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Effect of diallyl trisulfide-rich garlic oil on blood coagulation and plasma activity of anticoagulation factors in rats

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Abstract

Diallyl trisulfide (DAT)-rich garlic oil was fed to Sprague–Dawley rats and the effects of this DAT-rich garlic oil on bleeding time, clotting time and anticoagulation factors were examined. Garlic oil supplement at 5 or 50 mg garlic oil/kg bodyweight significantly prolonged bleeding time and thrombin time, and enhanced anticoagulation factor activity, such as antithrombin III and protein C ($P < 0.05$). These results suggested that the anticoagulant action of DAT-rich garlic oil was due to inhibition and/or inactivation of thrombin. In addition, DAT-rich garlic oil benefits blood anticoagulation factors, which might further prevent the development of thrombus formation. However, the intake of garlic oil at high dose significantly increased plasma fibrinogen concentration ($P < 0.05$), and affected the levels of several hematological parameters such as erythrocyte count, hemoglobin and platelets ($P < 0.05$). The adverse effect of high doses of garlic oil might further influence the hemostatic balance. Therefore, the concentration of DAT-rich garlic oil should be carefully considered in its application. Supplementation of garlic oil at 5 mg/kg bodyweight has anticoagulation effect in this animal study.

Keywords: Rat; Diallyl trisulfide; Garlic oil; Bleeding time; Antithrombin III; Protein C

1. Introduction

Thrombus formation plays an important role in the pathogenesis and progression of atherosclerosis, cardiovascular diseases and diabetic complications (Srivastava et al., 1994; Boos and Lip, 2006; Viles-Gonzalez et al., 2006). To prevent and control the thrombogenic state, possible mechanisms regarding blood coagulation and/or anticoagulation in human or animal have been widely studied (Muller-Berghaus, 1989; Rosenberg, 1989; Esmon, 2000).

The role of garlic in preventing cardiovascular diseases has been studied extensively over the last decade (Brace, 2002; Rajaram, 2003; Borek, 2006). Several studies have indicated that whole garlic and garlic aqueous extract were able to inhibit platelet aggregation through multiple mechanisms, and could be considered as an antithrombotic material (Harenberg et al., 1988; Steiner and Li, 2001; Allison et al., 2006). Bordia et al. (1998) further indicated that diallyl disulfide (DADS) and diallyl trisulfide (DAT), two major organosulfur compounds derived from garlic, could inhibit platelet thromboxane formation, and hence platelet aggregation. Our previous ex vivo study already observed that DADS and DAT could effectively protect platelet against oxidation and adenosine 5′-diphosphate (ADP)-induced aggregation, in which DAT was superior to DADS (Chan et al., 2003). However, up to the present, only few

Abbreviations: ADP, adenosine 5′-diphosphate; APTT, activated partial thromboplastin time; AT-III, antithrombin III; DADS, diallyl disulfide; DAT, diallyl trisulfide; Hb, hemoglobin; Ht, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PC, protein C; PLT, platelet count; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PT, prothrombin time; TT, thrombin time; WBC, white blood cell count.

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studies have addressed in garlic's effect on blood coagulation (Rahman and Lowe, 2006; Ohaeri and Adoga, 2006), therefore, an in vivo study was designed to further examine the effect of organosulfide-containing garlic oil on blood coagulation and anticoagulation factors.

In this present study, DAT-rich garlic oil was fed to Sprague–Dawley rats and the effects of this DAT-rich garlic oil on bleeding time, clotting time and anticoagulation factors such as antithrombin III (AT-III) and protein C (PC) activities, were examined. The purpose of this study was to examine the effect and possible action mode of DAT upon function blood coagulation and anticoagulation factors.

2. Materials and methods

2.1. DAT-rich garlic oil treatment

The steam-distilled garlic oil was purchased from Pharma-Rex Inc., (Los Alamitos, USA). The total sulfide content of this oil was 52.4–55.1 mg/g. The contents of diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DAT), analyzed by an HPLC method (Lawson et al., 1991), were 1.93 ± 0.28, 9.77 ± 1.64, and 29.95 ± 2.38mg/g, respectively. The sum of DAS, DADS and DAT was $81.6 \pm 3.5\%$ of total sulfides, in which DAT content was $61.3 \pm 2.3\%$ of total sulfides. The content of vitamin E in both garlic oil and lard (used as a diluent of garlic oil) was analyzed, and it was too low to be detected.

2.2. Animals

Thirty-six male Sprague–Dawley (SD) rats weighing 250–300 g were obtained from the National Laboratory Animal Center (National Science Council, Taipei City, Taiwan). These rats were housed individually in wire-bottom cages under controlled conditions of temperature 22 ± 2 °C, humidity (50–60%) and light (lights on from 0700 to 1900 h). They were given a regular rat chow (Fwusow Feed Industry Co., Shalu, Taiwan) and water ad libitum. All animals received humane care as outlined in the Chinese version of Guide for the Care and Use of Laboratory Animals and by the Providence University Animal Care Committee.

2.3. Experimental design

Three groups of twelve rats were used in this study. In order to avoid a dietary stress induced by gavage, all rats were gavaged daily with 1 mL lard containing 0, 5 or 50 mg garlic oil/kg bodyweight for 6 weeks and these diets represented control, low garlic oil (LGO) and high garlic oil (HGO) groups, respectively. The dosage of garlic oil used in LGO group was translated and calculated from the dosage for healthy adults (55 mg/70 kg bodyweight) as recommended by the manufacturer. Within the 6 weeks, bodyweight and food intake were measured. At the end of the feeding period, the rats were fasted overnight for 12 h, and then anesthetized with 50 mg/kg bodyweight sodium pentobarbital by intraperitoneal injection. Bleeding time was measured before the operation. Then, 6.3 mL of blood was collected from the inferior vena cava into syringes containing 0.7 mL of trisodium citrate (38 g/L) at a ratio of 9:1 for the following assays.

2.4. Hematological examination

A fully automated hematology analyzer (Sysmex K-1000, Toa Medical Electronics Co., Ltd., Hyogo, Japan) was used to examine the following parameters: erythrocyte count (RBC), white blood cell count (WBC), platelet count (PLT), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

2.5. Platelet aggregation

Blood samples were anticoagulated with 3.8% trisodium citrate solution (9:1, v/v). Platelet-rich plasma (PRP) was prepared by low speed centrifugation at $200 \times g$ for 15 min at 24 °C, whereas platelet-poor plasma (PPP) was prepared by centrifuging PRP at $2000 \times g$ for a further 15 min as described by Kinlough-Rathbone et al. (1983). Each PRP sample was counted for platelets and was standardized (approximately 4×10^5/mL) by adjusting the PRP with autologous PPP. Because there was limited amount of blood sample, and ADP was the most important aggregating agent in thrombus formation (Gachet and Cazenave, 1991; Hourani and Hall, 1994), ADP was selected as an agonist for platelet aggregation test. ADP-induced platelet aggregation in PRP was performed with a PACKS-4 aggregometer (Helena Lab, Beaumont, TX, USA) according to the manufacturer's instructions. In brief, 450 μL PRP was mixed with 50 μL of ADP to a final concentration of 5 μmol/L. Platelet aggregation kinetics were measured and platelet aggregation was expressed as a percentage of the PPP transmission value.

2.6. Bleeding time (BT) measurement

BT was measured by cutting the tail-tip as described by Chan et al. (1993). In brief, the tail of each anesthetized rat was cut 2 mm from the end using a sharp pair of surgical scissors and then immersed immediately into the cylinder with 100 mL isotonic saline at 37 °C. Bleeding time was measured from the moment the tail was surgically cut until bleeding completely stopped.

2.7. Measurement for prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT)

Blood samples were anticoagulated with 3.8% trisodium citrate solution (9:1, v/v). Plasma was separated by centrifugation at $2000 \times g$ for 10 min at 4 °C. PT, APTT and TT were measured by optical method on a coagulometer (COAG-A-MATE XM, Organon Tecknica, Durham, NC, USA). Briefly, PT was measured by incubating 100 μL plasma with 200 μL prewarmed thromboplastin agent. APTT was measured by incubating 100 μL plasma with 100 μL αPTT-SA agent for 3 min at 37 °C, and followed by adding 100 μL $CaCl_2$. TT was measured by incubating 200 μL plasma with 200 μL thrombin agent. Finally, the PT, APTT and TT were determined by measuring the difference in absorbance at 560 nm.

2.8. Activity of antithrombin III (AT-III) and protein C (PC)

The activity of AT-III and protein C in plasma was measured by commercial AT-III and protein C kits (Sigma Chemical Co., USA), respectively. The standard curve for these two measurements was prepared by SARP standard plasma. The absorbance at 405 nm was determined. The activity of AT-III and PC was expressed as a percentage related to the activity of standard plasma.

2.9. Fibrinogen (Fg) concentration and liver function tests

Plasma fibrinogen level was measured using a commercial kit based on the principle of salting out (Iatroset Fbg, Iatron Laboratory, Tokyo, Japan). The activities of serum glutamic-oxoloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) were measured by the method as described by Reitman and Frankel (1957).

2.10. Statistical analysis

The effect of each treatment was analyzed from twelve different preparations ($n = 12$). Data were subjected to analysis of variance (ANOVA) and

computed using the SAS General Model (GLM) procedure (SAS, 1990). Statistical difference was evaluated by t test at 95% of confidence level.

3. Results

The effect of garlic oil at two levels on bodyweight, average daily gain, feed efficiency and epididymal fat pad in rats is presented in Table 1. Garlic oil supplement significantly reduced bodyweight, feed intake and epididymal fat pad weight ($P < 0.05$). The influence of dietary garlic oil supplement on hematological analysis is shown in Table 2. The intake of high dose garlic oil significantly increased WBC ($P < 0.05$), and reduced RBC, Hb, Ht, MCV, PLT ($P < 0.05$). The effect of garlic oil supplement on platelet aggregation, BT, PT, APTT, TT and plasma fibrinogen concentration is presented in Table 3. Platelet aggregation was not affected by the intake of garlic oil, however, BT and TT were significantly prolonged after garlic oil supplement. In addition, fibrinogen level was increased significantly in HGO group ($P < 0.05$).

Table 1
Effect of two levels of garlic oil on bodyweight, average daily gain, feed efficiency and epididymal fat pad in rats

	Dietary group		
	Control	LGO[A]	HGO[A]
Initial bodyweight (g)	314.9 ± 4.0^{a}	320.2 ± 21.6^{a}	315.3 ± 15.6^{a}
Final bodyweight (g)	506.8 ± 26.8^{a}	464.7 ± 32.5^{b}	463.9 ± 14.2^{b}
Feed intake (g/day)	29.0 ± 2.2^{a}	25.9 ± 1.3^{b}	25.8 ± 0.8^{b}
ADG (g/day)[B]	4.4 ± 0.6^{a}	4.0 ± 0.5^{a}	3.7 ± 0.3^{b}
Feed efficiency (%)[C]	16.6 ± 1.3^{a}	13.8 ± 2.3^{b}	13.3 ± 1.0^{b}
Epididymal fat pad (g)	1.6 ± 0.5^{a}	1.2 ± 0.1^{b}	0.9 ± 0.1^{c}

Values are means ± S.D. $n = 12$. Means in row with different superscripts are significantly different, $P < 0.05$.
[A] LGO: Low garlic oil group (5 mg garlic oil/kg BW); HGO: High garlic oil group (50 mg garlic oil/kg BW).
[B] ADG: Average daily gain.
[C] Feed efficiency: (ADG/daily food intake) × 100%.

Table 2
Hematological parameters of rats fed with two levels of garlic oil

	Dietary group		
	Control	LGO[A]	HGO[A]
WBC ($\times 10^3/\mu L$)[B]	4.1 ± 0.6^{a}	4.4 ± 0.7^{a}	5.6 ± 1.2^{b}
RBC ($\times 10^6/\mu L$)	7.7 ± 0.5^{a}	7.5 ± 0.3^{a}	6.4 ± 0.4^{b}
Hb (g/dL)	14.2 ± 0.8^{a}	14.6 ± 0.6^{a}	12.7 ± 0.5^{b}
Ht (%)	37.3 ± 2.2^{a}	37.5 ± 1.1^{a}	34.4 ± 1.1^{b}
MCV (fL)	50.3 ± 0.8^{a}	51.3 ± 0.9^{a}	54.4 ± 1.1^{b}
MCH (pg)	19.0 ± 0.4^{a}	19.2 ± 0.2^{a}	20.0 ± 0.6^{a}
MCHC (g/dL)	37.0 ± 0.5^{a}	37.7 ± 0.6^{a}	36.9 ± 0.7^{a}
PLT ($\times 10^3/\mu L$)	980.6 ± 78.0^{a}	937.3 ± 63.2^{a}	890.0 ± 53.4^{b}

Values are means ± S.D. $n = 12$. Means in row with different superscripts are significantly different, $P < 0.05$.
[A] LGO: Low garlic oil group; HGO: High garlic oil group.
[B] WBC: white blood cell; RBC: Red blood cell; Hb: hemoglobin; Ht: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet count.

Table 3
Effect of two levels of garlic oil on platelet aggregation (PG), bleeding time (BT), prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and plasma fibrinogen (Fg) concentration in rats

	Dietary group		
	Control	LGO[A]	HGO[A]
PG (%)	53.2 ± 3.5^{a}	55.5 ± 4.1^{a}	57.2 ± 3.8^{a}
BT (s)	127.0 ± 12.0^{a}	268.0 ± 22.0^{b}	344.0 ± 31.0^{c}
PT (s)	13.3 ± 0.7^{a}	14.0 ± 0.7^{a}	13.8 ± 0.8^{a}
APTT (s)	36.9 ± 4.0^{a}	39.6 ± 4.0^{a}	40.6 ± 3.7^{a}
TT (s)	75.3 ± 9.9^{a}	100.3 ± 13.3^{b}	107.4 ± 17.2^{b}
Fg (mg/dL)	482.9 ± 42.5^{a}	$544.3 \pm 61.3^{a,b}$	647.1 ± 87.2^{c}

Values are means ± S.D. $n = 12$. Means in row with different superscripts are significantly different, $P < 0.05$.
[A] LGO: Low garlic oil group; HGO: High garlic oil group.

The effects of DAT-rich garlic oil on both AT-III and protein C activities are presented in Fig. 1 and Fig. 2. Garlic oil supplement significantly enhanced both AT-III and

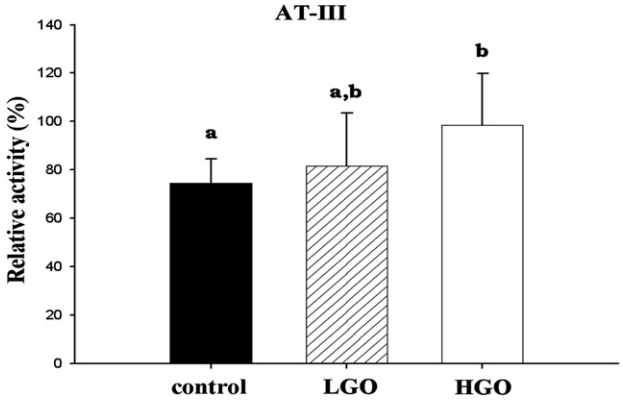

Fig. 1. Effect of two different levels of garlic oil on plasma activity of antithrombin III (AT-III) in rats. The % relative activity of AT-III on each group was calculated from the reference plasma. Values are means ± S.D. ($n = 12$). Columns not sharing a common superscript are significantly different, $P < 0.05$.

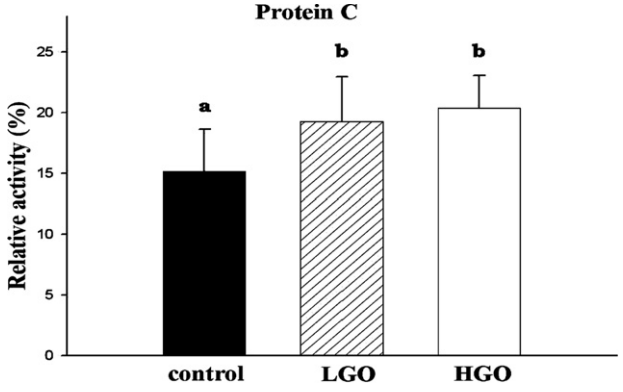

Fig. 2. Effect of two different levels of garlic oil on plasma activity of protein C in rats. The % relative activity of protein C on each group was calculated from the reference plasma. Values are means ± S.D. ($n = 12$). Columns not sharing a common superscript are significantly different, $P < 0.05$.

protein C activities ($P < 0.05$), both effects were dose-dependent ($P < 0.05$). Liver function as tested by SGOP and SGPT showed no difference between treatment groups (data not shown).

4. Discussion

Study on garlic oil's effect on food intake or satiety is rare. In this study, we have observed that food intake was reduced in those rats supplemented with garlic oil, which leads to a lower final bodyweight. Stress induced by gavaging of garlic oil to rats could result in a reduced food intake. However, because control rats also were gavaged with equal amounts of lard, therefore, DAT-rich garlic oil might have a suppressive effect on satiety. In addition, it has been reported that the supplementation of allyl-containing sulfides derived from garlic in rats resulted in lower epididymal fat pad weight because these agents enhanced thermogenesis (Oi et al., 1999). Because the amount of epididymal fat pad is highly correlated with total body fat content (LeHoux and Grondin, 1993), the observed lower bodyweight in garlic oil supplemented rats may be due to a decrease in body fat accumulation, which is resulted from the suppression of food intake and the enhanced thermogenesis.

Our previous ex vivo study has reported that DADS and DAT could markedly inhibited adenosine 5'-diphosphate (ADP)-induced human platelet aggregation (Chan et al., 1993). However, the administration of DAT-rich garlic oil to rats did not affect ADP-induced platelet aggregation in this in vivo study. It is reported that long-term administration of low dose garlic oil to healthy subjects and CAD patients inhibited platelet aggregation ex vivo (Bordia et al., 1996; Bordia et al., 1998). Other studies in humans also showed an inhibitory effect of aged garlic extract (GEX) at high dose on platelet aggregation (Rahman and Billington, 2000; Steiner and Li, 2001). Because the major sulfur compound in GEX is S-allylcysteine (Rahman, 2003), instead of diallyl sulfides in garlic oil, it is suggested that different kinds of garlic preparations, duration and dosage of garlic extract administered to the subjects may play crucial roles in their inhibitory effect on platelet aggregation. In addition, although garlic oil feeding did not affect ex vivo ADP-induced platelet aggregation, it prolonged TT and BT. Thus, the prolonged BT observed in vivo might be due to the inhibition of thrombin-induced platelet aggregation rather than ADP-induced platelet aggregation. This hypothesis may need further investigation.

In clinical tests of blood coagulation, PT is used to evaluate the overall efficiency of extrinsic clotting pathway, a prolonged PT indicates a deficiency in coagulation factors V, VII and X. On the other hand, APTT is a test of the intrinsic clotting activity; a prolonged APTT usually represents a deficiency in factors VIII, IX, XI, XII and Von Willebrand's factor (Laffan and Bradshaw, 1995). In this study, PT and APTT were not altered by garlic oil feeding, and the prolonged BT might result from an impaired activity of thrombin rather than altered activity of coagulation factors in both extrinsic and intrinsic clotting pathways. These results suggest that the anticoagulant effect of DAT-rich garlic oil in rats might be partially due to its antithrombin activity.

Our present in vivo study further observed that DAT-rich garlic oil could prolong BT and TT, as well as enhance the activity of AT-III and protein C in rats. These results suggest that DAT or DAT-rich garlic oil could benefit blood anticoagulation response, which could further prevent the development of thrombus formation and retard the pathogenesis and progression of atherosclerosis, cardiovascular diseases and macroangiopathy of diabetic complications. It is reported that activated AT-III could inhibit the activity of a number of proteases in the coagulation cascade, finally resulting in the inactivation of thrombin (Shimotori and Sakuragawa, 1990; Jeske and Fareed, 1993). On the other hand, the activation of protein C could convert the regulation signal generated by thrombin into an anticoagulant response via the thrombin–thrombomodulin complex, and also react with protein S to inactivate factors Va and VIIIa, two coagulation factors (Bourin et al., 1988; Butenas et al., 1999). Thus, activated AT-III and PC are considered as coagulation inhibitors because they could reduce thrombin generation and/or activation. In our present study, DAT-rich garlic oil supplements markedly elevate the activity of both AT-III and PC. These results suggest that the anticoagulant action of DAT-rich garlic oil is caused by its inhibiting and/or inactivating thrombin formation. This change in the activity of anticoagulation factors probably was not due to liver damage caused by the intake of garlic oil, because no signs of liver damage was observed, as judged by the SGOP and SGPT tests. Several studies have observed that the level of AT-III and/or PC was decreased in patients with cardiovascular events, septic shock and diabetes mellitus (Hughes et al., 1983; Vukovich and Schernthaner, 1986; Conlan et al., 1994; Fourrier et al., 1995). Since the DAT-rich garlic oil supplement could elevate AT-III and PC activities, DAT and DAT-rich garlic oil might provide beneficial effects to patients with these diseases.

In this study, we also found that DAT-rich garlic oil at high dose (50 mg/kg BW) increased fibrinogen level, which may conversely favor blood coagulation, because increased plasma fibrinogen level has been recognized as an independent risk factor in thrombogenesis (Shats et al., 1997). However, this increased fibrinogen level did not accelerate blood coagulation. Instead, it led to a prolonged TT in our study. One possible explanation is that in the final step of coagulation cascade, a fully activated thrombin is required for the transformation of fibrinogen to fibrin, however, the elevated activity of anticoagulation factors, AT-III and protein C, inhibits thrombin activity effectively, and hence results in a prolonged TT. These results indicate that anticoagulation factors may play an important role to counteract hypercoagulation.

On the other hand, DAT-rich garlic oil at high doses also changed hematological parameters, which might

further affect the hemostatic balance. The treatment of rats with high dose of garlic oil showed noteworthy reduction of erythrocyte and platelet counts. Ohaeri and Adoga (2006) also observed a reduction on erythrocyte and platelet counts when fed 50 mg/kg bodyweight garlic oil to streptozotocin-induced diabetic rats. Under hypercoagulation state, such as diabetes mellitus, the decreases in platelets may favor the control of thrombogenic events. Although the mechanism(s) of high dose garlic oil in inhibiting platelet production is not clear, when excess amount of garlic oil was consumed, a significant reduction in platelets might lead to bleeding problems in normal healthy subjects, because a normal platelet count is required for the maintenance of normal blood coagulation.

Although garlic has been used wildly and is ''generally regarded as safe, GRAS'' by the US FDA, excessive consumption of garlic may cause undesirable side effects such as burning sensations, diarrhea, gastrointestinal discomfort and mucosal irritation (Augusti, 1996; Amagase et al., 2001; Hoshino et al., 2001). Imada (1990) reports that allicin is one of the major irritants in raw garlic. Hydrophobic compounds (DADS and DAT) derived from allicin are more toxic than water-soluble compounds. Yeh and Liu (2001) also reports that both hydrophilic and hydrophobic compounds of garlic are good cholesterol-lowering agents; however, lipid-soluble compounds, such as DAT and dipropyl trisulfide, are highly cytotoxic at high concentrations (1–4mol/L). When fed 1.25 mL of garlic extract/kg bodyweight to dogs for 7 days, Lee et al. (2000) observed hematologic changes such as reduced erythrocyte count, hematocrit and hemoglobin concentration, and suggested that some constituents of garlic might oxidize erythrocyte membranes and hemoglobin, inducing hemolysis in those dogs. In our study, high dosage of garlic oil also found to decrease intact erythrocytes, however, no evidence of anemia or abnormal appearance of plasma induced by possible hemolysis were observed. Whether DAT or other constituents of garlic is responsible for causing these toxic effects needs further investigation. Therefore, the concentration of DAT-rich garlic oil should be carefully considered in its application.

In conclusion, the administration of DAT-rich garlic oil in rats favored blood anticoagulation via prolonging bleeding time and thrombin time, and increasing the activity of antithrombin III and protein C. These results suggest that DAT-rich garlic oil possesses an antithrombotic component. However, the concentration used must be carefully considered to avoid adverse effects such as increasing fibrinogen concentration or disturbing hemostatic balance.

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