ORIGINAL ARTICLE

Antioxidant activity and protective effect against oxidative hemolysis of *Azadirachta indica* A. Juss

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Abstract:

Toxic effects of free radical on various diseases have been reported. Increased oxidative stress level in cells led to severe damage to nucleic acid, lipids and proteins. Erythrocytes are highly susceptible to oxidative damage which implicate to hemolysis. *Azadirachta indica* (AI) is widely used as traditional medicine for remedy against many injuries. The present study was to investigate the antioxidant activity and protective effect of AI leaves extract against hydrogen peroxide induced hemolysis. Antioxidant activities of ethanolic extract of AI were determined by free radical (1, 1-diphenyl-2-picrylhydrazyl; DPPH and nitric oxide) scavenging activity and ferric reducing antioxidant power (FRAP). The protective effect of AI against hemolysis in hydrogen peroxide (H_2O_2) induced erythrocytes was also investigated. The extract was found to scavenge DPPH and nitric oxide with the IC_{50} of 680.20 ± 4.51 µg/ml and 636.0 ± 14.71 µg/ml, respectively. The reducing power value shown 47 mg of ascorbic acid is equivalent to one gram of AI extract. The extract demonstrated protection to erythrocytes against oxidative hemolysis with an IC_{50} of 415.56 ± 11.05 µg/ml. In conclusion, the AI leaves extract had an antihemolytic activity which mediated by its antioxidant properties.

Keywords: Antioxidants, *Azadirachta indica*, hemolysis, oxidative stress

1. INTRODUCTION

Oxidative stress refers to imbalance in the production of reactive oxygen species (ROS) and antioxidant defenses in the body [1]. Increasing evidence suggests that oxidative stress linked to the pathogenesis of various types of human diseases, including inflammatory disorders, ischemia, neurodegenaration, Parkinson's disease, etc [2]. Besides, oxidative stress may also be implicated in the pathogenesis of several hemolytic diseases such as thalassemia and hereditary spherocytosis [3].

Erythrocytes are more susceptible to oxidative stress due to the high polyunsaturated fatty acid content of their membrane and regularly exposed to oxygen than other body tissue [4]. Furthermore, high cellular concentration of oxygen and hemoglobin in the erythrocytes act as strong catalysts to oxidative hemolysis [5]. There are various harmful effects of ROS on oxidative stress related parameters in erythrocytes. The continuous exposure of erythrocytes to oxidant injury results in the changes of membrane behavior. Oxidation of lipid and protein membrane reduced the cells deformability and lead to the changes in cell morphology [6]. The destruction of these essential molecules cause the hemoglobin released in the plasma, which known as hemolysis [7].

More attentions have been given to the development of antioxidants from natural and botanical sources due to containing flavonoids, vitamins, polyphenols, etc [8]. *Azadirachta indica* (AI) belongs to Meliaceae family which is locally known as Neem or Semambu and have a wide spectrum of biological activity. In traditional medicine, AI is widely used for treating various human ailments. It has been documented that various parts of AI offer medicinal utilities which can be used for immunity booster, antifungal, anticarcinogenic activity, hepatoprotective activity and antiviral [9]. To our knowledge, the evaluation of antihemolytic activity of AI is yet to be fully studied. Thus, the present study was undertaken to investigate the antioxidant activity and protective effect of *Azadirachta indica* A. Juss. extract against hydrogen peroxide induced hemolysis.

2. MATERIALS AND METHODS

2.1 Chemicals

Hydrogen peroxide (H_2O_2), dimethyl sulfoxide, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), ethanol, ascorbic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride, acetic acid, sodium nitroprusside, Griess' reagent were obtained from Sigma Chemical Company (USA). All other chemicals and reagents used were of analytical grade.

2.2 Plant material and extraction

Leaves of *Azadirachta indica* were collected from Taman Botani, Shah Alam. Plant sample was then identified and authenticated from the Department of Forest Biodiversity, Forest Research Institute Malaysia (FRIM), Malaysia. The leaves were shade dried and coarsely powdered. The powdered then were extracted with 100% ethanol by soxhlet apparatus and evaporated to dryness using rotary evaporator.

2.3 *In vitro* antioxidant activity

2.3.1 DPPH scavenging activity assay

The DPPH radical scavenging activity of AI was measured by the method of Patel et al. [10]. Ethanolic extract of AI at various concentration (50-1000 µg/ml) were added to equal volume of ethanolic solution of DPPH (200 µM). Similarly, ethanolic solutions of ascorbic acid at various concentrations (2.5-25 µg/ml) were mixed with DPPH solution. A mixture of ethanol and ethanolic solution of DPPH was used as control. All the mixtures were left in dark for 20 minutes and the absorbance was measured at 517 nm. The measurements were performed in triplicate. The percentage of scavenging activity was calculated as follows:

DPPH scavenging activity (%) = [(Abs control - Abs sample) / (Abs control)] X 100

2.3.2 Nitric oxide scavenging activity assay

The nitric oxide radical scavenging activity of AI was carried out by the method of Green et al. [11]. Ethanolic extract of AI at various concentration (50-1000 µg/ml) were mixed to equal volume of sodium nitroprusside solution (5 mM). Similarly, ascorbic acid solutions at various concentrations (2.5-25 µg/ml) were mixed with sodium nitroprusside solution (5 mM). A mixture of phosphate buffer (pH 7.4) and sodium nitroprusside solution was used as control. The mixtures then were incubated at 25 ^{0}C for 5 hours. After incubation, the mixture was mixed with Griess' reagent and the absorbance was measured at 546 nm. The experiments were performed in triplicate. Percentage of scavenging activity was calculated using the same formula as described by DPPH scavenging assay.

2.3.3 Ferric reducing antioxidant power (FRAP) assay

The reducing power of AI was evaluated by the method of Benzie & Strain [12]. A working FRAP reagent was prepared using acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM Fe.Cl3.6H2O and the mixture were in a ratio of 10:1:1. Ten microliters ethanolic extract of AI at various concentration (50-1000 µg/ml) were mixed with 300 µL FRAP reagent. Similarly, ascorbic acid at various concentrations (2.5-25 µg/ml) were mixed with FRAP reagent and used to generate a standard curve for FRAP assay. All the mixtures were incubated at 30 ^{0}C for 4 minutes and the absorbance was measured at 610 nm. The tests were performed in triplicate.

2.4 Protective effect of AI against oxidative hemolysis

Blood samples from healthy volunteers were collected in heparinized tubes and centrifuged at 2000 rpm at 4 ^{0}C for 10 minutes. The plasma and buffy coat were removed and the erythrocytes were then washed three times with cold phosphate buffer saline (PBS, pH 7.4). A 5% erythrocytes suspension was freshly prepared and used for in vitro protective effect of AI against hydrogen peroxide induced hemolysis. In brief, the cell suspension was mixed with ethanolic extract of AI at different concentrations (50-1000 µg/ml), then 10 mM (H_2O_2) was added. The mixture was incubated at 37 ^{0}C for 3 hours. In an identical manner, ascorbic acid at various concentrations (2.5-25 µg/ml) was used as positive control. Following incubation, the mixture was added with PBS and centrifuged at 2000 rpm for 10 minutes. The absorbance of the supernatant was measured at 540 nm. The result was calculated as percentage of hemolysis inhibition.

2.5 Data analysis

The results were presented as mean ± S.E.M from three independent experiments. IC_{50} is the concentration of AI extract required to scavenge 50% radicals for scavenging activity and to inhibit 50% hemolysis for protective effect against hydrogen peroxide induced erythrocytes lysis.

3. RESULTS

3.1 DPPH scavenging activity of AI extract

The DPPH radical scavenging activity of AI extract and ascorbic acid were in a dose-dependent manner. IC_{50} of the AI extract and ascorbic acid were 680.20 ± 4.51 µg/ml and 19.23 ± 0.87 µg/ml respectively (Figure 1). The potency of AI extract was approximately 0.03 times of ascorbic acid.

3.2 Nitric oxide scavenging activity of AI extract

The nitric oxide radical scavenging activity of AI extract and ascorbic acid were dose dependent. IC_{50} of the AI extract and ascorbic acid were 636 ± 14.71 µg/ml and 20 ± 0.33 µg/ml respectively (Figure 2). The potency of AI extract was approximately 0.03 times of ascorbic acid.

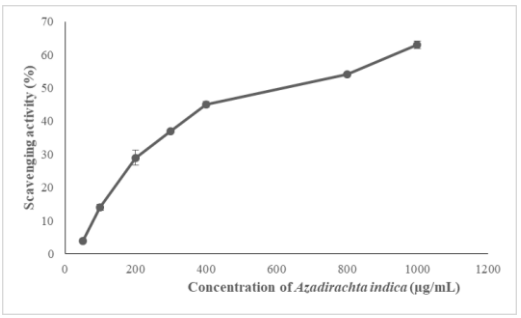
3.3 Ferric reducing antioxidant power (FRAP) activity of AI extract

The FRAP activity of AI extract and ascorbic acid were also in a dose-dependent manner and presented in Figure 3. The AI extract possessed reducing power with 1 g of AI extract being equivalent to 23.3 mg of ascorbic acid. The ferric reducing activity AI extract was 43 times less potent than ascorbic acid.

3.4 Effect of AI extract on H_2O_2 induced hemolysis

The protective effect of AI extract against H_2O_2 induced hemolysis was also dose dependent. IC_{50} of the AI extract and ascorbic acid were 415.56 ± 11.05 µg/ml and 19.67 ± 0.33 µg/ml respectively (Figure 4). The potency of AI extract was approximately 0.05 times of ascorbic acid.

a



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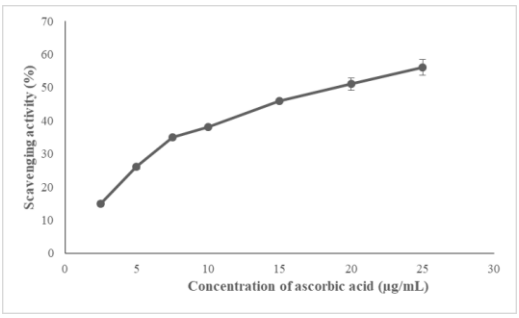

Figure 1: The DPPH scavenging activity of the (a) ethanolic extract of *Azadirachta indica* and (b) ascorbic acid.

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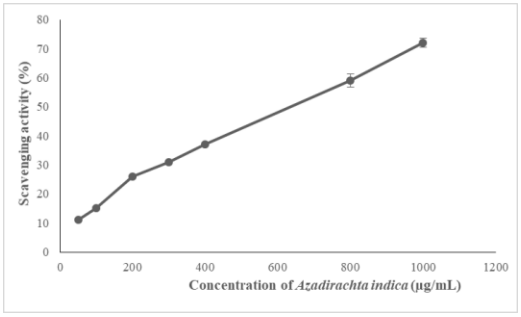

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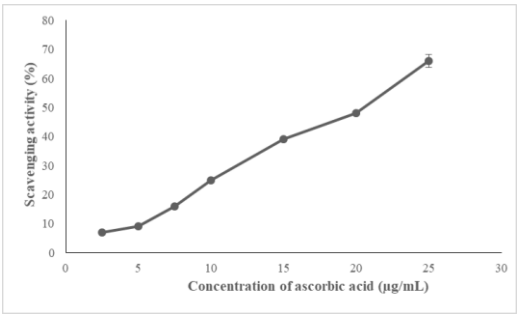

Figure 2: The nitric oxide scavenging activity of the (a) ethanolic extract of *Azadirachta indica* and (b) ascorbic acid.

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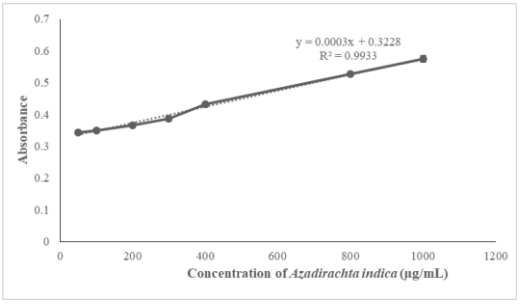

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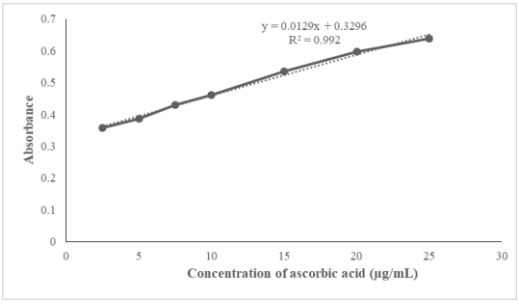

Figure 3: The ferric reducing antioxidant power of the (a) ethanolic extract of *Azadirachta indica* and (b) ascorbic acid.

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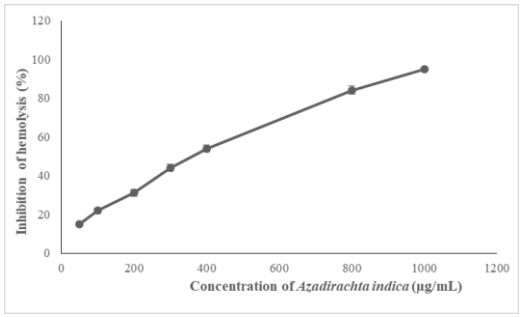

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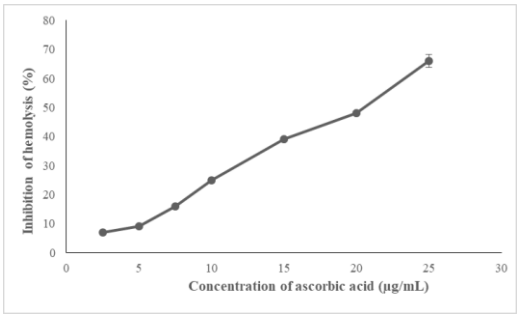

Figure 4: Effects of the (a) ethanolic extract of *Azadirachta indica* and (b) ascorbic acid against H_2O_2 induced hemolysis.

4. DISCUSSION

The findings of the present study exhibit the antioxidant activity of AI extract in different *in vitro* models. It was demonstrated that the capability of AI extract to scavenge free radicals and to reduce ferric ions were in a dose dependent manner. The AI extract was also found to be potent in the protection of erythrocytes against H_2O_2 induced hemolysis.

Investigation on medicinal plant extracts revealed the presence of naturally occurring antioxidants which provide benefits to health. The identified antioxidant compounds have the capacity to act as a free radical or reactive oxygen species scavenger [13]. Many biologically active compounds that can be extracted from AI extract including alkaloids, flavonoids, triterpenoids, phenolic compounds and others. Phytochemical investigation on the ethanolic extract AI leaves showed the presence of reducing sugar, flavonoids, saponin and tannin [14]. Other phytochemical study of AI leaves by using different solvent showed the presence of reducing sugars, gums, proteins, fats, steroids, saponin glycosides, flavonoid, alkaloids, tannins and phenolic compounds [15]. Phenolic hydroxyl group in polyphenols, tannins and flavonoids are reported to have redox properties and free radical scavenging activities [16].

In this study, the free radical scavenging activity of AI was measured by DPPH radical and nitric oxide radical scavenging activity assay. A review from Alam et al. [17] found that DPPH method was mostly used for the in vitro antioxidant assay. DPPH is a stable free radical and gets reduced when mixed with antioxidants which able to act as hydrogen donors or free radical scavengers [18]. Nitric oxide is important in the regulation of various physiological processes and its overproduction is implicated in several diseases [19]. In the nitric oxide scavenging activity, the scavengers will compete with oxygen to interact with nitric oxide and reduced the production of nitrite ions [20]. Following these two methods, the present study demonstrated the capacity of AI extract to scavenge the DPPH and nitric oxide radicals. Similarly, an earlier observation done by Patel et al. [10] showed a dose dependent scavenging activity of AI extract against DPPH and nitric oxide radicals. Previous study by Deka et al. [15] using different solvents has documented a lower IC_{50} value for DPPH radical scavenging activity of AI extract when compared with present findings. The ability of antioxidant to reduce ferric ions is measured by FRAP activity. The AI extract exhibited antioxidant activity by converting ferric ions to ferrous ions. Therefore, it is possible that the antioxidant activity of AI extract is due to presence of antioxidant compound in the leaves.

Frequently exposure of erythrocytes membrane to oxidative agent may lead to cell hemolysis. Invasion of the erythrocytes membrane by H_2O_2 lead to the formation of hydroxyl radical that can initiate lipid peroxidation and leakage of hemoglobin [1]. In addition, the radicals may cause the oxidation of protein membrane and reduced glutathione levels which lead to cellular deformability [4]. The results of the present study indicate that the AI extract could inhibit H_2O_2 induced erythrocytes lysis. Interaction of antioxidant compound in AI extract with biological membrane may maintain the membrane stability and fluidity [21, 22]. Therefore, it can be postulated that antioxidant activity of AI extract are responsible for the protective effect against oxidative hemolysis induced by H_2O_2.

5. CONCLUSION

From the present study, it can be concluded that ethanolic extract of AI leaves has *in vitro* antioxidant activity. Besides, investigation also demonstrated that the extract could protects the erythrocytes hemolysis against H_2O_2 induce oxidative stress. These findings suggest that the AI might be a valuable natural antioxidant in the amelioration of oxidative damage.

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