

Evaluation of fibrinolytic and antioxidant effects of *Allium affine* hydroalcoholic extract

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Abstract

Allium affine is a member of Amaryllidaceae family, which grows wildly in some western regions of Iran. Limited information is available about the pharmacological activities of this plant. The present study aimed to evaluate the fibrinolytic and antioxidant effects of hydroalcoholic extract of *A. affine* aerial parts. The *in vitro* antioxidant properties of the extract were evaluated by total phenolic content assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity and ferric reducing antioxidant power (FRAP) assay. The *in vivo* studies included the determination of hydroperoxides level and FRAP value in serum samples of rats receiving i.p. injections of the plant extract for 21 days. The fibrinolytic activity of the extract was quantitatively evaluated by measuring the clot weight. *In vitro* antioxidant analysis exhibited the promising potential of DPPH scavenging and total antioxidant capacity of *A. affine* extract. In the *in vivo* analysis, *A. affine* extract reduced the serum hydroperoxides level and increased the serum total antioxidant capacity in rats. *In vitro* fibrinolytic assay also elucidated notable thrombolytic activity of the plant extract. The results of this study revealed the valuable antioxidant and *in vitro* fibrinolytic activities of *A. affine* extract. Further studies are needed for better evaluation of anticoagulant and thrombolytic activities of this plant and understanding its detailed mechanisms.

Keywords: *Allium affine*; Antioxidant; Fibrinolytic agents

INTRODUCTION

The relationship between plants and medicine started with human civilization. Archeological evidences obtained from ancient Chinese and Egyptians indicate medicinal uses of herbs and ethno-medicine as early as 3,000 BC (1). Most of the people especially in developing countries use traditional or alternative medicine products (2). The total ratio of herbal medicines is equal to almost 25% of prescripational medicines around the world (3).

The genus *Allium* (Amaryllidaceae; Alliaceae) contains about 800 species, which are widely spread in northern hemisphere. The use of *Alliums* as nutrition and medicine started thousands of years ago. Garlic, onion,

leek and scallion are some of the most interesting members of the genus *Allium* which are used around the world as food, spice or medicine (4).

Ancient civilizations have used garlic for treating respiratory and urinary tract infections, leucorrhea and vaginitis, high blood pressure, diabetes and skin infections in traditional folk medicine (5). Pharmacological studies have shown various biological effects including antibacterial, antifungal, antiviral, antihypertensive, antihyperglycemic, anti-hyperlipidemic, anti-platelet aggregation, antithrombotic and anticarcinogenic activities for different *Allium* species (6).

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Because of their helpful effects, *Allium* species are often recommended for prevention and treatment of cardiovascular diseases (7).

Allium affine, a member of the genus *Allium*, is not well known to date. *A. affine* is highlighted with its fistular, semicylindrical, grooved leaves and ovated bulb (8). This plant is indigenous to the middle Asian countries and grows wildly in west regions of Iran like Chaharmahal and Bakhtiari Province (9,10). Although *A. affine* is widely used in its growth regions as an edible vegetable, a condiment and also for treatment of some diseases, limited information is available about the phytochemistry and pharmacological activities of this specie of *Allium*. Anticoagulant and fibrinolytic activities have been reported for several natural constituents like saponins (11,12). Isolation and identification of some steroidal saponin and sapogenins like diosgenin, tigogenin and ruscogenin in *A. affine* might propose potential of anti-thrombotic activity for this plant (10). Therefore, the present study was conducted to investigate the fibrinolytic and also *in vivo* and *in vitro* antioxidant properties of *A. affine* aerial parts extract.

MATERIALS AND METHODS

Chemicals

The assay kits for evaluation of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, hydroperoxides concentration and ferric reducing antioxidant power (FRAP) were purchased from Hakiman Shargh Research Co. (Isfahan, Iran). Streptokinase was prepared from Karma-Pharmatech GmbH (Germany). Folin-Ciocalteu reagent and all other chemicals were purchased from Merck Co. (Germany).

Plant material and preparation of hydroalcoholic extract

The aerial parts of *A. affine* were prepared from the local venders in Borujen, Chaharmahal and Bakhtiari Province, Iran, on May 2015. The plant was identified by a botanist and a voucher specimen (No. 3403) was deposited at the Herbarium of the Pharmacognosy Department, School of

Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences. For preparation of *A. affine* hydroalcoholic extract, the powdered air-dried aerial parts of the plant (1200 g) were extracted by maceration method with 4 liter hydroalcoholic solvent containing 70% ethanol for 72 h, three times at room temperature. The extract was concentrated under vacuum by rotary evaporator at 50 °C and freeze-dried to make a fine extract powder which was stored in the refrigerator till used for the assays. The yield of the plant extraction was 38.2% (w/w; dried residue after removal of chlorophyll).

Animals

Male Wistar albino rats weighing 180 to 220 g were obtained from the animal house of the School of Pharmacy and Pharmaceutical Sciences (Isfahan, Iran). The animals had free access to water and standard laboratory diet and were kept under standard laboratory settings with a 12 h light/12 h dark cycle. Rats were conditioned to the laboratory situation for 1 week before the research. The study protocol was approved by the Bioethics Committee of Isfahan University of Medical Sciences (Registration No. 394395), and performed in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals.

For *in vivo* study, 30 rats were randomly divided in to five groups of 6 each. Animals received daily intraperitoneal (i.p.) injections of *A. affine* extract at 100, 200 and 400 mg/kg or vitamin C (Vit C; 30 mg/kg, as a positive antioxidant control) for 21 days (13). The plant extract was dissolved in normal saline for preparation of an injectable solution and control animals received daily i.p. injection of vehicle. At the end of the experiment, the blood was collected from animals through direct cardiac puncture under mild ether anesthesia and serum samples were used for further experiments.

In vitro antioxidant assays

Determination of total phenolic content

Total phenolic content was estimated spectrophotometrically using Folin-Ciocalteu reagent. Briefly, the diluted reagent was mixed

with plant samples. After 5 min, sodium carbonate solution (20%) was added to the mixture followed by storing the samples at room temperature for 120 min. Subsequently, UV absorbance was measured at 765 nm using a UV-visible spectrophotometer (14). Total phenolics were quantified by standard curve obtained from various concentrations of gallic acid (50-500 µg/mL in methanol). The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram of dried extract.

Free radical scavenging assay

The free radical scavenging activity of *A. affine* hydroalcoholic extract was analyzed by DPPH method. According to the manufacturer's protocol, the methanolic solution of DPPH was added to 100 µL of different concentrations of plant extract and absorbance was measured at 517 nm after 30 min incubation in dark condition at room temperature. The ability of plant extract to scavenge the free radical was calculated from the formula $[(A_0 - A_1)/A_0] \times 100$, where, A_0 is the absorbance of the control, and A_1 is the absorbance of the sample. Vitamin C (Vit C) was used as the standard reference (14). The half maximal reduction concentration (RC_{50}) was estimated through a series of dose-response data and using an equation which was fitted to the curve.

Ferric reducing antioxidant power assay

The total antioxidant capacity of the *A. affine* hydroalcoholic extract was determined by FRAP method which measures the reduction of ferric-tripyridyl triazine (TPTZ) complex to ferrous form by colorimetric assay. Briefly, the FRAP reagent containing TPTZ/ferric chloride/acetate buffer was freshly prepared according to the manufacturer's protocol and was added to 10 µL of different concentrations of plant extract. After incubation for 40 min at 37 °C, the absorbance of colored solutions was measured at 570 nm using a microplate reader/spectrophotometer (Bio-Tek, PowerWave XS, USA). The FRAP value of the test samples were measured using a standard curve of $FeSO_4$ (15).

In vivo antioxidant experiments

Measurement of hydroperoxides concentration

The effect of administration of *A. affine* hydroalcoholic extract on serum hydroperoxides level in rat was determined based on the ferrous ion oxidation by xylenol orange reagent (FOX-1) assay. This reagent is consisted of ammonium ferrous sulfate and xylenol orange in aqueous medium containing sorbitol. After preparing the reagent according to the manufacturer's protocol, it was added to 10 µL of serum samples. The mixture was then incubated for 30 min in 37 °C and absorbance was measured at 540-560 nm using a microplate reader/spectrophotometer. The hydroperoxides concentration of serum samples were expressed as micromolar of hydrogen peroxide (H_2O_2) equivalents using a standard curve of different concentrations of H_2O_2 (16).

Ferric reducing antioxidant power assay

The effect of administration of hydroalcoholic extract of *A. affine* on serum total antioxidant capacity was evaluated by FRAP method as described above (15).

In vitro fibrinolytic assay

The fibrinolytic activity was quantitatively assessed by measurement of the clot weight. For this mean, the blood samples were collected from healthy human volunteers without a history of treatment with drugs affecting the hemostasis such as oral contraceptive or anticoagulants. The blood specimen (500 µL) was transferred to the pre-weighed micro-centrifuge tube and incubated at 37 °C for 30 min for clot formation. Then serum was completely removed without disturbing the clot and each tube was again weighed to determine the clot weight. After that, 100 µL of different concentrations of *A. affine* hydroalcoholic extract (0.005-50 mg/mL) or streptokinase were added to the micro-centrifuge tube containing the clots. The commercially lyophilized streptokinase vial (1500000 IU) was diluted with normal saline to prepare the working solution of positive thrombolytic control (8000 IU; equivalent to half maximal inhibitory concentration; IC_{50}) and normal saline was considered as a

negative control. After incubation of all tubes at 37 °C for 90 min, the released fluid was removed and the tubes were again weighed for calculation of the clot lysis percentage based on the difference between the initial and final weight of the clot (17).

Statistical analysis

Data were represented as the mean \pm SEM. One-way analysis of variance (ANOVA) followed by Tukey post-hoc test was used (SPSS software version 21.0) to compare the means. *P* values < 0.05 were considered as significant difference.

RESULTS

In vitro antioxidant experiments

Total phenolic assay

Based on the standard curve obtained through the evaluation of various concentrations of gallic acid, the total phenolic

content of the *A. affine* hydroalcoholic extract was determined as 19.62 ± 1.1 mg GAE/g of dried plant extract.

Free radical scavenging assay

DPPH scavenging test was used for evaluation of free radical scavenging activity of *A. affine* hydroalcoholic extract. RC_{50} for Vit C as a standard antioxidant was 43 μ g/mL. The scavenging effect of the plant extract is depicted in Fig. 1 where RC_{50} was found to be 201 μ g/mL.

Ferric reducing antioxidant power assay

FRAP method was used for evaluation of total antioxidant capacity of *A. affine* hydroalcoholic extract.

The results which were expressed as the equivalents of ferrous sulfate showed the increasing trend in total antioxidant capacity with increasing the extract concentrations (Fig. 2).

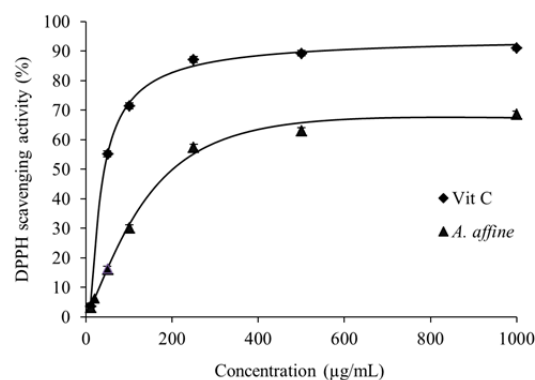


Fig. 1. Scavenging activity of *A. affine* hydroalcoholic extract and vitamin C (10-1000 μ g/mL) against 1, 1-diphenyl-2-picrylhydrazyl (DPPH). Data are presented as means \pm SEM of three independent experiments.

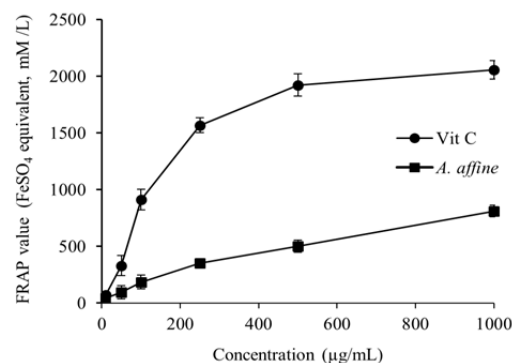


Fig. 2. Ferric reducing antioxidant power (FRAP) values of *A. affine* hydroalcoholic extract and vitamin C (10-1000 μ g/mL) measured as ferrous sulfate equivalents. Values are expressed as means \pm SEM from three independent experiments.

In vivo antioxidant experiments

Measurement of hydroperoxides concentration

FOX-1 method was used for determination of serum hydroperoxides level after administration of hydroalcoholic extract of *A. affine* and Vit C for 21 days in rats. Vit C resulted in a significant reduction in hydroperoxides level ($P < 0.001$). *A. affine* extract also significantly reduced the serum hydroperoxides concentration at all doses (Fig. 3).

Ferric reducing antioxidant power assay

The *in vivo* analysis showed significant increase in serum total antioxidant capacity after administration of *A. affine* hydroalcoholic extract at the doses of 200 and 400 mg/kg (Fig. 4).

In vitro fibrinolytic assay

Streptokinase as a positive control (8000 IU) resulted in 51.4% clot lysis. The thrombolytic activity of different concentrations of *A. affine* hydroalcoholic extract is exhibited in Fig. 5.

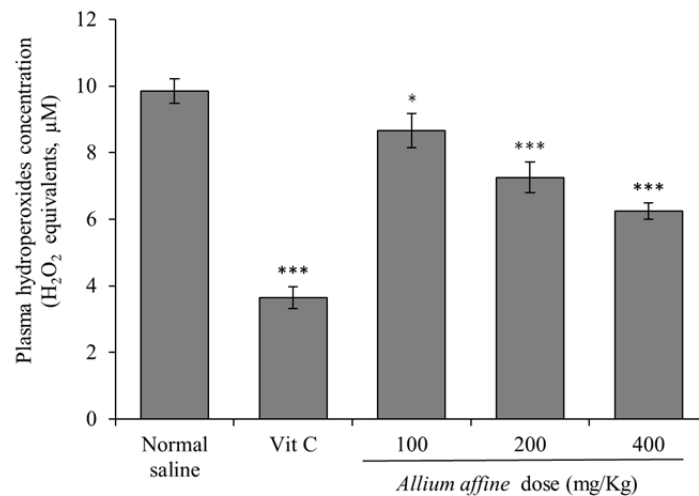


Fig. 3. Effect of 21-days administration of *A. affine* hydroalcoholic extract (100-400 mg/kg) and vitamin C (30 mg/kg) on plasma hydroperoxides concentrations determined as H₂O₂ equivalents by ferrous ion oxidation by xylenol orange reagent (FOX-1) method. Values are presented as means ± SEM for six rats. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ versus normal saline control group.

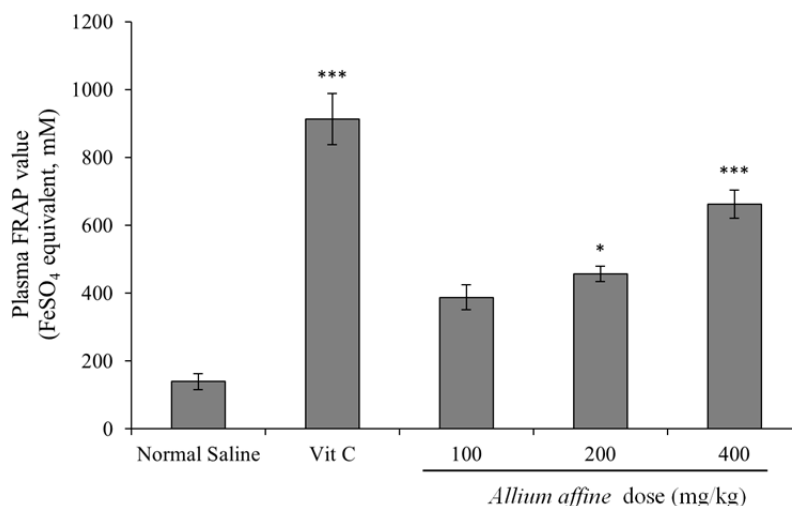


Fig. 4. Effect of 21-days administration of *A. affine* hydroalcoholic extract (100-400 mg/kg) and vitamin C (30 mg/kg) on plasma ferric reducing antioxidant power (FRAP) determined as ferrous sulfate equivalents. Values are presented as means ± SEM for six rats. *** $P < 0.001$ versus normal saline control group.

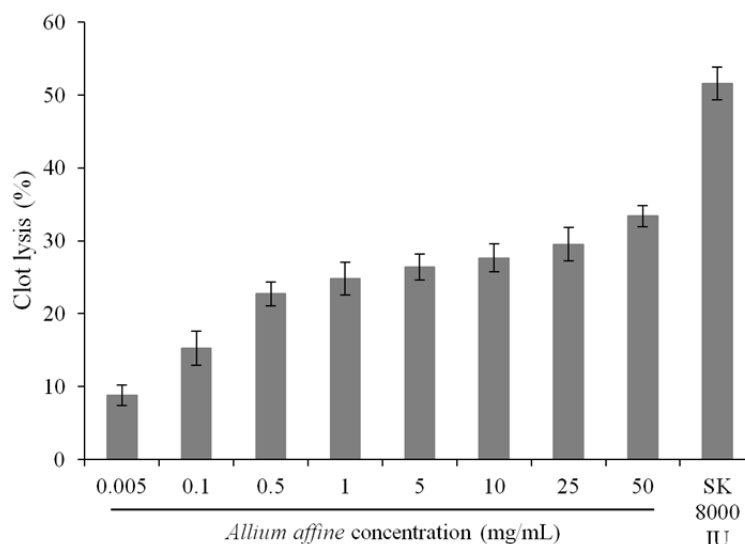


Fig. 5. Fibrinolytic activity of various concentrations of hydroalcoholic extract of *A. affine* and streptokinase (SK; 8000 I.U) determined by calculation of clot weight. Values are means \pm SEM from three independent experiments.

DISCUSSION

Allium vegetables have been well-established as the sources of bioactive compounds with beneficial effects on antioxidant capacity, plasma lipid profile, blood pressure and coagulation (6,7). *A. affine* is a subendemic plant of Iran, which grows wildly in western regions of the country and is used widely as a vegetable, condiment and herbal medicine. Despite the wide and different uses of this plant, there is not any comprehensive phytochemical or pharmacological study about *A. affine* to evaluate its constituents and activities and to the best of our knowledge, the current study is the first report on the *in vitro* and *in vivo* pharmacological activities of the plant. According to the results obtained through *in vitro* experiments, *A. affine* hydroalcoholic extract showed antioxidant effects by scavenging of DPPH radical and development of FRAP. In the *in vivo* analysis, the plant extract reduced the hydroperoxides level and increased the total antioxidant capacity in rat serum. Assessment of *in vitro* fibrinolytic activity also showed valuable thrombolytic potential for *A. affine* extract.

The antioxidant potential of *Allium* species has been described in various studies (18). Cardio-protective effects of garlic (*Allium sativum*), one of the regularly used vegetable of the genus *Allium* have been attributed to its

strong antioxidant properties (19). Various phytochemicals of *Alliums* including water- and lipid-soluble organosulfur compounds and flavonoids are involved in antioxidant activities through scavenging of free radicals, increasing the enzymatic and non-enzymatic antioxidants and preventing lipid peroxidation and LDL oxidation (20). Total phenolic content of *A. affine* aerial part extract being 19.62 mg GAE/g is comparable with data from other investigations in genus *Allium*. For example, the total phenolic content in the leaves of 10 cultivars of leek (*Allium ampeloprasum*) have been varied from 16 to 48 mg GAE/g (21). High DPPH scavenging activity has been reported for garlic bulbs (3.60% to 45.63%) compared to the other *Allium* members (5.07% to 11.36%) and this antioxidant activity was much higher in the garlic leaves (66.48%) than its bulb (45.63%) (22). *In vivo* and clinical studies have also shown the ability of garlic in decreasing oxidative stress and increasing total antioxidant capacities in animals and patients (6,14). Interestingly, our results showed great DPPH radical scavenging activity for *A. affine* aerial parts extract (67.2%) which is in accordance with the results of previous studies on *Allium* species (22).

Recent studies have shown the antithrombotic, fibrinolytic and anti-platelet aggregation effects of several *Allium* species (6). Some phytochemical compounds such as

allicin, adenosine, thiosulfinates, steroidal saponins and also flavonoids may be involved in the thrombolytic and anticoagulant activities of *Allium* spp (11,23). Some steroidal saponins and saponogenins with thrombolytic activity including diosgenin, tigogenin and ruscogenin have been isolated from *A. affine* (10). The mechanisms of this thrombolytic activity have been studied in some investigations, as in Zhang, *et al* study which reported anti-thrombotic activity for diosgenyl saponins via inhibition of factor VIII activities and platelet aggregation (24). Oral administration of ruscogenin has been able to inhibit venous thrombosis in mice in a dose-dependent manner (25). The phenolic compounds existing in *A. affine* may also be responsible for its fibrinolytic effect. Thrombolytic activity has been reported for some flavonoids like baicalein and kaempferol (26,27).

CONCLUSION

In conclusion, this study revealed the antioxidant and fibrinolytic effects of *A. affine* hydroalcoholic extract. Further investigations are needed to know more about the pharmacological activities of this plant and their mechanisms.

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