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Protective effects of aqueous and ethanolic extracts of *Nigella sativa* L. and *Portulaca oleracea* L. on free radical induced hemolysis of RBCs

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ABSTRACT

Background and the purpose of the study: It has been shown that *Nigella sativa* L. and *Portulaca oleracea* L. have many antioxidant components. In the present study, the cytoprotective effect of ethanolic and aqueous extracts of *N.sativa* and *P.oleracea* against hemolytic damages induced by free radical initiator, AAPH [2, 2' azobis (2- amidinopropane) hydrochloride] was evaluated.
Methods: Hemolysis was induced by addition of AAPH. To study the cytoprotective effect, aqueous (50, 200, 300, 400, 800 µg/ml) and ethanolic (25, 100, 150, 200 and 400 µg/ml) extracts of *N. sativa* and aqueous (25, 50, 100, 150, 200 and 400 µg/ml) and ethanolic (300, 600, 900, 1200 and 1800 µg/ml) extracts of *P. oleracea* were employed. RBCs were incubated with both extracts and AAPH at 37 °C for 6 hrs. In order to evaluate the impact of the time of addition, extracts were added one and 2 hrs after AAPH. Samples of suspensions were removed at different times and the degree of hemolysis was assessed spectrophotometrically by reading the absorption of supernatants at 540 nm.
Results: Aqueous (300, 400 and 800 µg/ml) and ethanolic (150, 200 and 400 µg/ml) extracts of *N.sativa* and also, aqueous (100, 150, 200 and 400 µg/ml) and ethanolic (1200, 1800 µg/ml) extracts of *P.oleracea* showed concentration-dependent cytoprotective effects. Addition of extracts one hour after AAPH reduced but did not eliminate protective activities of extracts.
Conclusion: Cytorotective effect of aqueous and ethanolic extracts of *N. sativa* and *P. oleracea* against AAPH- induced hemolysis may be related to antioxidant properties of these plants.

Keywords: Red blood cell, Antioxidant, AAPH.

INTRODUCTION

Reactive oxygen species (ROS) are formed in the body and a lack of balance in oxidant-antioxidant activity lead to several pathologic events (1). Oxidation of erythrocyte membranes is considered as a model injury of cell injuries (2). In intact isolated erythrocytes, AAPH [2, 2' azobis (2-amidinopropane) hydrochloride, a water-soluble radical generator] induce oxidation of membrane and cause hemolysis. The combined application of intact erythrocytes and AAPH can be considered as an appropriate experimental technique to evaluate in vitro ability of new potentially cytoprotective agents through free radical scavenger activities (3). Many studies have shown that antioxidants inhibit free radical oxidative damages (4).

Nigella sativa L. (black seed) is a member of Ranunculaceae family. Pharmacological studies have shown that the extract of seeds have antioxidant (5), anti-inflammatory (6) and anti-ischemic (7) effects. The antioxidant constituents of black seed are thymoquinone, carvacrol, t-anethole and 4-terpineol (5). Thymoquinone is the most important compound of *N. sativa* which is mostly responsible for antioxidant and free radical scavenging (8).

Portulaca oleracea L. is considered by the World Health Organization as one of the mostly used medicinal herbs. Recent investigation has demonstrated the antioxidant and free radicals scavenging activities of the *P. oleracea* (9). The plant has many antioxidant constituents such as omega-3 fatty acids, gallotannins, kaempferol, quercetin, apigenin, α-tocopherols, ascorbic acid and glutathione (10-12).

In the present study, possible cytoprotective activities of the aqueous and ethanolic extracts of *N. sativa* and *P. oleracea* against AAPH induced erythrocytes hemolysis, were evaluated.

MATERIAL AND METHODS

Preperation of the aqueous and ethanolic extracts of N. sativa and *P. oleracea*

N. sativa seeds were purchased from a local market

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in Mashhad, Iran. Aerial parts of *P. oleracea* were collected from campus of Ferdowsi University of Mashhad. The plants identified by Mr Joharchi at the Herbarium Center of Mashhad Ferdowsi University.

Aqueous and ethanolic extracts of of *N. sativa* seeds and the aerial parts of *P. oleracea* were prepared and used in this study (13).

Experimental procedure

A reported method with some modification was employed. (3). Briefly, blood was obtained from anaesthetized male wistar rats and quickly centrifuged (at 3000 rpm, 10 min, 22°C). Pellets of erythrocytes was subsequently used to prepare a 10% v/v erythrocyte suspension in phosphate buffer (pH=7.4). Hemolysis was carried out by addition of AAPH stock (50 mM) prepared in phosphate buffer. To assay the effect the aqueous and ethanolic extracts of *N. sativa* and *P. oleracea* on hemolysis, RBCs were incubated with different concentration of extracts and AAPH at 37 °C for 6 hrs.

In order to evaluate the impact of the time of exposure, the extracts were added 1 and 2 hrs after exposure to AAPH. Vitamin C (100 µg/ml) was selected as a reference inhibitor of hemolysis. Samples of the suspensions were removed at different time intervals and the degree of hemolysis was assessed spectrophotometrically by reading the absorption of supernatants at 540 nm (absorption A). Similarly, the reaction mixture was treated with 0.1% Triton X-100 solution to yield complete hemolysis, and the absorption of the supernatant after centrifugation was measured at 540 nm (absorption B). Percentage of hemolysis was calculated from the formula: absorption A/ absorption B ×100.

Statistical analyses

Data were presented as mean ± SEM. All data were analyzed using analysis of variance (ANOVA) followed by tukey-kramer test for comparison. Statisticsl significance was defined as $P<0.05$.

RESULTS AND DISCUSSION

Slight hemolysis (less than 6%) was observed in erythrocytes suspended in isotonic PBS which could be related to the mechanical stress of shaking. Both aqueous and ethanolic extracts of these plants did not induce any significant hemolysis in comparison with PBS. AAPH at concentration of 25 and 50 mM induced hemolysis in erythrocytes suspension in a concentration and time–dependent manner (Fig. 1). Results of this study demonstrated that the time required by AAPH to initiate the hemolysis inversely and the rate of the cell lysis induced by AAPH is directly proportional, to its extracellular concentration. According to these results, maximum hemolysis was found at concentration of 50 mM at incubation time of 6 hrs. The aqueous (300, 400, 800 µg/ml) and ethanolic (150, 200 and 400 µg/ml) extracts of *N. sativa* seeds and the aqueous (100, 150, 200 and 400 µg/ml) and ethanolic (1200 and 1800 µg/ml) extracts of *P. oleracea* decreased the rate of AAPH-induced hemolysis during the incubation period (Figs. 2-5).

Incubation of erythrocytes with AAPH decreased the level of glutathione (14). Some studies have shown that glutathione is in the first line of defense during oxidative damages (15). Several studies have demonstrated that *N. sativa* and *P. oleracea* increased cellular glutathione contents (10, 16). Data have shown that both plants increased the lag time of AAPH- induced hemolysis and decreased RBC damages. The protective effect of these plants may be due to prevention of AAPH induced decrease in intracellular glutathione. This is consistent with the results of another study that showed a commercial antioxidant mixture and Antrodia camphorate mycelia extract may prevent reduction of glutathione in erythrocytes (17).

Protective effect of *N. sativa* on AAPH induced hemolysis in RBCs is related to active components such as thymoquinone and carvacrol. It has been shown that thymoquinone and carvacrol are free radical scavenger and antioxidant (18-20). Also, *P. oleracea* extracts inhibited hemolytic damage induced by AAPH which is associated to antioxidant constituents such as omega-3 fatty acids, α-tocopherols, ascorbic acid and glutathione (10-12). The ability of *N. sativa* (18, 20) and *P. oleracea* (21, 22) to scavenge free radicals are related to reduction the net concentration of AAPH-derived peroxyl radicals generated during the test. It is likely that during the induction phase, antioxidant components of these plants are increasingly used by AAPH–derived peroxyl radicals. This scavenging property would decrease the extracellular amount of peroxyl radicals and this effect lead to the reduction in erythrocytes hemolysis. Results of other studies have shown that the hemolysis starts only after AAPH has reduced the erythrocyte membrane alpha-tocopherol level to critically low concentrations (23).

As shown in figure 2, RBCs hemolysis started 2 hrs after addition of 50 mM of AAPH, and the effect of extracts were evaluated 1 and 2 hrs after addition of AAPH. The effects of delay in addition of aqueous and ethanolic extracts (800 and 400 µg/ml, respectively) of *N. sativa* seeds and aqueous and ethanolic extracts (400 and1800 µg/ml, respectively) of *P. oleracea* on AAPH-induced erythrocyte lysis are shown in figures 6 and 7. The delay in addition of extracts by one hour was reduced relative to AAPH but did not eliminate protective effect of the extracts. However, addition of extracts 2 hrs after AAPH incubation had no effect on erythrocyte hemolytic, because RBCs hemolysis and oxidative stress started 2 hrs after AAPH addition.

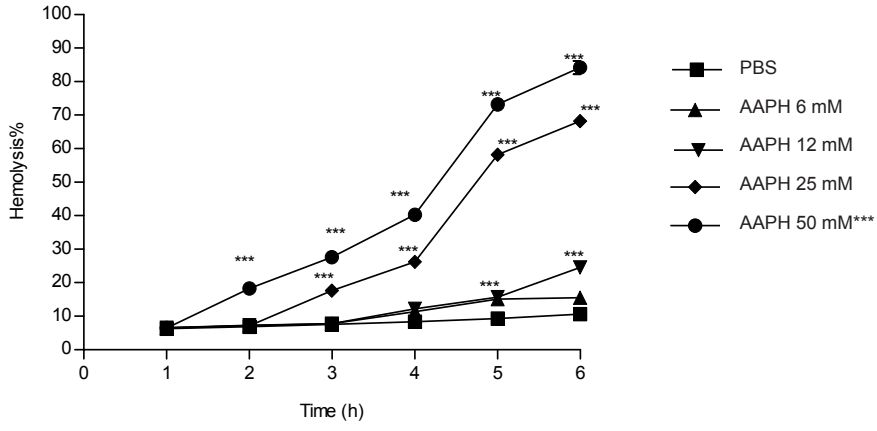

Figure 1. The effect of different concentrations of AAPH on hemolysis of RBCs.
Values are the mean ± S.D. n= 5. ***P < 0.001, compared to control (PBS), Tukey–Kramer.

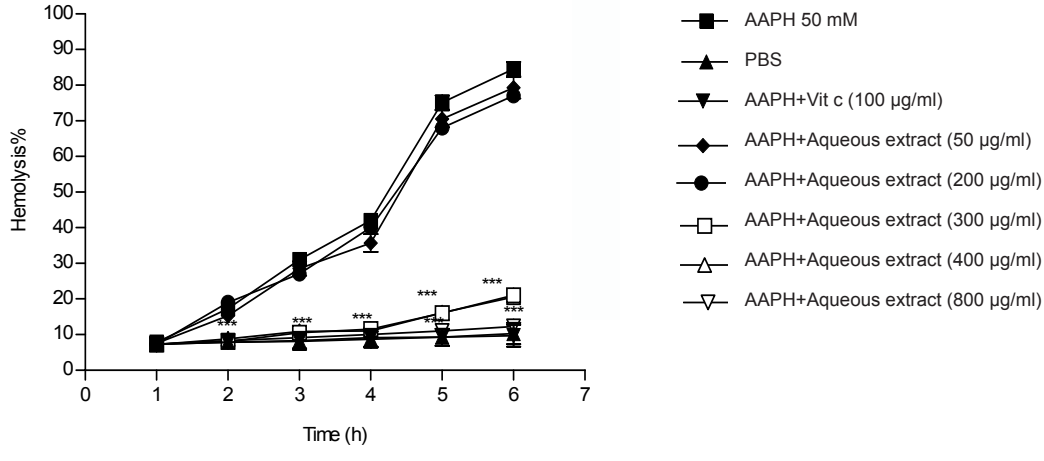

Figure 2. The effect of different concentrations of aqueous extracts of *N. sativa* seeds on the AAPH-induced erythrocyte lysis. Values are the mean ± S.D. n= 5. ***P < 0.001 , compared to AAPH, 50 mM, Tukey–Kramer.

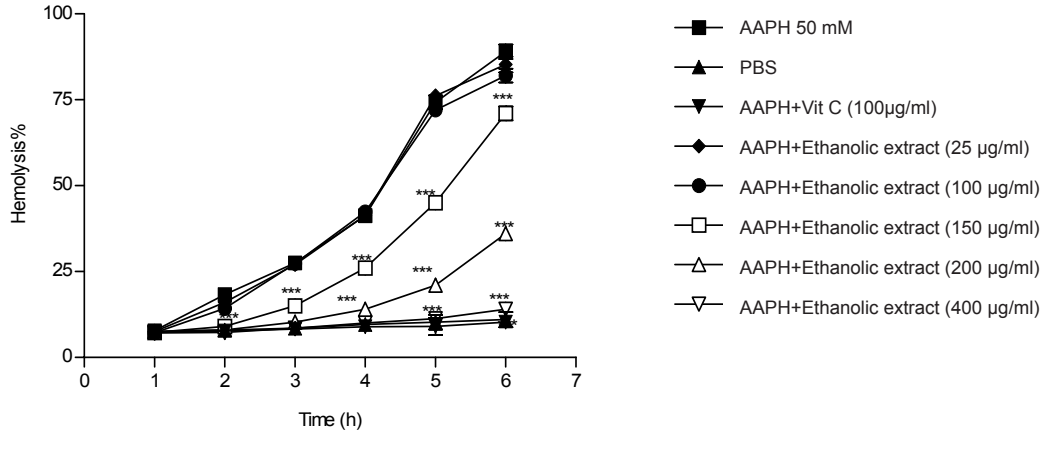

Figure 3. The effect of different concentrations of ethanolic extracts of *N. sativa* seeds on the AAPH-induced erythrocyte lysis.Values are the mean±S.D. n=5. ***P < 0.001, compared to AAPH, 50 mM , Tukey-Kramer.

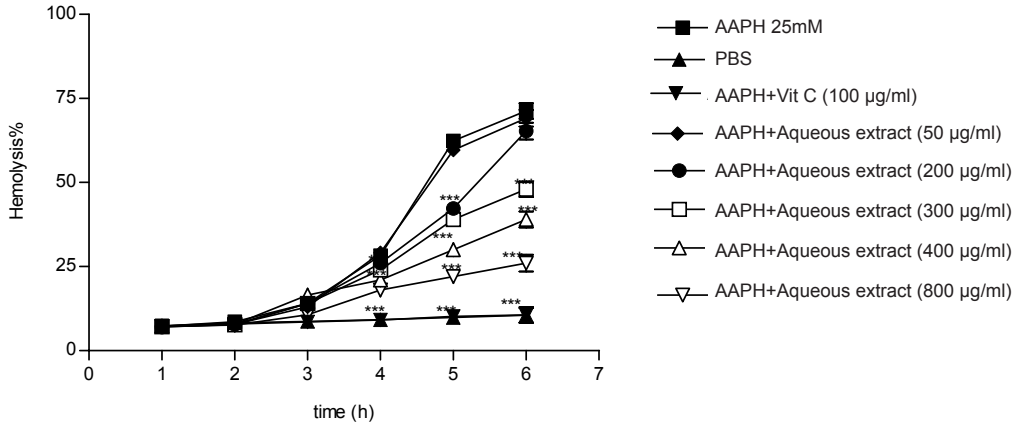

Figure 4. The effect of different concentrations of aqueous extracts of *P. oleracea* on the AAPH-induced erythrocyte lysis.
Values are the mean ± S.D. n= 5. ***P < 0.001, compared to AAPH, 50 mM, Tukey–Kramer.

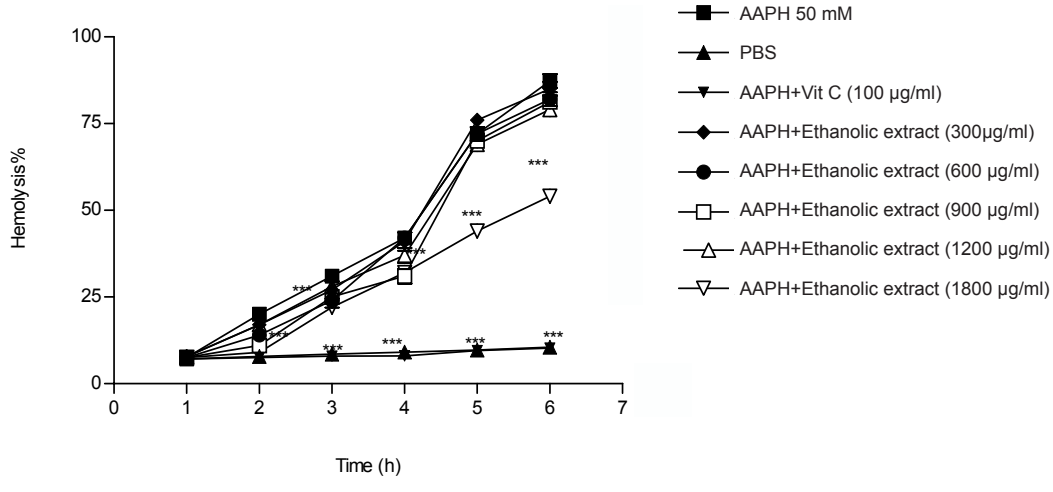

Figure 5. The effect of different concentrations of ethanolic extracts of *P. oleracea* on the AAPH-induced erythrocyte lysis.
Values are the mean ± S.D. n= 5. ***P < 0.001, compared to AAPH, 50 mM, Tukey–Kramer.

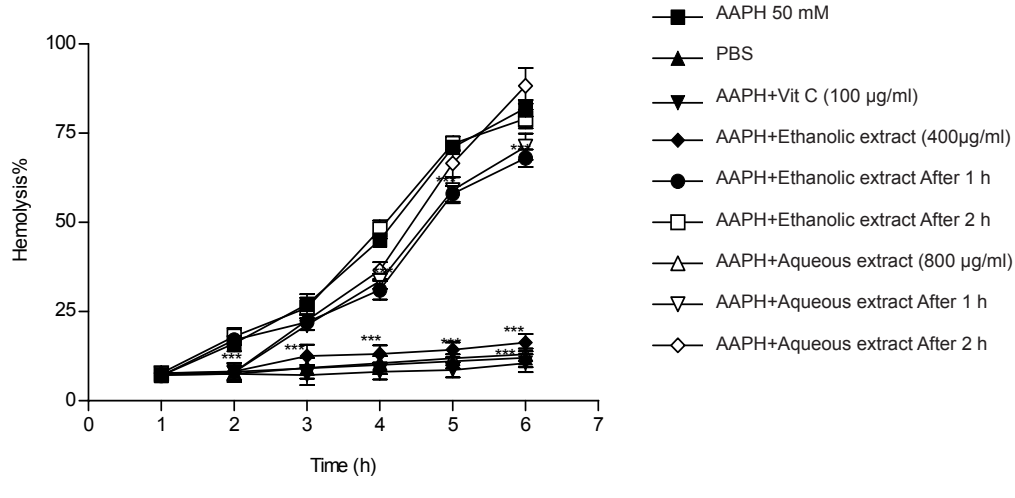

Figure 6. The effect of the delayed addition of aqueous and ethanolic extract (800 and 400 µg/ml, respectively) of *N. sativa* seeds on the AAPH-induced erythrocyte lysis.
Values are the mean ± S.D. n= 5. ***P < 0.001, compared to AAPH, 50 mM, Tukey–Kramer.

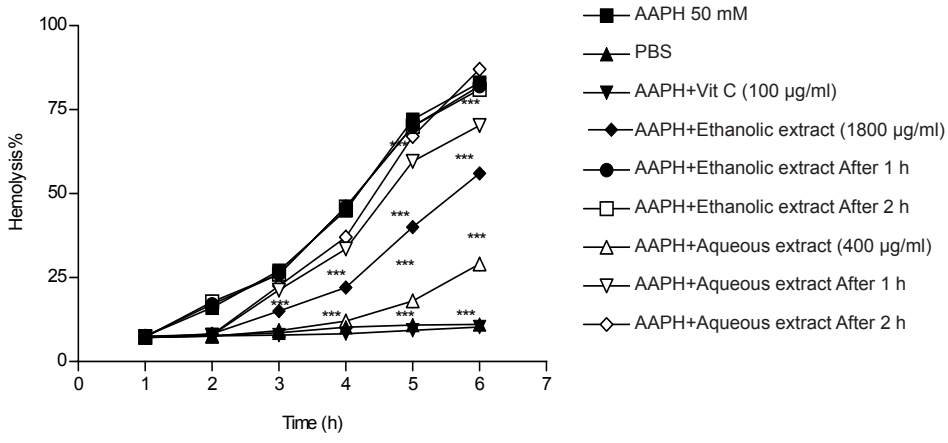

Figure 7. The effect of the delayed addition of aqueous and ethanolic extracts (400 and 1800, respectively µg/ml) of *P. oleracea* on the AAPH-induced erythrocyte lysis.
Values are the mean ± S.D. n= 5. ***P < 0.001, compared to AAPH, 50 mM, Tukey–Kramer

Considering the effects of both plants on AAPH induced hemolysis, they may be used as therapeutic agents to alleviate disturbances relevant to the oxidative stresses and related diseases.

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