EVALUATION OF IN VITRO HEMOLYTIC ACTIVITY OF DIFFERENT PARTS OF ABUTILON INDICUM (LINN.)

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ABSTRACT

In the present study different parts of (Leaf, Stem, flower and Pod) acetone extracts of Abutilon indicum (L.) were determine for the analysis of pytochemical and in vitro hemolytic activities. The evaluation of in vitro hemolytic activity was screened by using hypotonic induced saline and heat induced hemolysis method. The result of present study, maximum bioactive phytocompounds were present in the flower extract compared to leaf, stem and pod of A. indicum. The acetone extract of A. indicum leaf showed minimum hemolysis against human erythrocytes. In heat induced method, the mild RBC lysis was observed in acetone extract of leaf, when compared to other parts of A. indicum. In future to determine the active phytocompounds present in A. indicum, that to design the pharmaceutical drugs for the treatment of various diseases and less toxicity.

KEYWORDS: In vitro, Hemolytic activity, Heat induced hemolysis, Abutilon indicum, Erythrocyte, acetone extract.

INTRODUCTION

Medicinal plants are one of the most important contributors to the most of the medicinal preparations as raw plant materials, refined crude extracts and mixtures etc. Several thousands of plant have been identified containing medicinal values and are used to treat different ailments in various cultures worldwide.[1] Many plants contain chemical substances
that might have a hemolytic or anti-hemolytic effect on human erythrocytes. Several reports indicate that the membranes of human erythrocytes from blood types have varying stability as determined from the mean corpuscular fragility.\textsuperscript{[2]} Plant extracts can positively affect the red cell membrane and many plants have serious adverse effects, which include induction of hemolytic anemia.\textsuperscript{[3]}

\textit{In vitro} hemolytic activities are becoming a new area of research in drug lead discovery. Researchers are exploring ethno botanically important plants to find out potential natural products with antiaggregant action. These studies are important because some patients have become resistant to the already existing drug e.g. aspirin and/or conventional medication and in association with medicinal plant formulations. This is posing a serious problem to the society. Moreover the constant use of synthetic drugs is leading the society to face great danger.\textsuperscript{[4]} Erythrocytes, which are the most abundant cell in human body, possessing desirable physiological and morphological characteristics, are exploited extensively in drug delivery.\textsuperscript{[5]} Oxidative damage to the erythrocyte membrane (lipid/protein) may be implicated in hemolysis associated with some hemoglobinopathies, oxidative drugs, transition metal excess, radiation and deficiencies in some erythrocyte antioxidant systems.\textsuperscript{[6]}

\textit{Abutilon indicum} (Indian name: Atibala, family: Malvaceae) is extensively grown in Bangladesh, India, Pakistan, Srilanka. The plant is considered as astringent, antibacterial, anthelmintic, carminative and diuretic. It is used locally for cold, high fever, mumps, tuberculosis, bronchitis, diabetes, carbuncle, hemorrhoids, hernia, diarrhea and various type of worm infections.\textsuperscript{[7]} Perviously the phytochemical investigation of the plant revealed the presence of various phytochemical constituents namely luteolin, chrysoeriol, aspigenin 7-O-beta rhamnopyranosyl, quercetin, triacontanoic acid, uresol, methylstigmasterol, glucopyronoside.\textsuperscript{[8]} The plant possess many pharmacological studies such as analgesic,\textsuperscript{[9]} antioxidant, anti-microbial, anti-inflammatory, cancer preventive, anti-diabetic and hypertensive activities\textsuperscript{[10-11]}, anti-diarrhoeal\textsuperscript{[12]}, anti-convulsant\textsuperscript{[13]}, lipid lowering\textsuperscript{[14]}, wound healing\textsuperscript{[15]} and anti asthmatic activities.\textsuperscript{[16]} There is no detailed study on \textit{in vitro} hemolytic activity of acetone extracts of leaf, stem, flower and pod of \textit{A. indicum} (L.).

**MATERIALS AND METHODS**

**Collection of plant material**

The fresh plant of \textit{Abutilon indicum} was collected in November 2015 from the village of Thiruvali, Nagappattinam district, Tamilnadu, India. The collected plant was washed with tap
water and cleaned well then air dried under shade at room temperature; the sample was powdered in an electric grinder, sieved with coarse powder and stored in air tight container.

**Preparation of plant extracts**

20 g of powdered materials were soaked in the 200 ml of acetone for 4 days and then filtered through a cotton plug followed by Whatmann No. 1 filter paper. The filtrate was concentrated by boiling water and then crude extracts was stored at 4-8°C in air tight container.

**Qualitative phytochemical analysis (Sofowara, 1993)**

The preliminary chemical tests were carried out the acetone extracts of *A. indicum* (leaf, stem, flower and pod) to identify the presence of various phytoconstituents.

**Detection of Carbohydrate**

**Fehling’s test**

One ml of extract was boiled on water bath with 1 ml each of Fehling solutions A and B. The color change was observed. A red precipitates indicated presence of sugar.

**Barfoed’s test**

To 1 ml of extract, 1 ml of Barfoed’s reagent was added and heated on a boiling water bath for 2 minutes. The color change was noted and recorded. A red precipitates indicated presence of sugar.

**Benedict’s test**

To 0.5 ml of extract, 0.5 ml of Benedict’s reagent was added. The mixture is heated on a boiling water bath for 2 minutes and the result was observed. A red precipitates indicated presence of sugar.

**Detection of Phenols**

**Lead acetate test**

The extract (5 mg) was dissolved in distilled water and 3 ml of 10% lead acetate solution was added. A bulky white precipitates indicated the presence of phenols.
Detection of Tannins

Ferric chloride test
The extract (5 mg) was dissolved in 5 ml of distilled water and few drops of neutral 5% ferric chloride solution were added. The formation of blue green color indicated the presence of tannins.

Detection of flavonoids
An acetone solution of the extract was treated with ammonium hydroxide solution. The yellow fluorescence indicated the presence of flavonoids.

Detection of Saponins
Distilled water 2ml was added of each plant extracts and shaken in a graduated cylinder for 15 mins lengthwise. Formation of 1cm foam indicates the presence of saponins.

Detection of Glycosides

Legal's test
Chloroform (3ml) and ammonia solution (10%) was added to 2ml plant extract. Formation of pink color indicated the presence of glycosides.

Detection of Terpenoids
Chloroform (2ml) and concentrated sulphuric acid was added carefully to 0.5 ml of extract. Formation of red brown color at the interface indicated the presence of terpenoids.

Detection of Alkaloids
About 50 mg of Solvent free extract was stirred with 3 ml of dilute hydrochloric acid and then filtered thoroughly. The filtrate was tested carefully with various alkaloid reagents as follows:

Mayer’s test
To a 1 ml of filtrate, few drops of Mayer’s reagent are added by the side of the test tube. The white or creamy precipitate indicated test as positive.

Wagner’s test
To a 1 ml of filtrate, few drops of Wagner’s reagent are added by the side of the test tube. The color change was observed. A reddish-brown precipitates confirms the test as positive.
Dragendorff’s test
To a 1 ml of filtrate, 2 ml of Dragendorff’s reagent are added and the result was observed carefully. A prominent yellow precipitate confirms the test as positive.

Detection of steroids
To 0.5 ml of the plant extract equal volume of chloroform was added and subjected with few drops of concentrated sulphuric acid. Appearance of brown ring indicates the presence of steroids.

Detection of coumarins
10% NaOH (1ml) was added to 1 ml of the plant extracts formation of yellow color indicated presence of coumarins.

Detection of Quinone
Concentrated sulphuric acid (1ml) was added to 1ml of each of the plant extract. Formation of red color indicated the presence of Quinones.

Detection of Phlobatannins
Few drops of 10% ammonia solution was added to 0.5 ml of root extract. Appearance of pink color precipitates indicated the presence of phlobatannins.

Detection of Anthraquinones
Few drops of 2% HCL were added to 0.5 ml of seed extract. Appearance of red color precipitate indicated presence of anthraquinones.

In vitro hemolytic activity
Hemolytic assay was carried out by adopting the method of Bulmus et al.,[18]

Freshly collected human red blood cells were taken and allow clotting for 30 minutes. The serum was removed by 2500rpm for 15 minutes and then pellet was washed three times by 150 mM Nacl (2500 rpm for 10 minutes). 0.55% RBC cells were suspended in 100 mM sodium phosphate buffer. Four different concentrations (200, 400, 600, 800 and 1000 µg/ml ) of different extracts were mixed with 200 µg/ml (or) 0.2 ml of RBC solutions and the final reaction mixture volume was made up 1 ml by adding sodium phosphate buffer. The reaction mixture was then placed in water bath for 1 hour at 37°C. After the incubation time the reaction mixture was centrifuged again at 1500 rpm for 10 minutes. The supernatant was
collected and the optical density was measured at 541 nm keeping sodium phosphate buffer as blank. Aspirin was used as a positive control. The experiment was done in triplicate and mean ± S.E was calculated.

\[
\text{Percentage hemolysis} = \frac{(\text{Absorbance of sample} - \text{Absorbance of blank})}{\text{Absorbance of positive control}} \times 100
\]

**In vitro heat induced hemolysis**

The stabilization of human red blood cell (HRBC) membrane was carried out by the method of Chippada et al.\textsuperscript{[19]}

The assay mixture contains 1 ml phosphate buffer [pH 7.5, 0.15 mM], 2ml hypo saline [0.36%], 0.5 ml HRBC suspension [10% v/v] with 0.5 ml of various concentration of plant extracts (200, 400, 600, 800 and 1000 µg/ml) and negative aspirin, as positive control instead of hypo saline to produce 100% hemolysis were incubated at 37\textdegree{}C for 30 min and centrifuged respectively.

The hemoglobin content in the suspension was estimated using spectrophotometer at 560 nm.

The percentage of hemolysis of HRBC membrane can be calculated as follows:

\[
\% \text{ Hemolysis} = \frac{(\text{optical density of test sample} \div \text{optical density of control})}{\times 100}
\]

**RESULTS**

The pytochemical screening of acetone extracts of *A. indicum* (L.) (leaf, stem, flower and pod) showed the presence of bioactive phytocompounds such as alkaloids, carbohydrates, glycosides, tannins, phenols, flavonoids, coumarines, saponins, quinines, terpenoids and steroids were present in all the parts of plant Phlobatanins and anthraquinone was absent in all the parts of *A. indicum* (Table 1).

**Table 1- Phytochemicals analysis of acetone extracts of A. indicum.**

<table>
<thead>
<tr>
<th>Name of the phytocompounds</th>
<th>Different parts of <em>A. Indicum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>++</td>
</tr>
<tr>
<td>Glycosides</td>
<td>++</td>
</tr>
<tr>
<td>Tannins</td>
<td>++</td>
</tr>
<tr>
<td>Phenols</td>
<td>++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
</tbody>
</table>
In vitro hemolytic activity

The hemolytic activity of acetone extracts of (leaf, stem, flower and pod) of *A. indicum* (L.) were screened against normal human erythrocytes. The extracts exhibited low to high hemolytic effect exhibited toward human RBC. The activity of the plant extract were expressed in % hemolysis and reported as mean ± standard error of three replicates. The result indicated that acetone extract of leaf of *A. indicum* possess minimum hemolytic activity (45.61±3.65) at the concentration of 1000 µg/ml. The maximum lysis (70.24±4.9) was observed in flower extract of *A. indicum* at dosage of 1000 µg/ml.

**Table 2- In vitro hemolytic activity of acetone extracts of *A. indicum*.**

<table>
<thead>
<tr>
<th>Concentration of plant extracts (µg/ml)</th>
<th>Leaf</th>
<th>Stem</th>
<th>Flower</th>
<th>Pod</th>
<th>Control (Aspirin 1mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>10.52±2.26</td>
<td>19.6±1.26</td>
<td>17.85±3.50</td>
<td>7.66±1.04</td>
<td>-</td>
</tr>
<tr>
<td>400</td>
<td>21.04±4.28</td>
<td>32.35±1.26</td>
<td>24.89±3.45</td>
<td>19.20±1.64</td>
<td>-</td>
</tr>
<tr>
<td>600</td>
<td>28.06±8.78</td>
<td>37.25±2.04</td>
<td>39.14±3.43</td>
<td>30.71±1.65</td>
<td>-</td>
</tr>
<tr>
<td>800</td>
<td>35.08±3.65</td>
<td>48.91±2.32</td>
<td>40.47±1.53</td>
<td>42.30±1.04</td>
<td>-</td>
</tr>
<tr>
<td>1000</td>
<td>45.61±3.65</td>
<td>64.7±1.25</td>
<td>70.23±2.83</td>
<td>52.56±2.62</td>
<td>72.94±1.15</td>
</tr>
</tbody>
</table>

Values are expressed in mean± S.E of 3 replicates.

![Fig 1- In vitro hemolytic activity of acetone extracts of *A. indicum*.](image)
Heat induced hemolysis

The acetone extract of *A. indicum* at a concentration of 0.2 to 1 mg/ml significantly saved the lysis of human erythrocyte membrane by a temperature induced condition Table 3. This is similar to the standard drug aspirin as positive control (1mg). The maximum hemolysis was observed in acetone extract of pod of *A. indicum* at the concentration 1000 µg/ml. The minimum red blood cell destruction was showed in leaf (42.85 ± 1.85) and flower (43.47 ± 3.54) extracts of *A. indicum* which was compared to standard drug aspirin.

Table 3- Heat induced hemolysis activity of acetone extract of *A. indicum*.

<table>
<thead>
<tr>
<th>Concentration of plant extract (µg/ml)</th>
<th>% hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf</td>
</tr>
<tr>
<td>200</td>
<td>24.99 ± 0.88</td>
</tr>
<tr>
<td>400</td>
<td>31.52 ± 2.65</td>
</tr>
<tr>
<td>600</td>
<td>36.95 ± 3.55</td>
</tr>
<tr>
<td>800</td>
<td>41.3 ± 3.55</td>
</tr>
<tr>
<td>1000</td>
<td>42.85 ± 1.85</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± S.E of 3 replicates.

Fig 2 - Heat induced hemolysis activity of acetone extracts of *A. indicum*.

**DISCUSSION**

Plants have been associated with the human health from time immemorial and they are important source of medicines since human civilization. The secondary metabolites of plants are important sources of many food ingredients and phytocompounds. Plants produce several secondary metabolite compounds including alkaloids, glycosides, flavanoids,
saponins, steroids and terpenoids to protect themselves from the continuous attack of naturally occurring pathogens, insect pests and environmental stress.[22-23] Pytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties.[24] Plant produces phytochemicals to protect itself, but recent research demonstrates that many phytocompounds can protect humans against diseases.[25]

The various phytocompounds present in acetone extracts of leaf, flower, stem and pod of A. *indicum*. The phytocompounds are act as a many pharmacological activities. Phenolics are active in curing kidney and stomach problems as well as helpful as antimicrobial and anti-inflammatory in action.[26] Flavonoids are common within the plant kingdom more than 5000 flavonoids have been indentified in nature.[27] It has been considered to have effect on human nutrition and health as it shows antioxidant activity and their mechanism of action are through scavenging or chelating process.[28] These secondary metabolites activities of medicinal plants such as hypoglycemic, antidiabetic, antimicrobial, anti-inflammatory, anticarcinogenic, antimalarial, anticholinergic, antileprosy activities etc.[29] Terpinoids are also know to possess antimicrobial, antifungal, antiparasitic, antiviral, antiallergenic, antispasmodic, antihyperglycemic, anti-inflammatory properties.[30-31]

Saponins possess the unique property of precipitating and coagulating red blood cells[32,33] and steroids are responsible for cholesterol- reducing and anti-inflammatory properties. Steroids also help in regulating the immune response.[34] Phlobatannins have been reported to possess astringent properties.[35] Cumarins can be suggested to be beneficial for hyper proliferative skin diseases on the basis of their antimicrobial and anti inflammatory effects.[36]

Hemolysis is the breakage of the red blood cells (RBC’s) membrane, causing the release of the hemoglobin and other internal components into the surrounding fluid. Hemolysis is visually detected by showing a pink to red tinge in serum or plasma. Hemolysis is a common occurrence seen in serum samples and may compromise the laboratory’s test parameters. In-vivo hemolysis may be due to pathological conditions, such as autoimmune hemolytic anemia or transfusion reaction.[37] There has been growing demand for in *viv*

*In vitro* hemolysis tests have also been employed by several authors for the toxicological evaluation of different plants.[41]
The erythrocyte model has been widely used as it presents a direct indication of injectable formulation and erythrocytes are easy to isolate from the blood; moreover, its membrane has similarities with other cellular membranes.[42] Hemolysis is due to red blood cells destruction which resulted from lysis of membrane lipid bilayer. This hemolysis relates to concentration and potency of plant extracts. Furthermore hemolytic activity of each extracts is related to their chemical composition.[43] Erythrocytes are considered as major target for the free radicals owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the oxygen transport that associated with redox active hemoglobin molecules, which are potent promoters of acid oxygen species.[44]

The erythrocyte membrane matches to the lysosomal membrane and its stabilization implies that the extract may be well stabilizing lysosomal membranes. Mechanism of stabilization of lysosomal membrane is important in the inflammatory response by preventing the release and the action of intercessors such as serotonin, histamine, leukotrienes, and prostaglandins.[45] Exposure of red blood cell (RBCs) to injurious substances such as hypotonic medium and heat, results in the lysis of the membranes, accompanies by hemolysis and oxidation of hemoglobin.[46] The hemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Injury to red cell membrane will render the cell more susceptible to secondary damage through free radical induced lipid peroxidation.[47] Many reporters have shown that plant flavonoids possess potent anti-inflammatory and anti-oxidant properties.[48,49] The acetone extracts of leaf posses high stabilization of erythrocyte membrane. The phytocompounds present in leaf of A. indicum such as flavonoids, phenolic compounds, terpenoids and also less amount of form forming compounds may be responsible for minimum lysis of erythrocyte. Their anti-hemolytic activities are probably due to their inhibitory effect on enzymes involved in the production of the chemical mediators of inflammation and metabolism of arachidonic acid.[50, 51]

A possible explanation of the stabilizing activity of different extractives 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. The present investigation suggests that the less toxicity of A. indicum may be responsible for significant role in pharmaceutical drug preparations and it’s used for treatment of various type diseases.[52]
CONCLUSION
In this study, the phytochemical analysis of different parts of *Abutilon indicum* (L.) contains alkaloids, carbohydrates, glycosides, tannins, phenols, flavonoids, coumarins, saponins, quinine, terpenoids were present in acetone extract of flower when compared to leaf, stem and pod extracts of *A. indicum*. The *In vitro* hemolytic activity was screened against normal human RBC and membrane stabilization by heat induced hemolytic method. The less toxicity was observed in leaf extract of *A. indicum* compared than pod, stem and flower extracts of *A. indicum*. So plant posses less toxicity towards erythrocyte membrane which favours them for further research.

ACKNOWLEDGEMENTS
We would like to show our gratitude Dr.A. Malarvizhi, Asst. Professor, Head and Department of Biochemistry, D.G.Goverment Arts College (W) Mayiladuthrafor giving us a good guideline for assignment throughout numerous consultations. We would also like to expand our deepest gratitude to all those who have directly and indirectly guided us in writing this assignment.

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