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Article: **In Vitro Investigation of Therapeutic and Anti-Coagulant Properties of Allium Sativum L. on Human Plasma**

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In vitro Investigation of Therapeutic and Anti-Coagulant Effects of *Allium sativum L.* on Human Plasma

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Abstract

The current study was conducted to compare the anti-coagulant activity of different solvent-extracted fractions of garlic in a human blood sample *in vitro*. Two different solvents, namely rectified spirit and reverse osmosis (RO) water, were used to achieve the desired results. We used multiple extracting approaches to prepare different extract fractions of Allium sativum, both in ethanolic and aqueous extracts. These approaches included maceration, decoction, and soxhlet extraction methods. The concentration of each extract fraction was subjected to a primarily anti-coagulant screening method applied to a human blood sample *in vitro* by calculating the prothrombin time of human blood coagulation. The anti-coagulant activity of the extracts was determined by measuring the changes in prothrombin time with a null hypothesis value of $p< 0.05$. Additionally, a qualitative study of active phytochemical elements, such as alkaloids, flavonoids, steroids, proteins, carbohydrates, terpenoids, tannins, and glycosides was also conducted. The results indicated that all garlic extract fractions have a significant anti-coagulant potential. However, at 5 ppm concentration, soxhlet extraction extract showed the maximum anti-coagulant potential. Moreover, Garlic's aqueous extract also showed a significant anti-coagulant effect on human plasma. This observation conforms to the finding that the soxhlet extracted sample of garlic showed the highest activity of platelet aggregation inhibition. Furthermore, it was determined that aqueous and ethanolic extracts of Allium sativum showed a significant potential of anti-coagulation by comparing the current results with positively controlled EDTA and double oxalate acting as synthetic anti-coagulants.

1. Introduction

Cardiac disease and other heat related illnesses remain hazardous and deadly, worldwide. Thromboembolic disorders cause atherosclerosis, diabetes, cancer recurrence, pulmonary emboli, deep vein thrombosis, hypertension, strokes, and heart attack, among other cardiovascular ailments. These cardiac diseases play a leading role in mortality. According to WHO, about 1,400,000 people die from cardiovascular diseases, annually [1, 2]. The cardiovascular disorders that are the leading cause of death include strokes, thrombosis, and myocardial infarctions. They can arise from coagulation associated pathologies. Hereditary disorders and smoking habits can also cause a risk of blood coagulability [3-5].

In this context, natural herbal supplements are helpful because they contain essential secondary metabolites. Indeed, the availability of plants that may be used to prepare food and medicines is one of the countless divine blessings. Since ancient times, human beings have discovered innumerable ways to utilize herbal remedies as a source of treatment. Most people believe that herbal medicines are both effective and safe. However, in the United States, one out of every six persons who take natural remedies also utilizes pharmaceutical drugs [6-8].

Several scientific and clinical studies have showed that using natural herbs, secondary metabolites, and phytochemicals with anti-coagulant activity can effectively treat and prevent cardiovascular disorders [9, 10]. Garlic, which belongs to the onion family, is the most revered and valuable medicinal plant [11]. It is an essential plant due to its medicinal and nutritional value [12]. Garlic, an aromatic solid perennial vegetable bulbous plant that has been cultivated for hundreds of years and propagated through bulbs, is the therapeutic herb in the current study [13, 14].

Garlic contains 33 sulphur compounds, including 17 amino acids, enzymes, and minerals. It contains more sulphur compounds than any other Allium family member, including allicin, alliin, ajoene, diallyl disulfide, dithiin, and S-allyl cysteine [15, 16]. Its medicinal benefits, including its flavor, taste, and pungent odour, are due to these sulphur compounds. Also, its therapeutic effects, as well as its smell, taste, and pungent aroma are caused by these compounds. Dried, powdered garlic also contains 1% allicin (diallyl disulfide) [16, 17], which is derived from the amino acid cysteine. Allicin is a potent antibacterial and antifungal agent which also gives garlic its spiciness [18]. As a prophylactic agent, garlic is a unique indigenous therapeutic herbal plant. Therefore, Allium Sativum has been used as a resource for its research [19, 20].

Coagulation is a naturally occurring phenomenon that is an essential aspect of hemostasis. It aids in the development of blood clots which restrict or stop bleeding and eventually lead to wound/lesion healing. The coagulation cascade is composed of a series of operations or enzyme reactions. Extrinsic and intrinsic pathways of blood coagulation are the first two phases in this process [21, 22]. Garlic is the most effective anti-coagulant herb containing nine anti-coagulant chemicals. It also affects platelet aggregation by interfering with the production of

thromboxane and producing prostacyclin via the arachidonic acid pathway [23].

Keeping in view its medical value, the current study focuses on the therapeutic and anti-coagulant effects of Allium sativum on human plasma. Subsequently [23], data was analyzed and categorized according to its relevance and a table was created to summarize all of the findings [24]. These findings are expected to add value in addressing concerns about the impact of garlic ingestion on blood pressure and cardiovascular morbidity [25].

2. Methodology

Allium sativum L. ethanolic and aqueous extracts were prepared using rectified sprit and reverse osmosis (RO) water. Their phytochemical analysis was conducted using the existing stock standards. The collected sample was activated in its matured state gained by exposing it to sunlight (under shadow) for three days, for a total of eight hours per day. Following the color variation, samples were kept in the dark for 24 hours. Then, they were rinsed with purified water and packed in an air sealed packet at -4ºC until further procedure. UV spectroscopic and FTIR analysis on crude samples was conducted to assess the quality of Allium sativum. Phytochemical screening and proximate analysis was conducted as described by Safowra [26] and Trease and Millit [27] **.**

In this study, maceration, decoction, and soxhlet extractions were carried out to obtain Allium sativum extracts at varied solute-solvent ratios (V/V). We used 1 mg of crude garlic extract in 100 mL rectified spirit to make a stock solution of 10 ppm that was concurrently diluted at 1 ppm concentration. In 10 distinct blood samples of 5 mL as whole blood, 1 ppm garlic extract was applied using phosphate potassium buffer for pH adjustment. Plasma samples were taken from healthy individuals according to the methodology described earlier [28]. Healthy adults between the ages of 25 and 45, who did not have a family history of coagulation problems and were not on any medications, met the inclusion criteria. The Mayo Hospital Lahore Ethics in Human Research Committee (#24124) and the Lahore Garrison University Ethics Review Board (LGUERB #02/20) both gave their approval to the current study. Adult participants also gave their informed consent to participate in the study.

Blood samples were collected in tubes containing 0.105 mol/L (3.2%) in a ratio of nine volumes of whole blood to one volume of trisodium citrate, an anti-coagulant. Prothrombin time test was performed according to the procedure described in the literature [29-31]. For an appropriate coagulation investigation, a proper blood-to-anticoagulant ratio should be used. According to NCCLS standards [32], the value of blood specimens, that is, hematocrits (HCT) should be about 55% for prothrombin time screening, such that nine parts of freshly collected whole blood and one part anti-coagulant should be used for each sample [33, 34].

ANOVA with GraphPad Prism 7 software was used to examine the differences in prothrombin time to determine the anti-coagulant impact of various garlic extracts, followed by post hoc Tukey's Multiple Comparison Test. Mean values were considered as significantly different at $p \leq 0.05$. All experimental data was reported as the mean ± standard deviation (SD) of analysis in triplicate using SPSS software.

3. Results and discussion

UV spectra of garlic was examined in the wavelength range of 200-800 nm using Schimadzu double beam spectrophotometer. Absorption band at 220 nm is due to the transition of valance electron in the sulfonyl group [35, 36]. We used FTIR model number Nicolet 6700 to determine the major functional group in garlic extract at 400-4000 cm-1. The significant and larger peak at 3206 cm-1 in Figure 1 indicates the existence of a sulfonyl functional group in the tested sample, which suggests that allicin or ajoene are both organosulfonic compounds with active features of garlic therapeutic activity on human plasma. The disulfide functional group can be seen at 2926 cm-1, while the c=c, c-c, and c-h bonds can be seen at 1619, 1325, and 1006 cm-1, respectively in the near infrared area [31, 37].

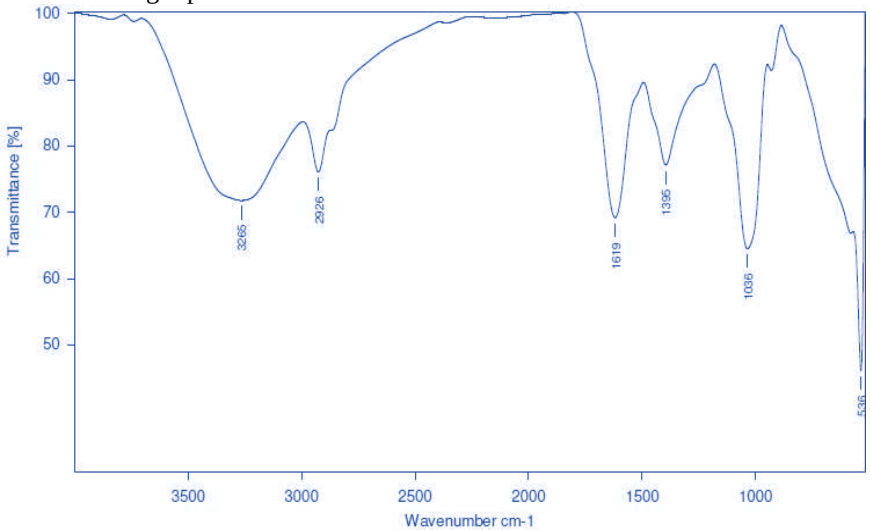

Figure 1. FTIR Spectrum of Allium sativum L. Extract at 1-ppm Concentration in Ethanol (96%)

3.1 phytochemical analysis

Allium sativum is rich in nutrients such as protein, carbohydrate, minerals, dietary fiber, and vitamins which play a significant role in preventing tissue damage because of free radicals. Hence, it is believed that garlic is a good source of antioxidants and anti-coagulant properties. The phytochemical screening of garlic for various phytochemical constituents, such as terpenoids, flavonoids, alkaloids, reducing sugars, steroid, glycoside, phenol, anthraquinones, saponin, and tannin was conducted using standard methods as described by Sofowora [26] and Trease and Evans [27] for both aqueous and ethanolic extractions (see Table 1).

Table 1. Qualitative Phytochemical Screening of Aqueous and Ethanol Extracts of A. sativum

Constituents of A.S.E.Et	Interface	Constituents of A.S.E.Aq	Interface	Constituents of A.S.E.Sx	Interface
Alkaloids	++	Alkaloids	+	Alkaloids	+++
Saponin	++	Saponin	+	Saponin	+
Tannin	+++	Tannin	++	Tannin	+++
Frothing	+++	Frothing	++	Frothing	++
Flavonoids	++	Flavonoids	+	Flavonoids	+++
Glycosides	+	Glycosides	++	Glycosides	++
Anthraquinone	+	Anthraquinone	**NO**	Anthraquinone	++
Cardiac glycosides	+	Cardiac glycosides	+	Cardiac glycosides	++
Saponin Glycoside	**NO**	Saponin Glycoside	**NO**	Saponin Glycoside	**NO**

***Key**: +++= strong; ++= Adequate; +=negligible; NO=Not Observed*

Phytochemicals, such as saponin, flavonoid, tannin, reducing sugar, steroid, and terpenoid were found in both aqueous and ethanolic Allium sativa extracts. The findings of this study regarding the phytochemistry of garlic corroborated those of Deresse [38], who discovered that garlic extracts were active against both Gram-negative (E. coli, Salmonella sp., Citrobacter Enterobacter, Pseudomonas klebsiella) and Gram-positive (S. aureus, S. pneumonia, Streptococcus, and Bacillus anthrax) bacteria due to the presence of phytochemicals such as saponin and tannins.

3.2 proximate estimation

Proximate estimation is a semi-quantitative analysis used to evaluate the organic matter of samples and their accuracy in subsequent estimation analyses. We determined the organic and dry weight of the sample and estimated the nutrient and constituent ratio in it using proximate determination. This technique estimates the moisture, protein, lipid, fat, and carbohydrates in the sample and compares them to standard statistics. Proximate analysis employs a variety of methodologies and adaptations. However, in the current study, we used the preliminary method. Three samples of data were collected and statistically analyzed to assess the outcomes. Data was provided in standard error of mean (SEM) (n = 3) format. Table 2 shows the mean ±standard deviation (SD) analysis of all experimental data performed in triplicate using SPSS software (IBM, PASW 117 Statistics 254 19, USA). Previous studies discovered that A. sativum has a low fat content. Low fat diets are known to reduce cholesterol levels [39].

Table 2. Proximate Analysis of Allium Sativum L. extract

Observed parameters	Finding ratio %
Moisture	65.16761±1.271
Ash content	1.429136±0.017
Crude Fiber	0.789892±0.007
Crude lipid	0.613374±0.018
Carbohydrate	31.01279±0.736

shown significant SD± in the comparison among three groups

3.3 prothrombin time and anti-coagulation investigation

To investigate the anti-coagulant effects of Allium sativum extracts, a 5 ppm dose of garlic extract was extracted with maceration in an aqueous and soxhlet extractor. This dose has an anti-coagulant therapeutic effect on human blood plasma,. In a healthy person, the usual prothrombin time is 12-14 seconds [40, 41]. In a double-blind trial, we investigated the effect and efficiency of our garlic crude extract using a positive and controlled EDTA 5 mg combination and a negative control. We used the laboratory anti-coagulant chemical compound ethylene diamine tetraacetic acid (EDTA) as a positive active control to compare and verify the null hypothesis regarding garlic's anti-coagulant action on human blood plasma. Table 3 compares the anti-coagulant effects of *Allium sativum L.* (1 ppm extract of human whole blood to the other two controls groups).

Table 3. Anticoagulant Effects of Allium sativum L. 1-ppm Extract on Human Whole Blood Compared with Other Two Controlled Groups.

	Allium St. Extract 1mg/1 L	EDTA As positive control	Controlled as negative Control
	16.34	17.1	12.93
	15.66	17.02	13.03
	15.9	16.92	11.14
	15.46	16.9	14.17
	15.7	16.83	11.22
	16	16.73	10.66
	15.99	16.81	11.52
	17.9	16.86	12.02
	15.99	16.99	10.1
	16.89	16.09	11.91
Mean	15.99	16.88	11.715
SD±	0.684851	0.265754	1.160095

Table 4 shows the prothrombin time, extrinsic route, and coagulation time in seconds with two independent groups serving as positive and negative controls to verify garlic's anti-coagulant capabilities. To test and verify the null hypothesis regarding garlic's anti-coagulant activity on human blood plasma, we included laboratory anti-coagulant chemical product double oxalate (a combination of ammonium and potassium oxalate in the ratio 3:2) as a positive active dose. In a healthy person, the usual prothrombin time is 12-14 seconds [40]

Table 4. Prothrombin Time Investigation of Human Plasma Compared with Three Random Groups Samples

	Aq. Ext. by Maceration 5 ppm	**Thermal EtOH Soxhlet Ext. 5 ppm**	**Controlled group as negative**	**Double Oxalate as positive control group 5 mg/L**
	16.01	17.10	11.20	14.71
	16.10	17.02	11.40	14.81
	16.76	16.92	10.90	14.91
	16.89	16.90	11.03	14.11
	16.83	16.83	11.07	14.97
	16.71	16.73	11.70	15.01
	16.76	16.81	12.01	15.99
	17.11	16.86	11.90	14.09
	17.99	16.99	11.61	11.51
	17.98	16.09	12.00	14.93
Mean	16.795	16.88	11.505	14.86
SD±	0.625223	0.265753645	0.39783057	1.115994624
Average	16.914	16.825	11.4818	14.504
Max.	17.99	17.1	12.01	15.99

To find out the prevailing difference between the groups and to rule out the null hypothesis (Ho), we plotted the statistical results in standard error of mean and analyzed the data using one-way analysis of variance (ANOVA). An alpha value of 0.05 or a p-value of 0.05 or 5% general population factor was considered as statistically significant. However, data analysis resulted in a significant value less than the p-value of 0.05 which we considered significant, making our findings categorical and rejecting the null hypothesis. Figure 2 shows the standard deviation graph of *Allium sativum l.* anti-coagulation time in comparison to the other two controlled groups.

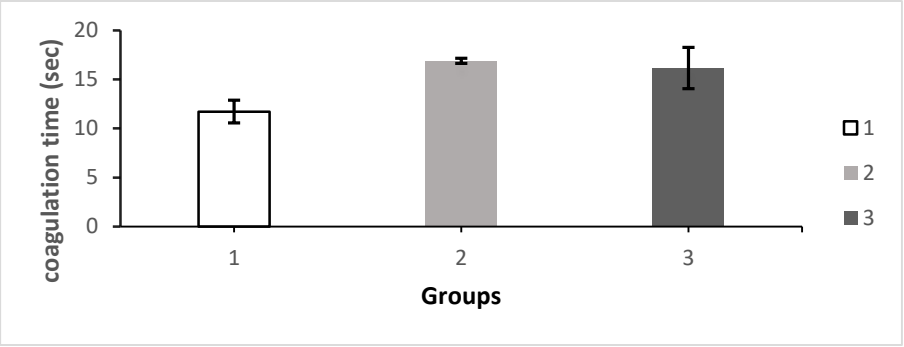

Figure 2. *Standard Deviation Graph of Allium sativum L. Anticoagulation Time Compared with Other Two Controlled Groups*

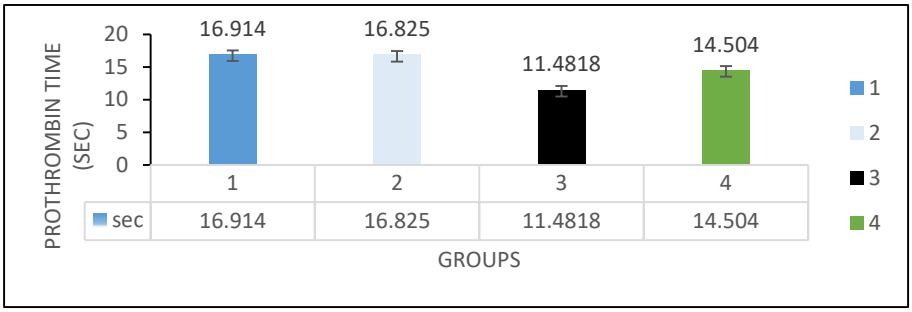

Figure 3. Prothrombin Time Coagulation Standard Deviation Bar among Random Groups and Allium sativum L. Extract

The observations and data given in Table 3 and Table 4 reveal that the garlic extract mixture acts as an anti-coagulant on whole blood, which is countered by a controlled positive result. However, a closer study of Table 3 and Table 4 reveals that when we use 1mg of garlic aqueous extract in 1000 ml with a concentration of 1ppm as a positive control, we get the results presented in Table 3. These results indicate that garlic aqueous extract performed better as a blood anti-coagulant against EDTA. In the second experiment, the efficiency of garlic extract from both aqueous and ethanolic soxhlet extractions at 5 ppm against double oxalate as a positive control was examined. It was found that garlic extract at both 1 and 5 ppm concentrations surpassed the positive control group. However, when the data from garlic extracts in aqueous solution was compared with ethanolic extracts obtained using soxhlet extraction, it was found that the ethanolic extract yielded a better result. The standard deviation graph given in Figure 3 depicts the mean standard error among the random groups selected in the current study In this in vitro investigation, the results of

statistical tests and ANOVA determined that Allium sativum L. has beneficial anti-coagulant properties and anti-coagulant effect on humane plasma.

Conclusion

The experiment was conducted to evaluate the clotting of blood in an individual by the addition of garlic ethanolic extract fractions to human blood samples. PT test was used to determine the number of seconds it take for a clot to form in a person's plasma sample after the addition of thromboplastin reagent. Among all the fractions of garlic extract tested in this study, ethanolic extract fraction showed significant anti-coagulant activity. PT prolongation specified the inhibition of the extrinsic coagulation cascade. The phytochemical constituents of garlic can actually decrease fibrin formation. It was, however, not possible to determine exactly which constituents in the extracts were accountable for the said activity. Herbal products are a symbol of safety in contrast to synthetic drugs that are regarded as unsafe, both for human beings and the environment. Although herbs have been priced for their medicinal, flavoring, and aromatic qualities for centuries, synthetic products of the modern age have surpassed their importance and usage. However, the era of blind dependence on synthetics is over and people are returning to the naturals with the hope of retaining safety and security. Hence, it is time to promote them globally.

Conflict of interest

The authors declare no conflict of interest.

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List of abbreviations

A.S.E.Et	*Allium sativum L.* Extract in Ethanol 95%
A.S.E.Aq	*Allium sativum L.* Extract in Aqueous
A.S.E.Sx	*Allium sativum L.* Extract by Soxhlet Extraction
EDTA	Ethylene diamine Tetra Acetic Acid
Aq. Ext.	Aqueous Extract
EtOH	Ethanol
Ext.	Extract
St.	*Sativum*