considerably low temperature (not more than 40°C) for better grinding.

The dried leaves were then grounded in course powder using a high capacity grinding machine. The coarse powder were then stored in air-tight plastic container with necessary markings for identification and then kept in cool, dark and dry place for further investigation.

Extraction procedure

The powdered plant parts (30 gm) were successively extracted in a soxhlet extractor at elevated temperature using 300 mL of distilled methanol (40-60) °C which was followed by ethanol, and chloroform. All extracts were then filtered individually through filter paper and rotary evaporator was used to evaporate the liquid solvents from the extracts to get the dry extracts. After extraction all extracts were kept in a refrigerator 4 for future investigation with their necessary markings for identification.

In vitro Antioxidant activities

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Thus, interest in natural antioxidants has increased considerably. Therefore, it is essential to develop and utilize effective natural antioxidants so that they can protect the human body from free radicals (Shanta *et al.*, 2013). *In vitro* investigation of antioxidant activity of the plant extracts were done by five methods following standard procedure.

DPPH free radical scavenging assay

The free radical scavenging capacity of the extracts was determined using DPPH (Braca *et al.*, 2001). one mL of plant extract or standard of different diluted (6.25µg/mL to 200µg/mL) concentration solutions were taken in test tubes and freshly prepared 2 mL of 0.004% DPPH solution was added to each test tube to make the final volume of 3 mL. The mixture was incubated at room temperature for 30 min, the absorbance was read at 517 nm using UV-VIS spectrophotometer. Ascorbic acid was used as standard. Control sample was prepared containing the same volume without any extract or standard.

Methanol was served as blank. Free radical scavenging activity of the extracts was evaluated as % inhibition and/or IC_{50} using the following equation:

$$\% \text{ inhibition} = (1-A_1/A_0) \times 100\%$$

Here, A_1 = Absorbance of the extract or standard

A_0 = Absorbance of the control

IC_{50} is the concentration at which 50 % of the total DPPH free radical is scavenged or neutralized and can be determined by linear regression method from plotting % inhibition against corresponding concentration.

Determination of total phenolic contents (TPC)

Total phenolic contents in the extracts were determined by the Folin-Ciocalteu reagent method (Velioglu *et al.*, 1998; Demiray *et al.*, 2009). Gallic acid was used as standard.

All of the extracts and standard were diluted by serial dilutions as 6.25µg/mL to 200µg/mL. Then 5 mL Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 mL (7.5% sodium carbonate) of sodium carbonate were added to each test tube containing 1 mL of diluted solution of sample or standard. Samples were incubated at 20 ^{0}C temperature for 60 min and standard diluted solution–reagent mixture was incubated at 20 ^{0}C temperature for 30 min. Absorbance of samples and standard were measured at 765 nm using UV-VIS spectrophotometer against blank. A typical blank solution containing the solvent used to dissolve the plant extract. The total content of phenolic compounds in plant extracts in Gallic acid equivalents (GAE) was calculated using the following equation:

$$C = (c \times V)/m$$

Where, C = total content of phenol compounds, mg/gm plant extract, in GAE

c = the concentration of Gallic acid established from the calibration curve (mg/mL)

V = the volume of extract in mL

m = the weight of crude plant extract in gm

Determination of total flavonoid contents (TFC)

Aluminum chloride colorimetric method was used for flavonoids determination (Wang and Jiao, 2000). To 1 mL of plant extract or standard of different diluted (6.25µg/mL to 200µg/mL) concentration solutions was taken in a test tube and 3 mL of methanol, 0.2 mL of aluminum chloride, 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water were added. It was incubated at room temperature for 30 min, and then absorbance of the reaction mixture was measured at 415 nm with UV-VIS spectrophotometer against blank. Methanol served as blank. The total content of flavonoid compounds in plant methanol extracts in Quercetin equivalents was calculated with same equation used in total contents of phenol.

Nitric Oxide (NO) scavenging assay

Nitric oxide scavenging assay was carried out using sodium nitroprusside (Sreejayan and Rao, 1997).This can be determined by the use of the Griess Illosvoy reaction. Two mL of 10mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of extract or sub-fraction of different diluted (6.25µg/mL to 100µg/mL) concentration solutions and the mixture was incubated at 25°C for 10 min. From the mixture 0.5 mL was taken out and added into 1.0 mL sulphanilamide solution (0.33% in 20% glacial acetic acid) and further incubated at room

temperature for 5 min. Finally, 1.0 mL Naphthyl ethylenediamine dihydrochloride (0.1% w/v) was mixed and maintained at room temperature for 30 min. The absorbance was measured at 546 nm with UV-VIS spectrophotometer. Typical control solutions contain the same solution mixture without plant extract or standard. Percentage inhibition of the NO free radical was measured by using the equation of % inhibition of the DPPH free radical.

Reducing power capacity assessment

The reducing power of the extract of *Hoya parasitica* Wall. was determined by the method of Oyaizu, 1986. In this method, 1 mL of various concentrations of the plant extracts was taken in a test tube and mixed with 2.5 mL of phosphate buffer (0.2M, pH 6.6) and potassium ferricyanide (1% w/v) and incubated at 50 ^{0}C for 20 min. Then 2.5 mL of trichloroacetic acid (10%) were added to the mixture, which was then centrifuged at 3000 rpm for 30 min whenever necessary. Then 2.5 mL of the upper layer of solution was mixed with 2.5 mL of distilled water and a freshly prepared 0.5 mL of ferric chloride solution (0.1%). The absorbance was measured at 700 nm with UV-VIS spectrophotometer. A blank was prepared without adding extract. Ascorbic acid at various concentrations was used as standard. Increased absorbance of the reaction mixture indicates increase in reducing power. According to Arulpriya *et al*., 2010 percent increase in reducing power was calculated.

$$\% \text{ increase in reducing power} = (A_{test}/ A_{blank} - 1) \text{ X } 100$$

Where,

A_{test} = Absorbance of the extract or standard (sample) and

A_{blank} = Absorbance of the control.

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 microcentrifuge tubes and was allowed to form clots.

Membrane stabilizing activity

The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane (Omale and Okafor, 2008). The membrane stabilizing activity of the extractives was assessed by using hypotonic solution and heat induced methods (Shinde *et al.,* 1999). To prepare the erythrocyte suspension, whole blood was obtained from healthy human volunteer and was taken in syringes containing anticoagulant EDTA (3.1% Na- Citrate). The blood was centrifuged and blood cells were washed three times with solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 g.

Hypotonic solution-induced haemolysis

The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extracts (1.0 mg/mL) or acetyl salicylic acid (0.1 mg/mL). The control sample consisted of 0.5 mL of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm by UV-VIS spectrophotometer. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

$$\% \text{ inhibition of haemolysis} = 100 \text{ x } (OD_1\text{-}OD_2/OD_1).$$

Heat-induced haemolysis

Isotonic buffer containing aliquots (5 mL) of the different extracts were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension was added to each test tube and mixed properly. One pair of the tubes was incubated at 54 ^{0}C for 20 min in a water bath, while the other pair was maintained 0 ^{0}C to 5 ^{0}C in an ice bath. The reaction mixture was centrifuged for 3 min at 1500g and the absorbance of the supernatant was measured at 560 nm by UV-VIS spectrophotometer. The percentage inhibition or acceleration of hemolysis in tests was calculated according to the equation:

$$\% \text{ Inhibition of hemolysis} = 100 \text{ x } [1\text{- } (OD_2\text{-}OD_1/ OD_3\text{-}OD_1)]$$

Where, OD_1 = optical density of unheated test sample

OD_2 = optical density of heated test sample

OD_3 = optical density of heated control sample

Experimental animal

For the experiment, Swiss albino mice of either sex, 4-5 weeks of age, weighing between 10-24 gm were collected from ICDDR,B, Dhaka. Animals were maintained under standard environmental conditions (temperature: (27.0 ±1.0) ºC, 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.

***In vivo* gastrointestinal (GI) motility determination**

Intestinal diseases are one of the main causes of death in infants, particularly in developing countries (Keusch *et al.,* 2006). It is therefore important to identify and evaluate commonly available natural drugs as an alternative to currently-used anti-diarrheal drugs (Marona and Lucchesi, 2004). Medicinal plants are usually preferred to treat gastrointestinal disorders, for example, constipation and diarrhea, because they contain multiple constituents with effect-enhancing and/or side effect-neutralizing potential, and, hence are considered relatively safe in prolonged use (Bashir *et al.,* 2011).

The gastrointestinal motility test was performed according to Marona and Lucchesi, 2004 with slight modifications where necessary. This new protocol intended to conform to the 3Rs (Replacement, Reduction, and Refinement) principle, using

animals fasted for 3 hours to control intestinal motility which reduced stress in the animals. In this protocol, mice were deprived of food for a short time (3 hours) and are not killed and observed until evacuation containing charcoal was observed. The experimental results were based on the charcoal evacuation time.

All the mice were weighed, marked and divided in 8 groups containing 6 mice and were kept in food deprivation for 3 hours. Control solution (5 mL/kg), standard solution (5 mg/kg), sample solutions of 100 mg/kg and 200 mg/kg of all the extracts were administered by oral gavages. Ninety min later, 0.3 mL of charcoal suspension was administered to all the mice. Sixty min later, mice were provided with free access to food. The mice were observed at 5 min intervals until feces with charcoal were eliminated (maximum time of observation was 360 min). Charcoal was observed on the feces using normal light when it was easily visible, or using a microscope to help the identification of the black spots. The time for charcoal defecation was recorded.

In vivo acute toxicity study

Toxicity is the fundamental science of poison. The Organization for Economic and Development (OECD) mentioned acute toxicity as the advance effect occurring within a short time of oral administration. It includes observational data gathering and data utilization to predict outcome of exposure in human and animals (Priyadarshini *et al.*, 2014).

Acute oral toxicity test was performed as per OECD guidelines 423. The acute toxic class method is based on biometric evaluations with fixed doses, adequately separated to enable a substance to be ranked for classification purposes and hazard assessment. The method as adopted in 1996 was extensively validated *in vivo* against LD_{50} data (Walum, 1998).

Mice were kept fasting for 1-2 hours but water was provided and were divided in 7 groups. Each group contained 6 mice. Then all the mice were marked and weighed. Control solution (0.9% NaCl), sample solution of each extract of 1000 mg/kg b.w, 2000 mg/kg b.w and 3000 mg/kg b.w were administered orally to the mice. Then the mice were kept on food deprivation for another 1-2 hours with access to water. The animals were then observed for any mortality or any other sign of toxicity for 1 every 1 hour for next 12 hours. All the mice were kept under observation for one week.

RESULTS AND DISCUSSIONS

Antioxidant activity

DPPH free radical scavenging activity

The free radical scavenging activity of different extracts of *Hoya parasitica* leaf was studied by DPPH method. The DPPH free radical scavenging activity is usually described as IC_{50} which is the concentration of samples to produce 50% reduction of free radical (Hassan *et al.*, 2011). The IC_{50} values of different leaf extracts of *Hoya parasitica* Wall are presented in (table 1). Different concentration (200, 100, 50, 25, 12.5, 6.25, µg/mL) of leaf extracts of this plant were subjected to this investigation and the methanol, ethanol and chloroform extracts showed maximum activity of 65.54 %, 64.70% and 95.79% inhibition at 200 µg/mL concentration respectively whereas standard L-ascorbic acid showed maximum 96.63% inhibition at the same concentration (figure 1). IC_{50} of ascorbic acid was found 3.1 µg/mL. Whereas methanol, ethanol and chloroform extracts showed IC_{50} values of 3.8, 3.9 and 3.2 µg/mL respectively (table 1). In DPPH free radical scavenging assay, the crude extracts of the plant showed dose dependent scavenging of DPPH radicals in a way similar to that of the reference antioxidant ascorbic acid. The extracts of *Hoya parasitica* Wall. have the ability to scavenge the free radical by donating a hydrogen molecule.

Table 1: IC_{50} value for DPPH scavenging assay of leaf extracts of *Hoya parasitica* Wall.

Sample	IC_{50} Value (µg/mL)
L-ascorbic acid	3.1
Methanol extract	3.8
Ethanol extract	3.9
Chloroform extract	3.2

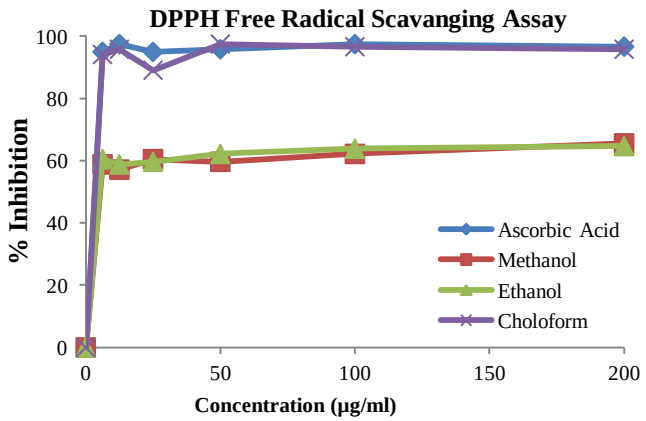

Fig. 1: DPPH free radical scavenging activity of different leaf extracts of *Hoya parasitica* Wall.

Total phenolic contents (TPC)

Total phenolic content of the different extracts of *Hoya parasitica* Wall. were determined by using the Folin-Ciocalteu reagent and were expressed as gallic acid equivalents (GAE) per gram of plant extract. The total phenolic contents of the test fractions were calculated using the standard curve of Gallic acid ($y= 1.0769x - 0.8088$; $R^2 = 0.9533$). Ethanol extract of leaves of the plant was found to contain the highest amount of phenolic content (2.03 mg/gm) (table 2).

Table 2: Total phenolic contents of different extracts of *Hoya parasitica* Wall.

Sample	Total Phenolic Contents (mg/gm, Gallic Acid equivalents
Methanol Extract	1.89 ± 0.49
Ethanol Extract	2.03 ± 0.09
Chloroform Extract	1.77 ± 0.26

[Values represent Mean ± SEM]

Polyphenols have been shown to block LDL oxidation, decrease the formation of atherosclerotic plaques and reduce arterial stiffness, leaving arteries more responsive to endogenous

stimuli of vasodilatation (Moline *et al.*, 2000; Zheng and Wang, 2001). Moreover, polyphenols have been shown to exert anti-carcinogenic effects and inhibit the cytochrome P_{450} superfamily of enzymes that metabolizes many pro-carcinogens to reactive compounds, thus reducing the formation of reactive intermediates (Shahriar *et al.*, 2015). In addition, they have been shown to inhibit lipoxygenase and cyclogenase activity leading to lower aggregation of platelets and a reduction of thrombotic tendency (Moline *et al.*, 2000). The results strongly suggest that phenolics are important components of the tested plant extracts. Literature reveals that antioxidant activity of plant extract is mainly due to presence of phenolic compounds, which may exerts antioxidant effects as free radical scavengers, as hydrogen donating sources or as singlet oxygen quenchers and metal ion chelators (Sadeghi *et al.*, 2015).

Total flavonoid content (TFC)

Aluminum chloride colorimetric method was used to determine the total flavonoid contents of the different extracts of *Hoya parasitica* Wall. Total flavonoid contents were calculated using the standard curve of quercetin ($y = 0.7754x - 0.7697$; $R^2 = 0.812$) and expressed as quercetin equivalents (QE) per gram of the plant extract. Ethanol extract of *Hoya parasitica* leaves was found to contain the highest amount of flavonoids content (2.22 mg/gm) (table 3). Flavonoids play an important role in antioxidant system in plants. The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper and inhibition of enzymes responsible for free radical generation. Depending on their structure, flavonoids are able to scavenge practically all known ROS (Shahriar *et al.*, 2013). According to the study, the high contents of flavonoids in *Hoya parasitica* can explain its high radical scavenging activity.

Table 3: Total flavonoid contents of different extracts of *Hoya parasitica* Wall.

Sample	Total Flavonoid Contents (mg/gm, Quercetin equivalents
Methanol Extract	2.03 ± 0.07
Ethanol Extract	2.22 ± 0.08
Chloroform Extract	2.21 ± 0.24

[Values represent Mean ± SEM]

Nitric Oxide Scavenging Assay

NO is very unstable species and reacting with oxygen molecule produce stable nitrate and nitrite which can be estimated by using Griess reagent. In the presence of a scavenging test compound, the amount of nitrous acid will decrease which can be measured at 546 nm.

Different concentration (200, 100, 50, 25, 12.5, 6.25 µg/ml) of leaf extracts of the plant were subjected to investigate the nitric oxide scavenging activity. Methanol, ethanol and chloroform extracts showed maximum activity at different concentrations in comparison to standard L-ascorbic acid. Methanol extract showed 55.95% inhibition at 200 µg/ml, ethanol extract showed 45.23% inhibition at 12.5 µg/ml and chloroform extract showed 57.34% inhibition at 100 µg/ml) and ascorbic acid showed 73.41% inhibition at 200 µg/ml (Figure 2). IC_{50} of ascorbic acid was found 3.9 µg/ml whereas methanol, ethanol and chloroform extracts showed IC_{50} values of 6.0, 6.2 and 6.9 µg/ml respectively (table 4).

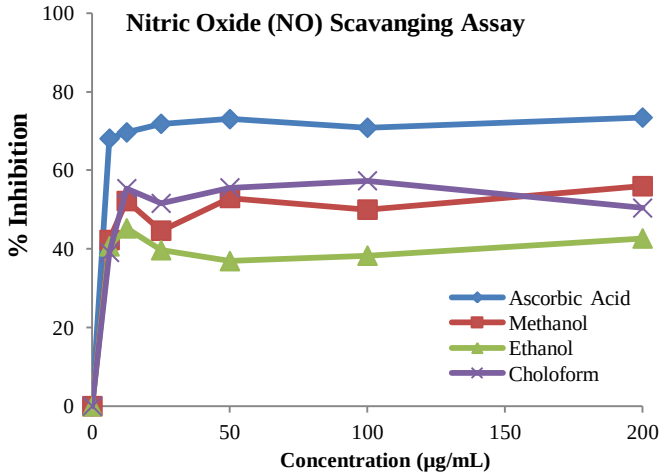

Fig. 2: NO scavenging activity of different leaf extracts of *Hoya parasitica* Wall.

NO acts as a free radical and inhibits several physiological processes such as smooth muscle relaxation and neuronal signaling inhibition of platelet aggregation and regulation of cell mediated toxicity (Banerjee *et al*., 2011; Singh *et al*., 2012). NO scavenging assay is based on the scavenging ability of the extracts as well as ascorbic acid, which is used as standard. The scavenging of NO was found to increase in dose dependent manner. Overproduction and accumulation of NO are associated with cytotoxic effects observed in various disorders like cancer, alzheimer's disease, arthritis and AIDS (Sainani *et al.*, 1997). Excess generation of NO can mediate toxic effects such as DNA fragmentation, cell damage and neuronal cell death (Dawson *et al.*, 1992).

Table 4: IC_{50} value for NO scavenging assay of leaf extracts of *Hoya parasitica* Wall.

Samples	IC_{50} Value (µg/mL)
L-ascorbic acid	3.9
ME	6.0
EE	6.2
ChE	6.9

Different extracts of *Hoya parasitica* was able to scavenge NO produced from nitroprusside at a considerable level. Based on these we speculate that nitric oxide scavenging activity of *H. parasitica* may have great relevance in the prevention and control of disorders where NO is thought to play a key role.

Reducing power capacity assessment

Reducing power of the fractions was assessed using ferric to ferrous reducing activity as determined spectrophotometrically from the formation of Perl's Prussian blue color complex (Shahriar *et al.*, 2013). In this assay the yellow

color of the test solution changes to various shades of green and blue, depending upon the reducing power of each compound (Arulpriya *et al.*, 2010).

The extracts were found to display high to moderate reducing power. Reducing power was found to increase with increasing concentration of the extracts in all cases and was comparable to the standard L-ascorbic acid (figure 3). Among the extracts, the chloroform extract exhibited the most reducing power (69.10% at 200µg/mL) compared to standard that showed 74.23% reducing power at the same concentration (figure 3). IC_{50} of ascorbic acid was found 4.2 µg/mL. In comparison to standard, methanol, ethanol and chloroform extracts showed greater IC_{50} values of 5.6, 5.3 and 5.4 µg/mL respectively (table 5).

Table 5: IC_{50} value for Reducing Power Capacity Assessment of leaf extracts of *Hoya parasitica* Wall.

Sample	IC_{50} Value (µg/mL)
L-ascorbic acid	4.2
Methanol extract	5.6
Ethanol extract	5.3
Chloroform extract	5.4

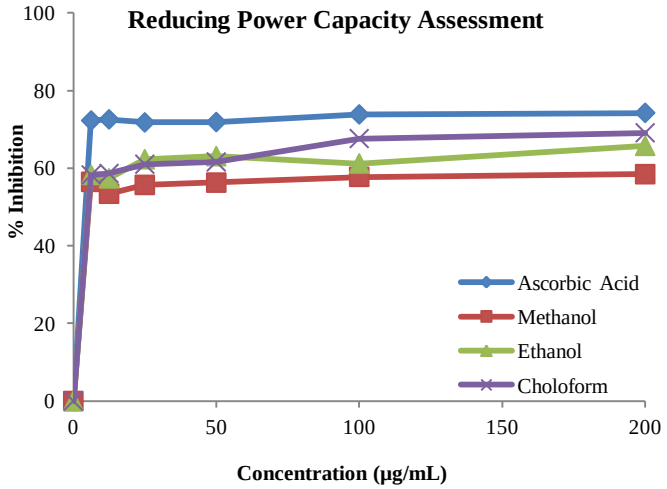

Fig. 3: Reducing power of leaf extracts of *Hoya parasitica* Wall.

These results indicate that the extracts may consist of polyphenolic compounds that usually show great reducing power. The reducing power of the extracts may be due to the biologically active compounds in the extract which possess proton donating abilities. This assay further confirmed the antioxidant properties of the extracts.

Membrane stabilizing activity

In the study of membrane stabilizing activity, the leaf extracts of *Hoya parasitica* Wall. at a concentration of 1.0 mg/mL were tested against the lysis of human erythrocyte membrane induced by hypotonic solution as well as heat, and compared with the standard acetyl salicylic acid (ASA) at a concentration of 0.1 mg/mL. In hypotonic solution induced haemolysis, standard acetyl salicylic acid (0.1 mg/mL) showed 57.07% inhibition of RBC haemolysis whereas methanol extract, ethanol extract and chloroform extract, at a concentration of 1 mg/mL, showed 21.54%, 17.74% and 32.62% inhibition of RBC heamolysis respectively. On the other hand, in heat induced haemolysis, methanol extract, ethanol extract and chloroform extract showed 58.86%, 45.91% and 51.20% inhibition of RBC heamolysis respectively whereas standard acetyl salicylic acid showed 61.40% inhibition of RBC haemolysis (table 6).

Table 6: Effect of different extractives of *Hoya parasitica* Wall. on hypotonic solution and heat induced haemolysis of erythrocyte

Samples	Concentration (mg/mL)	% Inhibition of Haemolysis	
		Hypotonic solution induced	Heat induced
Control	–	–	–
Acetyl salicylic acid	0.1	57.07 ± 0.009	61.40 ± 5.35
Methanol Extract	1.0	21.52 ± 0.015***	58.86 ± 11.01
Ethanol Extract	1.0	17.74 ± 0.013***	45.91 ± 7.38
Chloroform Extract	1.0	32.62 ± 0.021*	51.20 ± 7.01

Values represent mean ± SEM (n=6), ∗ Significant ($p < 0.05$), ∗∗ Significant ($p < 0.01$), ∗∗∗ Significant ($p < 0.001$) when compared with the corresponding value of the standard (acetyl salicylic acid)

The results revealed that although all the leaf extracts have very good potential of membrane stabilizing activity, chloroform extract showed good potential in hypotonic induced haemolysis and methanol and chloroform extracts showed better potential in heat induced haemolysis as compared to the standard. No significant value was observed in heat induced haemolysis, but all of the extracts showed significant ($p<0.001$, $p<0.05$) result in hypotonic solution induced haemolysis (table 6). A possible explanation of the stabilizing activity of different extractives was due to an increase in the surface area/volume ratio of the cells which could be brought about by an expansion of the membrane or shrinkage of the cell and an interaction with membrane proteins. As lysosomal membrane stabilization contributes to protect cells from inflammation, the present investigation suggests that the membrane stabilizing activity of *Hoya parasitica* Wall. leaf extracts may play a very significant role in development of anti-inflammatory drugs (Shahriar *et al.*, 2013).

Gastrointestinal (GI) motility test

In-vivo gastrointestinal motility test was conducted on methanol, ethanol and chloroform extracts on the doses of 100 mg/kg and 200 mg/kg. The duration between charcoal administration and charcoal defecation is measured for gastrointestinal motility determination. The gastrointestinal motility test results are shown in (figure 4). Among all the leaf extracts, methanol extracts of 100 mg/kg and 200 mg/kg and ethanol extracts of 100 mg/kg and 200 mg/kg showed better GI motility or antispasmodic effect as compared to standard. Charcoal defecation time after administration of Ethanol extracts of 100 mg/kg and 200 mg/kg took 190 min and 195 min respectively. On the other hand methanol extracts of 100 mg/kg and 200 mg/kg showed charcoal defecation time of 220 min and 221 min respectively (figure 4). In both cases defecation time is greater than butapan (hyoscine butyl bromide). However hyoscine butyl bromide is an anti-cholinergic drug with high affinity for muscarinic receptors located on the smooth-muscle cells of the GI

tract and exerts a smooth-muscle relaxing/spasmolytic effect (Tytgat, 2007). So our current results indicate that both the ethanol and methanol extract of *Hoya parasitica* Wall. have potential effect on muscarinic receptor.

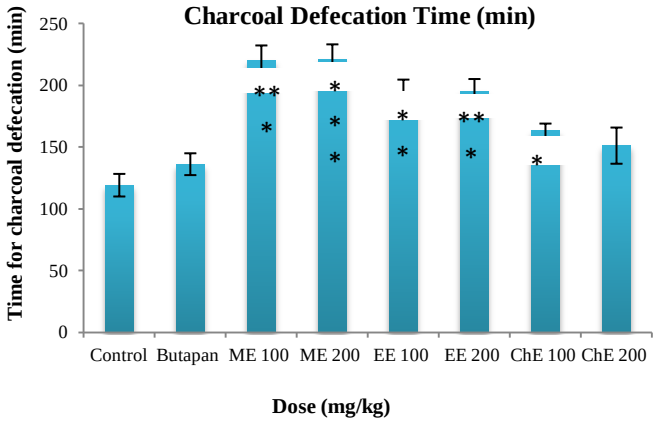

Fig. 4: Graphical presentation of Charcoal defecation time (min) after administration of leaf extract of *Hoya parasitica* Wall. [Methanol Extract = ME, Ethanol Extract = EE, Chloroform Extract = ChE, Values are mean ± Standard error mean (S.E.M), n=6, ∗Significant ($p< 0.05$), ∗∗ Significant ($p < 0.01$) when compared with the corresponding value of the standard (Butapan).]

Methanol extract (100 and 200 mg/kg b.w.) and ethanol extract 200 mg/kg b.w. showed significant ($p<0.001$) result. Defecation time decreased significantly ($p<0.05$, $p<0.01$, $p<0.001$) in case of chloroform extracts administration. Chloroform 100 mg/kg and 200 mg/kg extracts needed 163 and 151 minutes for charcoal defecation (figure 4). The result revealed that the stimulating effect of the methanol and ethanol extracts of the plant on GI motility is dependent on its concentration. This increase in GI motility indicates the potential of antispasmodic effect of the extracts. It has been shown that the fruits of *Hoya parasitica* Wall. are used to treat constipation among the Tripura tribe of Bangladesh (Hossan *et al.*, 2009). However, our current result showed that the leaf extract can also be used as antispasmodic agent especially in constipation. Biochemical screening of the leaf extract would reveal more comprehensive and conclusive result.

Acute toxicity study

Plants or drugs must be ensured to be safe before they could be used as medicines. A key stage in ensuring the safety of drugs is to conduct toxicity tests in appropriate animal models, and acute toxicity studies are just one of a battery of toxicity tests that are used.

Experimental screening method is imperative in order to establish the safety and efficacy of traditional and herbal products and also to set up the active components of the herbal products (Baghel *et al.*, 2011). In the acute toxicity test of the leaf extracts of *Hoya parasitica* Wall., there was no mortality or any signs of behavioral changes or toxicity observed after oral administration of extract up to the highest dose level of 3000 mg/kg body weight in mice during one week of observation (table 7).

This indicated the absence of any toxic material in the leaf of *Hoya parasitica* Wall. The non-toxic nature of methanol, ethanol and chloroform extract of the plant is evident by the absence of mortality of the test animals at oral treatment of 3000 mg/ kg body weight. The leaf extracts of this plant is completely safe up to 3000 mg/kg body weight.

Table 7: Results of acute toxicity study of the leaf extracts of *Hoya parasitica* Wall.

Sample	Dose(s)	Number of death
0.9% NaCl saline	1 mL/kg	0
Methanol Extract	1000 mg/kg	0
	2000 mg/kg	0
	3000 mg/kg	0
Ethanol Extract	1000 mg/kg	0
	2000 mg/kg	0
	3000 mg/kg	0
Chloroform Extract	1000 mg/kg	0
	2000 mg/kg	0
	3000 mg/kg	0

CONCLUSION

The current study clearly indicates that the crude extracts of *Hoya parasitica* Wall. may be a very important contributor in different drug discovery including antioxidant, antispasmodic, anti-inflammatory and cardio protective drugs. In present study, the acute toxicity study emphasizes the call for carrying out toxicity studies even in natural plant products and drug of indigenous medicinal system. The findings of the present study evidenced that, methanol and ethanol extracts of leaves showed potent antioxidant activity. So the present study indicated a better chance of anti-tumor potential of the plant that might be revealed in near future. Since the plant has also been used for treating diabetes, further study would be focused on the investigation of anti diabetic drug.

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 about the findings of the present study. Therefore, further investigation on *Hoya parasitica* Wall. to isolate new bioactive compounds responsible for the specific pharmacological action of this study might be the next step to be followed to eventually find new led compounds.

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