PROTECTIVE EFFECT OF Hibiscus polyphenol-rich extract AGAINST H$_2$O$_2$ INDUCED OXIDATIVE DAMAGE OF HUMAN ERYTHROCYTES

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Abstract: In the present study, Hibiscus rosasinensis polyphenol-rich extract (HPE) was isolated from the flowers and evaluated for its protective effect against hydrogen peroxide (H$_2$O$_2$) induced oxidation in normal human erythrocytes. RBCs pretreated with different concentrations of HPE extract showed the same hemolysis as that of control samples, demonstrating that the tested HPE extract did not have a harmful effect on erythrocytes. RBCs, preincubated with micromolar quantities of HPE and challenged with H$_2$O$_2$ were analyzed for oxidative hemolysis, met hemoglobin production, and lipid peroxidation. The preincubation of RBCs with the polyphenol-rich extract significantly reduced the oxidative modifications. The inhibition of lipid peroxidation was found to be concentration-dependent up to 100µl of the extract, which contained 1.0 mM gallic acid equivalent (GAE) of polyphenolic compounds. In conclusion, HPE appears to be beneficial in preventing hydrogen peroxide induced oxidative RBC damage in human system and can improve RBC membrane permanence.

Key words: Hibiscus rosasinensis, H$_2$O$_2$ oxidation, Human erythrocytes

INTRODUCTION

Hibiscus rosasinensis Linn (Family: Malvaceae) is an evergreen woody glabrous showy shrub. The genus Hibiscus contains 220 species distributed around the world. It is an interesting source of potential bioactive molecules, as phenolic compounds, triterpene derivatives, phytosteroids, with antioxidant, cardio protective, antihypertensive, anti convulsant and antiproliferative activities.

In recent times, focus on plant research has increased all over the world, and several evidences show the immense potential of medicinal plants used in traditional systems. Various medicinal plants have been identified and studied using modern scientific approaches. The results revealed the potential of medicinal plants in the field of pharmacology [1-3]. Natural antioxidants, such as α--tocopherol (vitamin E), L-ascorbic acid (vitamin C) and α--carotene, could inhibit lipid peroxidation, and hence may have beneficial effects in preventing the diseases and keeping the aged healthy [4]. Many compounds in food have antioxidant properties, which are capable of interacting with reactive molecules. The vitamins C and E, provitamin α--carotene, and the trace element selenium are the major antioxidant nutrients. Besides these, other compounds found in plant foods such as flavonoids and polyphenols are also powerful antioxidants. Polyphenolic compounds have a wide range of protective effects such as anti-inflammatory responses, prevention of low-density lipoprotein oxidation, and antihypertensive, antithrombic, and carcinostatic actions [5].

Hibiscus rosasinensis is a glabrous shrub widely cultivated in the tropics as an ornamental plant, since it presents several forms with varying flower colours.
In South Asian traditional medicine, various parts of the plant are used in the preparation of a variety of foods [6]. Phytochemical studies revealed the presence of several chemicals, including flavonoids, flavonoid glycosides, hibiscetin, cyanidine, cyanidin glucosides, taraxeryl acetate, α-sitosterol, campesterol, stigmasterol, ergosterol, citric, tartaric and oxalic acids, cyclopropenoids and anthocyanin pigments [7,8]. The flowers of *H. rosasinensis* contain substantial quantities of flavonoids which are associated with antioxidant, fever-reducing (antipyretic), pain-relieving (analgesic) and spasm-inhibiting (spasmolytic) activities. *H. rosa sinensis* also reduces blood pressure and cholesterol level in blood due to the presence of saponins [9]. The plant also exhibits wound healing property [10] due to the occurrence of tannins and terpenoids [11]. The presence of terpenoids in *H. rosa sinensis* soothes the irritated tissues and the mucous membranes that line the respiratory tract, which eases hacking coughs. Looking into so many properties of this plant, the protective role of Hibiscus polyphenol rich extract against H2O2-induced oxidative damage in normal human RBCs has been evaluated.

**MATERIALS AND METHODS**

**Chemicals:** Hydrogen peroxide was purchased from Universal Laboratories Pvt. Ltd., Gallic acid was obtained from Hi-media laboratories and haemoglobin was obtained from SISCO Research Laboratories (SRL) Pvt. Ltd., Mumbai. All other chemicals and reagents used were of highest purity available and analytical grade.

**Collection of blood and isolation of RBC:** The study included 10 normal healthy volunteers, determined to be healthy on the basis of their medical history. Informed consent was obtained from each subject and the Institutional Ethical Committee approved this study design. Venous blood samples were collected, with heparin as the anticoagulant. The blood samples were centrifuged at 4 °C for 10 minutes at 1000×g to remove plasma and buffy coat. The red blood cells were washed thrice with phosphate-buffered saline (PBS: 138 mM NaCl, 5 mM KCl, 6.1 mM Na2HPO4, 1.4 mM NaH2PO4, 1 mM MgSO4 and 5 mM glucose, pH 7.4) [13] and a 5% V/V RBC suspension in PBS (pH 7.4) was used for in vitro studies.

**Preparation of polyphenolic extract from plant:** *Hibiscus rosasinensis* flowers (wild-type) were collected from a local garden in Sholinganallur, Chennai. They were cleaned, shade dried and then powdered. Polyphenols were extracted from ground flowers by the method of Xia et al. [14]. About 100 g of the powder was mixed with 2 volumes of 80% methanol and kept for 5 days at room temperature. After 5 days it was filtered and evaporated under the hood. The residue was dissolved in water and the aqueous layer was washed with petroleum ether several times until a clear upper layer of petroleum ether was obtained. The lower layer was then treated with ethyl acetate containing glacial acetic acid (10 ml/l). Extraction of polyphenols was carried out for 36 hours at room temperature and the combined ethyl acetate layer was concentrated. The residue was dissolved in water and was used for in vitro studies.

**Estimation of polyphenolic content:** Polyphenolic content was estimated by the method of Singleton and Rossi [15] with some modifications. Briefly, 1 ml of sample was mixed with 1 ml of Folin-Ciocalteau’s phenol reagent. After 3 min. 1 ml of saturated sodium carbonate solution was added to the mixture and made up to 10 ml with distilled water. The reaction was kept in the dark for 90 min. Thereafter, the absorbance was read at 725 nm spectrophotometrically. Gallic acid was used for constructing the standard curve. The total polyphenol content in the extract was expressed as mM gallic acid equivalents (mM GAE).

**In vitro hemolysis with polyphenol extract and determination of met-Hb content:** To evaluate the hemolysis induced by extracts, aliquots of 2.0 ml of RBC suspension was preincubated with 20- 100 µl of the extract for 1 hour at 37 °C under aerobic conditions. Cells, centrifuged and washed twice, were resuspended in PBS and the percent of hemolysis determined spectrophotometrically at 540 nm. The extent of hemolysis induced by addition of hydrogen peroxide was determined by the method of Grinberg et al. [16]. Briefly, aliquots of 2.0 ml of RBC suspension were preincubated with 20 -100 µl of the polyphenol extract for 1 hour at 37 °C under aerobic conditions. The cells were centrifuged, washed, and then resuspended in phosphate buffered saline (PBS). To this H2O2 was added to obtain a final concentration of 100 µM. The RBCs were then incubated at 37 °C for 150 minutes. Aliquots (0.2 ml)
Fig. 1: Time-dependent rate of H$_2$O$_2$-induced hemolysis of red blood cells with and without preincubation with the Hibiscus Polyphenol rich Extract (HPE).

Fig. 2: Methemoglobin (met-Hb) content in control and HPE pretreated red blood cells. At the end of incubation with extracts, parallel sets of samples were treated with or without 100 mM hydrogen peroxide for 3 hours. The data presented are mean ±SD.

Fig. 3: Effects of varying amounts of polyphenol extract (20–100 µl) on H$_2$O$_2$-induced lipid peroxidation of RBCs. Values shown are means ± SD.
were obtained, diluted in 2 ml of PBS and centrifuged. The extent of hemolysis was measured spectrophotometrically at 540 nm by comparing the extra cellular hemoglobin content of the aliquots with that of a fully hemolyzed reference sample, which was prepared in the same way except that H$_2$O$_2$ solution was replaced by 1% Triton X solution.

To study the time-dependent rate of H$_2$O$_2$-induced hemolysis of red blood cells, an aliquot of (2.0 ml) erythrocytes was preincubated with 100 µl of the polyphenol extract for 1 hour at 37 °C under aerobic conditions. The cells were centrifuged, washed, and then resuspended in phosphate buffered saline (PBS). To this H$_2$O$_2$ was added to obtain a final concentration of 100 µM. The RBCs, were then incubated at 37 °C. Aliquots (0.2 ml) were obtained at 30-min intervals ranging from 0 (corresponding to the time of mixing RBCs with H$_2$O$_2$ solution) to 240 min. The samples were diluted in 2 ml of PBS and centrifuged. The extent of hemolysis was measured spectrophotometrically at 540 nm. Erythrocyte samples without preincubation with polyphenol extract were also tested for hemolysis. Percentage hemolysis was measured according to the following equation:

\[
\text{Percentage Hemolysis} = \frac{A \times 100}{B}
\]

Where A is the absorbance of the sample aliquot at 540 nm and B is that of the fully hemolyzed reference sample at 540 nm.

The retarding effect of HPE on H$_2$O$_2$ induced oxidative hemolysis of RBCs was obtained according to the following equation:

\[
\text{Percentage Retardation of Hemolysis} = \frac{A - B}{A} \times 100
\]

A- % hemolysis of H$_2$O$_2$ induced RBC
B- % hemolysis of HPE pretreated RBC

The met-Hb content was determined as a percentage of total hemoglobin (Hb), from the ratio of Hb released from the cells to the total Hb in samples incubated for 3 hours with 100 µM H$_2$O$_2$, according to the method of Winterbourn [17].

**Measurement of lipid peroxidation:** The effect of HPE on hydrogen peroxide-induced lipid peroxidation in RBC was determined by the method of Tedesco et al. [18]. Aliquots of saline washed RBCs was preincubated with 20-100 µl of polyphenol extract for 1 hour at 37 °C under aerobic conditions. The cells were centrifuged, washed, and then resuspended in phosphate buffered saline. The RBCs were then incubated with 200 µM H$_2$O$_2$ for 2 hours at 37 °C. Control samples without polyphenol extract were also incubated. After the requisite time of incubation, the mixtures were treated with 10% TCA and centrifuged. 0.5 ml of 1% thiobarbituric acid in 0.05 M sodium hydroxide was added to the supernatants removed from the tubes. The mixtures were boiled for 1 hour, cooled, and the absorbance at 535 nm was determined [19]. A standard curve was prepared using 1, 1, 3’, 3’ tetra methoxypropane. Lipid peroxidation levels were expressed as nmoles thiobarbituric acid reactive substances/mg Hb (nmol TBARS/ mgHb).

**RESULTS**

**Polyphenolic content:** The total polyphenol content of HPE was determined to be 0.69 g / 100g on dry weight basis in this study. The stock aqueous solution prepared for the in vitro studies had a concentration of 1.0 mM GAE, {the molar concentration of total polyphenols in gallic acid molecular weight equivalents reported as (GAE)}.

**Protective effect of polyphenols against cellular damage:** RBCs pretreated with different concentrations of HPE extract showed the same hemolysis as that of control samples (Table 1) which demonstrated that the tested HPE extract did not have a harmful effect on erythrocytes. The protective effect of HPE extracts on RBC hemolysis was evaluated by oxidative stress induced experimentally using H$_2$O$_2$. Under the given conditions, H$_2$O$_2$ caused considerable RBC lysis that was significantly inhibited by HPE extracts (Table 2). It is worthy to note that, the polyphenol rich extract of hibiscus, exerted the strongest protective effect at the concentration of 1.0 mM of GAE, providing 26.5 % inhibition of RBC hemolysis.

Figure 1 represents the time-dependent rate of H$_2$O$_2$-induced hemolysis of red blood preincubated with and without Hibiscus polyphenol rich extract (HPE). Under the given conditions, H$_2$O$_2$ caused considerable lysis in RBC that was not preincubated with HPE.
However, the curve of the RBC preincubated with the HPE was shifted more towards right than RBC which was not pretreated with HPE, suggesting its protective effect. From the obtained curves the time required to achieve 50% hemolysis (T$_{50}$, min) was calculated. T$_{50}$ values are significantly decreased in RBC that was not preincubated with HPE compared with HPE pretreated RBC. (RBC without HPE T$_{50}$ = 143 min; RBC with HPE T$_{50}$ = 159 min)

**Methemoglobin:** Figure 2 represents the methemoglobin (met-Hb) content in control and red blood cells pretreated with HPE. At the end of incubation with extracts, parallel sets of samples were treated with or without 100 mM hydrogen peroxide for 3 hours. The Met Hb content of RBCs preincubated with HPE was significantly decreased compared to control.

**Lipid Peroxidation:** Figure 3 shows the protective effect of polyphenol extract as an antioxidant. We determined lipid peroxidation as a parameter of oxidative stress. The protective effect was found to be dose dependent and significant inhibition was observed with addition of 100 µl of the extract containing 1.0 mM GAE, the highest amount evaluated.

**DISCUSSION**

The *in vitro* oxidative hemolysis of human red blood cells (RBC) was used as a model to study the free radical induced damage of biological membranes and the inhibitory effect of natural antioxidants. Potential sources of antioxidant compounds have been searched in many types of plant materials such as fruits, leaves, seeds, etc. [21].

RBCs are directly exposed to molecular oxygen, have high polyunsaturated fatty acid content in their membranes, and a high cellular concentration of hemoglobin. This makes the RBCs particularly susceptible to oxidative damage. The hemoglobin released from erythrocytes is potentially dangerous because in reacting with H$_2$O$_2$ it is not only converted to oxidized forms, but the free hemoglobin exposed to H$_2$O$_2$ causes heme degradation with the release of iron ions [22]. H$_2$O$_2$ can initiate the formation of free radicals in the presence of iron, described by the Haber–Weiss reaction, and converts the polyunsaturated fatty acids to radicals, which propagate a chain reaction of lipid peroxidation in the presence of oxygen.

In studies investigating dietary influences on cell function erythrocytes are often isolated from subjects and the production of lipid peroxidation products following hydrogen peroxide-induced oxidative stress is frequently measured as TBARS [23].

Polyphenolic flavonoids are the possible candidates that might explain the antioxidant activity of the extract. Phytochemical studies revealed the presence of several chemicals, including flavonoids, flavonoid glycosides, hibiscetin, cyanidine, cyanidin glucosides, taraxeryl acetate, ß-sitosterol, campesterol, stigmasterol, ergosterol, citric, tartaric and oxalic acids, cyclopropenoids and anthocyanin pigments [24, 25].

The data presented in this study show that *Hibiscus* polyphenol rich extract behave as potent scavenger of reactive oxygen species (ROS). In fact, when intact human RBCs were preincubated with polyphenol rich extract, a strong protective effect against

Table 1: Hemolysis of red blood cells preincubated with 20 to 100 µl of HPE for 1 hour at 37 ºC under aerobic conditions. * Molar concentrations of total polyphenols in gallic acid molecular weight equivalents

<table>
<thead>
<tr>
<th>Volume of HPE extract (µl)</th>
<th>mM GAE</th>
<th>% Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Nil</td>
<td>2.6</td>
</tr>
<tr>
<td>20</td>
<td>0.20</td>
<td>3.1</td>
</tr>
<tr>
<td>40</td>
<td>0.40</td>
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</tr>
<tr>
<td>60</td>
<td>0.60</td>
<td>2.8</td>
</tr>
<tr>
<td>80</td>
<td>0.80</td>
<td>2.7</td>
</tr>
<tr>
<td>100</td>
<td>1.00</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Table 2: Hemolysis of red blood cells preincubated with 20 to 100 µl of HPE for 1 hour at 37 ºC under aerobic conditions. The oxidative stress was induced by adding 100 mM hydrogen peroxide and incubated for 150 minutes. Hemolysis was evaluated as described in Materials and methods. Values are means ± SD

<table>
<thead>
<tr>
<th>Particulars</th>
<th>HPE (mM of GAE)</th>
<th>H$_2$O$_2$ (µM)</th>
<th>% Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
<td>57.3 ± 0.23</td>
</tr>
<tr>
<td>Test</td>
<td>0.20</td>
<td>100</td>
<td>54.8 ± 0.62</td>
</tr>
<tr>
<td>Test</td>
<td>0.40</td>
<td>100</td>
<td>49.5 ± 0.45</td>
</tr>
<tr>
<td>Test</td>
<td>0.60</td>
<td>100</td>
<td>47.3 ± 0.41</td>
</tr>
<tr>
<td>Test</td>
<td>0.80</td>
<td>100</td>
<td>43.6 ± 0.71</td>
</tr>
<tr>
<td>Test</td>
<td>1.00</td>
<td>100</td>
<td>42.1 ± 0.28</td>
</tr>
</tbody>
</table>
H$_2$O$_2$-generated hemolysis and lipid peroxidation was observed. However, further studies are needed to document whether the extract is capable of protecting against other oxidants in vitro and oxidation in vivo.

REFERENCES


