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Anti-platelet aggregation assay and chemical composition of essential oil from *Allium atroviolaceum* Boiss growing in Iran

Zahra Lorigooini[1,4], Farzad Kobarfard[2,3*], Seyed Abdolmajid Ayatollahi[1,3]

[1]*Department of Pharmacognosy, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[2]*Department of Medicinal Chemistry, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[3]*Phytochemistry Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[4]*Students' Research Committee, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

Key words: *Allium atroviolaceum*, essential oil, GC-MS, antiplatelet aggregation, steam distillation.

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Abstract

Plants belonging to genera *Allium* have widely been acquired as food and medicine. Their wide use was mainly due to the medicinal properties attributed to these plants over the centuries, lately supported by epidemiological and research studies. In this study, essential oil constituents of *Allium atroviolaceum* growing in Shahr-e-kord, Iran, were investigated through gas chromatography/mass spectrometry (GC-MS) technique. In this essential oil two major constituents were trisulfide, di-2-propenyl (26.85%) and diallyl disulphide (10.98%) while trans-2-(2-pentenyl) furan (0.02%) and Limonene (0.06%) have been identified in lower amounts. The *in-vitro* antiplatelet activity of essential oil was evaluated, using arachidonic acid (AA) and adenosine diphosphate (ADP) as the platelet aggregation inducers. The results showed that essential oil of *Allium atroviolaceum* with IC_{50}; 0.25 mg/ml and 0.47 mg/ml inhibited *in-vitro* platelet aggregation induced by AA and ADP respectively.

* **Corresponding Author:** Farzad Kobarfard ✉ Kobarfard@sbmu.ac.ir

Introduction

The genus *Allium* is a very diverse and taxonomically complicated group belonging to the family Alliaceae (Fritsch and Maroofi). The genus *Allium* comprises of around 750 species according to Stearn (Hirschegger *et al.*, 2010). It is well known that the *Allium* genus is rich of flavonoids, saponins, sapogenins and volatile sulfur compounds. The later compounds are responsible for their characteristic pungent aroma and taste, though they are unstable and easily transform to other compounds (Lanzotti, 2006). However, only a few of them have been used for their pungency and flavoring value and in some parts of the world, for religious connotations. Since ancient times, species in genus *Allium* have been used in folklore of many cultures as food, preventive and therapeutic medicinal agents (Fenwick *et al.*, 1985). There has been an increase in awareness and usage of all forms of alternative medical therapies often mentioned as complementary medicine (CAM) (Rahman, 2007).

Allium species are reported to have several positive health effects on immune functions, antibacterial, antifungal, antivirus, anticancer and practically cardiovascular activities. They are known to have direct effect on vessel wall, hypotensive and cholesterol- and triglyceride-lowering properties (Iciek *et al.*, 2009).

Cardiovascular disease is the main reason of morbidity and mortality in the world, particularly in developing countries (21.9% of total death) (Amidi *et al.*, 2013). Cardiovascular disease is a complex and multifactorial disease. Among these factors increased platelet aggregation and thrombus formation plays a significant role in the etiology of cardiovascular disease (Rahman, 2007). Clot formation, decreased or interrupted blood supply to vital organs such as the heart and brain lead to cardiovascular disorders such as myocardial infarction, unstable angina, stroke, venous thromboembolism (Weller *et al.*, 1994).

Platelets are activated by a variety of metabolic pathways. The mechanism of platelet aggregation pathway is very complex and involves multiple components and it can be controlled by heterogeneous group of endogenous compounds such as ADP, ATP, collagen, thrombin, tryptophan, epinephrine, thromboxane A2 and calcium. Each can independently and together begin the process leading to platelet aggregation. These compounds on platelets have specific receptors. Their effect on platelet aggregation is applied through binding to these receptors (Steinhubl *et al.*, 2007).

The aim of this study was to analyze and clarify the medicinal constituents of essential oils of *Allium atroviolaceum* and to determine its *in-vitro* antiplatelet activity, using AA and ADP as the platelet aggregation inducers.

Materials and methods

Plant material

Aerial parts of *Allium atroviolaceum* (The local name; Sirdeng) were collected in May 2010 in Rig mountain, Lordegan, Shahr-e-kord province, at 2610 m above sea level, A voucher specimen (SBMU-8013) has been deposited in the Herbarium of Departmen of Pharmacognosy, Faculty of Pharmacy of Shahid beheshti University of medical science, Tehran, Iran.

Isolation of essential oil

Aerial parts were carefully left to dry in controlled temperature (22˚C) without exposure to the light and moisture. They were chopped and then passed from sieve size 60 (25/0 mm) and 142g subjected to hydrodistillation for 4 h, using a Clevenger-type apparatus. The oils were dried over anhydrous sodium sulfate and stored at 4°C.

Gas chromatography-Mass spectrometry

The GC-MS analyses were carried out on a Hewlett Packard GC-MS system, model 5973, fitted with a 30m long, cross-linked 5% phenylmethyl siloxane (HP- 5MS 5% Phenyl Methyl Silox, Agilent 19091S-433) (30 m x 250 µm x 0.25 µm). The source temperature was 230°C, the quadrupole temperature 150°C, the initial oven temperature was 60°C; this was then raised to 260°C at 4°C/min and the final temperature maintained for 20 min. The injector and detector temperatures were 200°C and 250°C,

respectively. The carrier gas was helium at 1.0 mL/min. The sample was injected using a split ratio of 1:100. The carrier gas helium, adjusted to a linear velocity of 34 m/s. the ionization energy was 70 ev, and the scan range 40-650 amu at 3.9 scans/s. The injected volume was 1.0 µl of a 2% dilution of oil in n-heptane. The identification of the oil components was based on calculated relative retention time to those of C8-C24 n-alkanes, and compared with values reported in the literature and Wiley MS data library (6th ed).

Blood collection

Blood was obtained from healthy volunteers who did not take any medication for 14 days and were fasting overnight prior to the study. Blood collected at falcon tube containing 0.1 volume of 2.2% sodium citrate. Platelet rich plasma (PRP) was prepared by the centrifugation of citrated blood at 100g for 10 min. The residual blood was centrifuged at speed of 1500 g for 15 min to give platelet poor plasma (PPP). Platelets were counted under microscope and the platelet count was adjusted to (250 ± 25) ×109/L by diluting the supernatant PRP with PPP.

Platelet aggregation studies

Platelet aggregation responses were monitored with a turbidmetric method using an optical aggregometer. Aliquots of 200 µl of PRP were distributed in the test cuvettes and placed in incubation chamber of APACT-4004 aggregometer (LABiTec, Ahrensburg, Germany), at 37°C. Platelet aggregation was measured using PRP after activation by the addition of ADP or AA according to Born method. The essential oil was dissolved in DMSO (at 0.05% final concentration) and added to the PRP, 5 min prior to the activation with ADP or AA. The extent of aggregation was quantified by determining the maximum height of the curve. The platelet aggregation inhibitory activity was expressed as percent inhibition by comparison with that measured for the vehicle (DMSO) alone (Amidi *et al.*, 2013).

Statistical analysis

The anti-aggregation value of each compound was expressed as either % inhibition or IC_{50} values (the concentration of the compound causing 50% inhibitory effects). The IC_{50} values were determined from the Graph pad Prism version 3.02.

Result and discussion

Essential oil of aerial part (0.7 mL; 0.49%) of *Allium atroviolaceum* analyzed by GC/MS/MS showed the presence of Forty-two components. A list of the identified compounds, along with their percentages of the total oil, Kovats index and retention time is given in Table 1. Forty-two compounds were identified, representing 84.95% (w/w) of the total oil. The two major constituents of the oil samples were trisulfide, di-2-propenyl (26.85%) and diallyl disulphide (10.98%) while trans-2-(2-pentenyl)furan (0.02%) and Limonene (0.06%) were detected in lower amounts The presence of compounds showed mono-sulfur (5.15%), disulfide (19.38%), tri-sulfur (36.82%) and tetra-sulfur compounds (7.43%). The results indicated that the highest amount of sulfur compounds is related to tri-sulfur compounds. Differences were observed in the sulfur content of the constituents of this plant with other *Allium* species (Lazarevic *et al.*, 2011).

Table 1. Inhibitory effect of quercetin as positive control on *in-vitro* platelet aggregation induced by arachidonic acid (AA) and ADP.

Compound	AA (1.35mM)		ADP (5µM)	
	0.15mg/ml	0.07mg/ml	0.15mg/ml	0.07mg/ml
QUERCETIN (Inhibition%)	36%	1%	2%	-
QUERCETIN IC_{50} (mg/ml)	0.1		-	

Also, it could be related to differences in the composition of the essential oil from aerial parts of our study with the bulb in the other studies. Remarkably, the presence of sulfur compounds in the aerial part such as bulb in a significant amount.

Table 2. Effect of essential oil of *A. atroviolaceum* on *in-vitro* platelet aggregation induced by AA and ADP.

Concentration of essential oil	AA (1.35mM)		ADP (5µM)	
	%Inhibition	%Aggregation	%Inhibition	%Aggregation
2 mg/ml	99.85	0.11± 1.31	98.74	0±2.21
1 mg/ml	98.35	1.28±2.13	91.88	7.78±1.45
0.5mg/ml	97.80	1.71±2.35	58.95	39.32±3.14
0.33mg/ml	97.68	1.79±3.57	3.26	92.69±1.17
0.28mg/ml	97.43	1.98±3.11	-	-
0.25mg/ml	37.41	48.32±2.61	-	-
0.2mg/ml	9.05	70.22±1.78	-	-
Solvent	-	77.84±4.7	-	95.82±4.8
IC_{50} (mg/ml)	0.25		0.47	

Table 3. Chemical composition of essential oil of *A. atroviolaceum*.

Peak No.	RT	KI	Area%	
1	4.094	808	0.21	Norbornene,5-methylene-2>
2	5.898	907	0.09	Heptanal
3	6.059	914	0.15	3,4-dimethylthiophene
4	6.309	924	2.06	Isocitronellene
5	6.663	938	1.92	Disulfide,methyl 1-propenyl
6	7.694	979	5.69	Dimethyl trisulfide
7	8.153	997	0.33	Furan,2-pentyl-
8	8.354	1004	0.11	Pyrazine, 2-ethy-6-methyl-
9	8.427	1006	0.02	Trans-2-(2-pentenyl)furan
10	8.668	1014	0.17	Terpinene<alpha>
11	8.966	1024	0.06	Limonene
12	9.474	1040	0.13	Ocimene<-beta->
13	9.715	1048	0.89	Terpinene<Gamma->
14	10.754	1082	0.13	Pyrazine, 2,6-diethyl-
15	10.827	1084	10.98	Diallyl disulphide
16	11.004	1090	0.29	Pyrazine,2-ethyl-3,5-dimethyl-
17	11.737	1113	0.24	1,3-dithiane, 2,2-dimethyl-
18	11.963	1119	0.37	1,2-dithiolane
19	12.341	1131	0.67	Disulfide,methyl (methylthio)methyl
20	13.146	1155	3.78	2-thiatricyclo[3.3.1.1(3,7)]decane
21	14.588	1198	0.32	Tetradecane
22	15.289	1219	1.51	Dimethyl,tetrasulfide
23	15.74	1233	2.22	4,6-dimethyl-[1,2,3]trithiane
24	17.729	1292	0.92	Methane,(methylsulfinyl)methylthio)-
25	18.019	1302	26.85	Trisulfide,di-2-propenyl
26	18.857	1328	5.57	1,2,4-trithiolane,3,5-diethyl-
27	20.532	1382	3.46	Tetrasulfide, di-2-propenyl
28	20.943	1395	1.23	Bicyclo[3,2,1]oct-2-ene, exo-4-(phenylthio)-
29	22.618	1450	0.22	5,9-undecadien-2-one, 6,10-dimethyl-(Z)
30	23.351	1474	1.19	1,1'-thiobis3-(methylthio)-propane
31	23.697	1486	0.97	3-buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-
32	23.749	1487	0.25	5-methyl-2-phenyl-2hexenal
33	24.647	1518	0.51	Cyclohexanebutanal,2-methyl-3-oxo-cis
34	24.849	1525	1.10	1,2,4-cyclopentanetrione,3-(2-pentenyl)-
35	25.324	1541	2.46	Tetrasulfide, di-2-propenyl
36	25.614	1551	0.47	Formic acid,2-methyl-[1,3]dithian-2-ylmethyl ester
37	27.732	1624	2.25	1-(2-ethyl-[1,3]dithian-2-yl)-3-methyl-butan-1-ol
38	27.966	1633	0.17	Eudesmol<beta>
39	28.441	1650	0.54	1,2-dithiolane, 1.1-dioxide
40	31.123	1750	0.36	Tetradecanoic acid
41	31.654	1771	1.78	1-(2-ethyl-[1,3]dithian-2-yl)-3-methyl-butan-1-ol
42	33.329	1838	2.34	6,10,14-trimethylpentadecan-2-one

Currently, there is a growing attention both in industry and scientific investigation in spices and aromatic herbs because of their strong antioxidant and antimicrobial properties. These properties are in line for many substances, including some terpenoids, flavonoids, vitamins, carotenoids, phytoestrogens,

etc. (Bareemizadeh *et al.*, 2014). Moreover *Allium* species are reported to have several effects on immune functions and antibacterial, antifungal, antivirus, anticancer and practically effect on cardiovascular diseases. In view of that we examined the anti-platelet aggregation activity of essential oil of *A. atroviolaceum* (Table 2, 3).

Essential oil of *A. atroviolaceum* showed a dose-dependent inhibitory effect against AA and ADP-induced aggregation with IC50 values of 0.25 and 0.47 mg/mL, respectively. Platelet aggregation inhibitory effect of essential oil of *A. atroviolaceum* is about two times weaker that of quercetin when ADP is used as aggregation inducer and five times weaker when AA is used as the inducer.

The antiplatelet activity of *A. atroviolaceum* oils may not be due solely to any individual components could be due to the synergistic effects a group of contituents. Further studies need to be carried out to identify anti-platelet aggregation activity of major components of this essential oil and comparing the IC50 values with IC50 values obtained for the total essential oil used in this study. Previous studies have reported that the antiplatelet properties of *Lavandula hybrida* and *Goniothalamus* oil could be due to the synergistic effect of their components (Ballabeni *et al.*, 2004, Moharam *et al.*, 2010).

Plants of the genus *Allium* such as onion and garlic are often consumed as a source of compounds which inhibit human platelet activity, with the goal of decreasing vascular diseases. Antiplatelet activity of these plants is in part due to the concentrations of organosulfur compounds. Goldman IL *et al* (1996) demonstrated antiplatelet activity is genotype dependent and correlated with bulb sulfur content(Goldman *et al.*, 1996).

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