International Journal of KIU

Journal home page : https: //ij.kiu.ac.lk/
DOI: https://doi.org/10.37966/ijkiu2022032032

Original Article

Comparison of *In Vitro* Anticoagulant Activity of Raw, Boiled, and Honey Fermented *Allium sativum* (GARLIC)

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Article history:
Received: 15.08.2022
Received in revised form - 08.12.2022
Accepted - 08.12.2022

Cite as: Zam Hareera M. N. F., Gunasena M. D. C. L., Wijesekara G. U. S., Bandara E. M. S., Wanniarachchi D., (2022) COMPARISON OF IN VITRO ANTICOAGULANT ACTIVITY OF RAW, BOILED AND HONEY FERMENTED Allium sativum (GARLIC) USING DIFFERENT EXTRACTION METHODS ' International Journal of KIU, 3. (2), 17-135. https://doi.org/10.37966/ijkiu2022032032
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Abstract

Garlic (*Allium sativum*) is one of the medically beneficial spices consumed by Sri Lankan people in different ways. The study aimed to determine the bioactive compounds and *in vitro* anticoagulant activity of aqueous and methanolic extract of raw, boiled, and honey fermented preparations of garlic. Different concentrations of aqueous and methanolic extract of raw, boiled, and honey fermented garlic were prepared by grinding different weights of garlic (2.5×10^{-3} kg for 500 mgmL^{-1}, 1.25×10^{-3} kg for 250 mgmL^{-1}, 0.25×10^{-3} kg for 50 mgmL^{-1} and 0.05×10^{-3} kg for 10 mgmL^{-1}). For aqueous extract, the crude extract had been collected whereas for methanolic extract preparation, maceration had been done. *In vitro* anticoagulant activity was analysed using prothrombin time (PT) of pooled plasma diluted with different concentrations of garlic extract. Bioactive compounds in garlic extracts were analysed by Gas Chromatography-Mass Spectrometry. Methanolic extract of all 3 preparations and aqueous extract of honey fermented garlic had significantly prolonged PT at all concentrations compared to the control ($p<0.05$). Aqueous extract of raw and boiled garlic showed significant prolongation in PT only at high concentrations compared to the control ($p=0.008$). Prolongation in PT was increased with increasing concentration of garlic extract. Honey fermented garlic had the highest prolongation in PT compared to the other two preparations. Moreover, methanolic garlic extract exhibited the a higher prolongation in PT compared to aqueous garlic extract. The content of Dodecanoic acid methyl ester and Methyl tetradecanoate in boiled garlic extract was much higher than in raw garlic extract. Diallyl disulphide, Methyl thiourea and S-Methyl methanethiosulfinate were only found in aqueous raw garlic extract. Beta sitosterol was only detected in methanolic raw garlic extract. All three consumption methods of garlic have an inhibitory effect on blood coagulation. Honey fermented garlic is the most effective preparation for anticoagulant activity.

Keywords: Boiled garlic, Gas Chromatography-Mass Spectrometry, Honey fermented garlic, *In vitro* anticoagulant activity, Raw garlic

Introduction

Garlic (*Allium sativum*, Family: Amaryllidaceae) is a seasoning plant cultivated all over the world. It produces bulbs, each bulb has 5 to 10 cloves. It is utilized as a spice for flavouring food during the cooking process[1]. Garlic is one of the most popular spices used by Sri Lankan people. Moreover, several parts of garlic are used in traditional folk medicine. Leaves and cloves are mostly used for medicinal purposes[2]. Since ancient times, garlic is used to treat cardiac diseases, hypertension, arthritis, respiratory infections, cold, diarrhoea, headache, bites, skin diseases, wounds, ulcers, and tumors[1,3]. Consumption of 1-2 garlic cloves per day is good for health[3].

Garlic contains several bioactive compounds including organosulfur compounds, saponins, phenolic compounds, and polysaccharides. Organosulfur compounds are major compounds found in garlic. There are four major organosulfur compounds which are alliin (S-allyl cysteine sulfoxide), S-allylcysteine (SAC), S-methylcysteine (SMC), and S-ethylcysteine (SEC). Allicin (Diallyl thiosulfinate) is the principal bioactive compound, responsible for most activities of garlic, released by crushing or chopping garlic. The crushing in garlicwhich is released an alliinase enzyme that catalyzes the formation of unstable allicin from alliin. Allyl methyl disulfide (MADS), diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), E/Z-ajoene, and dithiins found in garlic are produced due to breakdown of allicin[2,4]. Compounds in garlic have several pharmacological properties such as anticoagulant, antimicrobial, antioxidant, anti-inflammatory, anticancer, antidiabetic, anti-obesity, antihypertensive, hypolipidemic, and fibrinolytic activities[5,6].

Garlic has been reported to have an anticoagulant property in many studies. Organosulfur compounds in garlic play a major role in the anticoagulant effect of garlic. Blood coagulation is prevented by allicin in garlic by enhancing fibrinolytic activity and arresting the coagulation system. Fibrinolytic activity is enhanced by increasing tissue-type plasminogen activator (t-PA) which mediates plasminogen activation. The coagulation system is arrested by inhibiting thrombin formation. DADS and DATS in garlic also show antiplatelet activity by resisting thromboxane formation. Ajoene is another important molecule in garlic that prevents platelet aggregation by interfering with presumptive fibrinogen receptors[2]. Allyl methyl trisulfide, vinyldithiins, and other sulfur compounds produced by the breakdown of allicin in garlic bulb also inhibit platelet aggregation and enhance fibrinolytic activity[7]. The fluidity of blood is preserved by Garlic[8]. Due to the above mentioned properties, garlic can be used to reduce the risk of thrombosis[9].

Garlic is consumed by people in raw form or processed form. Even though there are several processed products of garlic are available commercially, boiling, frying, roasting, and fermentation are some commonly used methods at home. *In vitro* platelet aggregation is inhibited by aqueous extract of raw garlic, garlic oil, and other preparations of garlic. Consumption of raw garlic, garlic oil, garlic powder, and aged garlic extract for a long period reduces the platelet aggregation in vivo[10].

This study was conducted to determine and compare the bioactive compounds and *in vitro* anticoagulant activity of aqueous and methanolic extract of raw, boiled, and honey fermented preparations of garlic. Since there are many garlic studies in literature, this comparison study will be useful to determine the most effective preparation of garlic for *in vitro* anticoagulant activity.

Materials and Methods

Study design and setting

Laboratory-based experimental study was conducted at Haematology Laboratory, Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences and Instrument

Center, Faculty of Applied Sciences, University of Sri Jayewardenepura

Preparation of aqueous and methanolic extracts of garlic

Different concentrations of aqueous and methanolic extract of raw, boiled, and honey fermented garlic were prepared by grinding different weights of garlic (2.5×10^{-3} kg for 500 $mgmL^{-1}$, 1.25×10^{-3} kg for 250 $mgmL^{-1}$, 0.25×10^{-3} kg for 50 $mgmL^{-1}$ and 0.05×10^{-3} kg for 10 $mgmL^{-1}$).

The raw, boiled, and honey fermented garlic was ground using a motor and pestle separately by adding 5 mL of distilled water gradually. The extracts obtained were filtered separately using a clean cloth and centrifuged at 2000 rpm for 5 minutes. The supernatant was separated to assess Prothrombin Time (PT). For aqueous extract, the crude extract had been collected whereas for methanolic extract preparation.

Maceration had been used based on previous literature. Methanolic extract was prepared by soaking raw, boiled, and honey fermented garlic in 5×10^{-3} L of methanol separately for 24 hours. The prepared methanolic extracts were evaporated using a water bath at 55ºC for 45 minutes. The residue (gel) that remained in the beakers was redissolved with 5×10^{-3} L of 0.5% (v/v) dimethyl sulfoxide (DMSO)[6] (Figure 1).

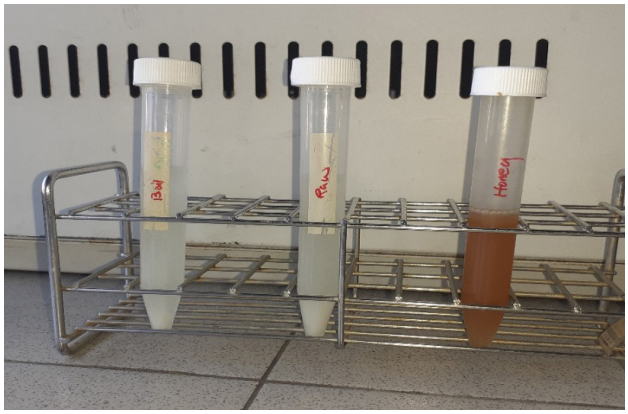

Figure 1: Aqueous extracts of raw, boiled, and honey fermented garlic

Preparation of pooled plasma for prothrombin time assessment

Blood was collected into a container whereas 0.5×10^{-3} L of 3.2% trisodium citrate was added. Platelet poor plasma (PPP) was prepared by centrifugation of each sample at 2000rpm for 15 minutes. Pooled plasma was prepared by pooling all the PPP which had normal prothrombin time. The pool was gently mixed and prothrombin time of pooled plasma was measured. Finally, pooled plasma was aliquoted into eppendorf tubes (1×10^{-3} L) and stored in a freezer at -20ºC until processing.

PT value for diluted plasma sample with aqueous and methanolic garlic extracts

An equal volume of pooled plasma and garlic extract of each consumption method were added together to prepare a mixture of 1:1 ratio. PT was measured by using 100×10^{-6} L of mixtures and 200×10^{-6} L of PT reagent separately. The procedure was replicated four times and mean PT was recorded. 0.9% normal saline was used to replace the extract solution for control.

Preparation of sample for Gas Chromatography - Mass Spectrometry

A 500 $mgmL^{-1}$ aqueous extract of raw and boiled garlic were centrifuged at 2000rpm for 5 minutes and the supernatant was mixed with 5×10^{-3} L of 10% NaCl. The above extract solution was then mixed with 10×10^{-3} L of chloroform and supernatant (garlic extract) was separated. It was mixed with 10×10^{-3} L of chloroform two more times and the supernatant was separated and all of three chloroform layers were pooled together. 500 $mgmL^{-1}$ methanolic extract of raw and boiled garlic were centrifuged at 2000rpm for 5 minutes. The supernatant was evaporated in a water bath at 55ºC for 1 hour and the remaining gel was mixed with 30×10^{-3} L of chloroform. Anhydrous Na_2SO_4 was added into chloroform extracts until anhydrous Na_2SO_4 was freely moved. The solution was filtered using a glass syringe with PVDF filter diameter of

s0.22×10^{-6} m into GC-MS vials. Filtration was repeated, until all the water bubbles and particles were removed from the sample and the final volume of filtrate was about about 1×10^{-3} L.

Gas Chromatography Mass spectrometry

The Gas Chromatography Mass Spectrometry (GC-MS) analysis was performed on Agilent 7890A Gas chromatography coupled to 5975C Mass spectrometer with Triple-Axis Detector using a split /splitless inlet with Helium as the carrier gas with 1 mL/min rate. Operating conditions were as follows: HP 5MS column, length 30m, diameter 0.25×10^{-3} m and film thickness 0.25×10^{-6} m, initial temperature of column 90 ºC, injector temperature 270 ºC, total run time 39 minutes, mass range 50-550 m/z, compound identification by NIST database Chemstation® software.

Statistical analysis

All the statistical analysis was conducted by using SPSS version 26.0. Independent sample t-test was performed to find out mean and p-values to compare the prothrombin time values with each consumption method. A p-value of <0.05 was considered statistically significant.

Ethical approval

Ethical approval was obtained from ethics review committee, Faculty of Medical Sciences, University of Sri Jayewardenepura for blood collection (MLS/01/20).

Results

Prothrombin time values obtained with different garlic preparations

The mean PT of pooled plasma was 15.7±1.494s. Aqueous extract of raw and boiled garlic showed significant prolongation in PT compared to control (24.00s) at concentrations of 250 mgmL^{-1} and 500 mgmL^{-1} ($p<0.05$). Aqueous extract of honey fermented garlic showed significant prolongation ($p<0.05$) in PT compared to control at all the concentrations (Figure 2) (Table 1). Methanolic extract of raw and honey fermented garlic showed a significant prolongation in PT compared to control at all the concentrations ($p<0.05$). Similarly, methanolic extract of boiled garlic showed a significant prolongation in PT compared to control at the concentrations of 10 mgmL^{-1}, 250 mgmL^{-1} and 500 mgmL^{-1} ($p<0.05$). The prolongation in PT at the concentration of 50 mgmL^{-1} was not significant (pº0.127) when compared to the control (Figure 3) (Table 1).

Honey fermented garlic showed significantly high PT values compared to raw and boiled garlic in both aqueous and methanolic extracts. (pº0.015 & pº0.011) (Figure 1 & 2). Boiled garlic had significantly high PT values compared to raw garlic in methanolic extract (pº0.015). In aqueous extract, boiled garlic had significantly high PT values compared to raw garlic only at low concentrations, while raw garlic at high concentrations showed significantly high PT values compared to boiled garlic (pº0.011). Methanolic extract of garlic showed a significantly high prolongation in PT compared to aqueous extract with almost all preparations of garlic (pº0.015).

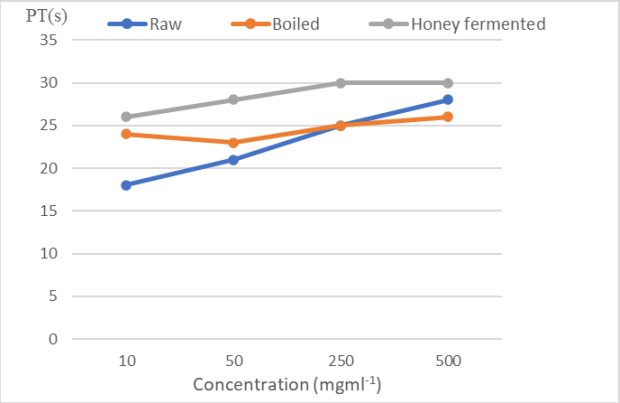

Figure 2: Graphical illustration of median PT against concentration of aqueous extract of garlic

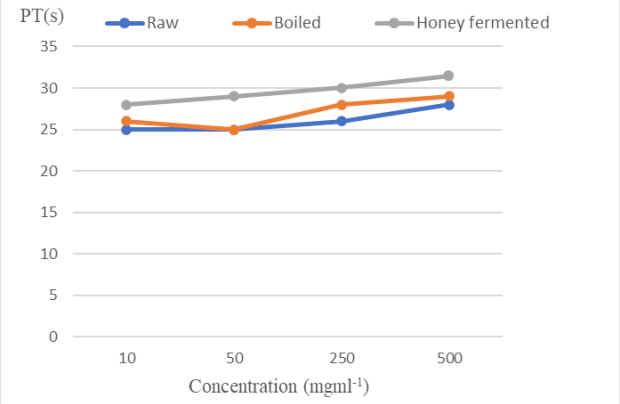

Figure 3: Graphical illustration of median PT against concentration of methanolic extract of garlic

Table 1: Comparison of PT values of garlic preparations with control at different concentrations

Extract	Concentration ($mgmL^{-1}$)			
	10	50	250	500
Aqueous Raw Garlic Extract	P=0.011	P=0.011	P=0.008	P=0.011
Aqueous Boiled Garlic Extract	P=0.317	P=0.011	P=0.011	P=0.008
Aqueous Honey Fermented Garlic Extract	P=0.011	P=0.011	P=0.008	P=0.008
Methanolic Raw Garlic Extract	P=0.040	P=0.011	P=0.011	P=0.011
Methanolic Boiled Garlic Extract	P=0.011	P=0.127	P=0.008	P=0.008
Methanolic Honey Fermented Garlic Extract	P=0.011	P=0.011	P=0.008	P=0.013

Bioactive compounds responsible for anticoagulant activity in different garlic extracts

Bioactive compounds responsible for anticoagulant activity in garlic extracts are given in the table 2.

Bioactive Compounds	Aqueous Raw garlic extract (%)	Aqueous Boiled garlic extract (%)	Methanolic Raw garlic extract (%)	Methanolic Boiled garlic extract (%)
Diallyl disulphide	0.20	-	-	-
Methyl thiourea	0.19	-	-	-
S-Methyl methanethiosulfinate	0.18	-	-	-
Dodecanoic acid methyl ester	0.29	0.55	0.47	0.52
Methyl tetradecanoate	0.34	3.13	1.30	1.68
Beta-Sitosterol	-	-	1.33	-

Discussion

The goal of this study was to describe the *in vitro* anticoagulant activity of aqueous and methanolic extract of raw, boiled and honey fermented preparations of garlic in Sri Lanka. In the current study, a significant prolongation in PT with aqueous raw garlic extract was observed only at high concentrations and the PT was significantly low at low concentrations when compared to the control. This finding deviates from the findings of previous studies in which an increase in PT was observed with aqueous raw garlic extract at very low concentrations (100-500 μgmL^{-1})[11]. This deviation may be due to differences in the method of extract preparation. Prolongation in PT with aqueous extract of raw garlic also was reported in the previous studies[12].

Our results showed a significant prolongation in PT with methanolic extract of raw garlic at all concentrations including at low concentrations when compared to control. Prolongation in PT at low concentrations with methanolic extract of raw garlic also reported in an another study[11].

In our study, significantly high values for PT were observed with all the concentrations of aqueous and methanolic honey fermented garlic extracts compared to the control. The effect of fermented garlic on platelet aggregation was analysed in previous studies. It was found that fermented garlic significantly inhibits *ex vivo platelet* aggregation induced by collagen and adenosine diphosphate (ADP) and also blocks granule secretion[13]. Anticoagulant activity of garlic extract is due to the presence of organosulfur compounds, Dithiins, Saponins, Galactolipid, Phytosterol, and etc[2,14].

In the current study, the compounds which possesses anticoagulant properties were Diallyl disulphide (DADS), Methyl thiourea (MT), S-Methyl methanethiosulfinate (SMMT), Dodecanoic acid methyl ester (DDME), Methyl tetradecanoate (MTD), and Beta-Sitosterol (BS). Furthermore, DDME and MTD were reported in all the extracts. Some previous studies did not report DDME and MTD in the methanolic garlic extract[15]. DADS, MT and SMMT are organosulfur compounds which were detected only in aqueous raw garlic extract in our study. SMMT and DADS were previously detected in the aqueous fresh garlic extract[16]. However, Beta-Sitosterol, was a phytosterol detected only

in methanolic raw garlic extract in this studys, which may be due to its less polarity.

Among the above the compounds which identified in the current study, DADS was reported to have anticoagulant activity in many studies. DADS in garlic show antiplatelet activity by resisting thromboxane formation[2]. Thiosulfinates was reported to have a major role in antiaggregatory activity of garlic by another study[17]. Both DDME and MTD were reported to have an inhibitory effect on blood clotting and preventing stroke and MTD mainly affects platelet aggregation[18]. Platelet aggregation induced by arachidonic acid is retarded by antiplatelet thiourea and novel thiourea compounds which have an effect on platelet aggregation and no effect on coagulation cascade which were reported in previous studies[19]. It was reported in previous studies that Beta-Sitosterol inhibits coagulation factor IIa (Thrombin) and promote thrombolytic activity by triggering plasminogen[20].

The detection of Hexadecanoic acid methyl ester (HDME) in aqueous garlic extract is a new finding reported in this study. It promotes blood coagulation by preventing the fibrinolysis. In previous studies this was detected in methanolic extracts[21].

Our GC-MS findings were supported by prolongation of PT values. In current study, aqueous raw garlic extract showed significantly higher PT than aqueous boiled garlic extract at the concentration of 500 $mgmL^{-1}$ which may be due to the presence of SMMT and DADS. Both compounds have major role in the anticoagulant effect of garlic in aqueous raw garlic extract[17]. Boiling destroys enzyme alliinase, therefore prevent the formation of DADS[22]. In the current study methanolic boiled garlic extract had significantly higher PT value compared to methanolic raw garlic extract which may be due to higher content of DDME and MTD in the methanolic boiled garlic extract when compared to methanolic raw garlic extract. Even though indings of this study revealed significant differences in anticoagulant activity due to boiling, some previous studies reported that boiling of garlic has negligible effect on anticoagulant activity and there was no significant difference between boiled and un-boiled extracts in the prolongation of thrombin induced clotting time[6]. Aqueous raw garlic extract was found to have more effect on inhibition of platelet aggregation induced by collagen than boiled garlic extract[23]. Boiled garlic extract has little effect on TXB_2 synthesis and cyclooxygenase activity in rabbit tissue compared with raw garlic[24]. Boiling for 10 or more minutes reduce the ability of garlic to inhibit platelet aggregation[10].

Even though there were no organosulfur compounds detected in methanolic extract of garlic in our study, highest PT values were observed with methanolic extract of garlic compared to aqueous extract. In the current study, the presence of HDME in aqueous garlic extract may have contributed to reduce the strength of anticoagulatory effect of aqueous garlic extract. The findings of the current study were well supported with similar results which were found study in which plasma incubated with alcoholic extract of *Allium sativum* highly reduced *in vitro* platelet aggregation induced by agonists than aqueous extract[25].

In the current study, honey fermented garlic preparations showed significantly more prolongation in PT than other two preparations. It may be due to the effect of honey on blood coagulation. Flavonoids and hydrogen peroxide in honey have an effect on platelet aggregation. Synthesis of prostacyclin by endothelial cells is triggered by flavonoids. Prostacyclin increases the formation cAMP which blocks GPIIb/IIIa receptors[26]. Flavonoids also interfere with coagulation factors like fibrinogen and factor VII[27]. Fermented garlic has highest effect on the inhibition of platelet aggregation than unfermented garlic[13]. However, In the current study honey fermented garlic was not analysed by GC-MS. Therefore, it will be worthwhile to analyse honey fermented garlic for its scientific validity.

Conclusions

According to the findings of this study, it can be concluded that raw, boiled, and honey fermented garlic preparations have inhibitory effect on blood coagulation while honey fermented garlic has more effective anticoagulant activity than raw and boiled garlic. Further, methanolic extract of garlic exhibited comparatively higher anticoagulant effect than aqueous extract which may be due to high yield of decanoic acid compounds.

Conflict of Interest

There is no conflict of interest

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