In Vitro Investigation of Therapeutic and Anti-Coagulant Properties of Allium Sativum L. On Human Plasma

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ABSTRACT

Cardio vascular illnesses are increasing worldwide and among these illnesses Thromboembolic disorders are very prevalent. The anticoagulant properties of many herbs have been reported. Garlic is a common herbal supplement. The current study was conducted to compare the in vitro anti-coagulant activity of different solvent-extracted fractions on human blood samples. For this purpose, rectified spirit and reverse osmosis (RO water), were used as solvents. Multiple extracting approaches were applied to prepare different extract fractions of Allium sativum, both in ethanolic and aqueous extracts by maceration, decoction, and soxhlet extraction methods. The concentration of each extract fraction was subjected to a primarily anti-coagulant screening method in human blood sample in-vitro by calculating their prothrombin time of 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. The results indicated that all garlic extract fractions had significant
anti-coagulant potential. However, at 5 ppm concentration, soxhlet extraction extract had the maximum anti-coagulant potential. Moreover, Garlic's aqueous extract also showed a significant anti-coagulant effect on human plasma. This observation agrees with the obtained data that the soxhlet extracted sample of Garlic showed the highest activity of platelet aggregation inhibition. Both ethanolic and aqueous extracts of *A. sativum* showed significant anti-coagulation properties as compared to positive controlled EDTA and Double oxalate as a synthetic anti-coagulants in our study.

Keywords: Allium Sativum L., Anti-Coagulant, Human Plasma, Prothrombin Time, Rectified Sprit

1. Introduction

Cardiac disease and other heat-related illnesses are widespread. Thromboembolism, or clotting of blood venous or arterial blood causes many cardiovascular illnesses and also influence atherosclerosis [1], diabetes mellitus [2], cancer complications [3], pulmonary emboli [4], deep vein thrombosis, hypertension [5], strokes and heart attacks [6]. These cardiac diseases play a leading role in global burden of disease and mortality. Hereditary disorders and smoking habits can also cause coagulation of blood [7]. According to the reports of WHO lives of about 17.9 million people are claimed by cardiovascular disease annually [8].

Natural herbal supplements are reported to be effective against many diseases. They have revolutionized the medicine industry. They act as secondary metabolites and modulate several biosynthetic pathways. Many plants have been proved effective in clinical trials against many diseases [9]. Several scientific clinical studies have shown that using natural herbs, secondary metabolites, and phytochemicals with anti-coagulant activity can effectively treat and prevent
cardiovascular disorders [10, 11]. Garlic is a natural, which belongs to the onion family, is on of the most revered and valuable medicinal plant [12]. It is an essential plant due to its medicinal and nutritional values [12]. Garlic, an aromatic solid perennial vegetable bulbous plant that has been farmed for hundreds of years and propagated through bulbs, is our therapeutic herb in the present study [13, 14].

Garlic contains 33 sulphur compounds, including seventeen 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]. Garlic's medicinal benefits, including its flavor, taste, and pungent odour, are due to sulphur compounds. Garlic's therapeutic effects mainly occur due to sulfur compounds, smell, taste, and pungent aroma. Allicin is derived from the amino acid cysteine, and dried, powdered Garlic contains 1% allicin [16, 17]. Garlic's spiciness is driven by allicin (diallyl disulfide), a potent antibacterial and antifungal agent [18]. As a prophylactic agent, Garlic is unique in the therapeutic indigenous herbal plant. Therefore, Avesta has been used as a resource for its research. This report on research references was published [19, 20].

Coagulation is a naturally occurring phenomenon that is an essential aspect of hemostasis. It aids the development of clots in the blood, which restricts or stops bleeding and eventually leads to wound/lesion healing. The coagulation cascade is composed of a series of operations or enzyme reactions. The extrinsic and intrinsic pathways of blood coagulation are the first two phases in the process [21, 22]. Garlic is the most effective anti-coagulant herb, with 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].
With its medical value in mind, the current study focuses on the therapeutic and anti-coagulant effects of Allium Sativum on human plasma. As a result[23], the data was analyzed and categorized according to their relevance and a table was created to summarize all of the findings[24]. The findings are expected to add value in addressing concerns about the impact of garlic ingestion on blood pressure and cardiovascular morbidity [25].

2. Methodology

2.1 Extract Preparation

_Allium sativum_ L. ethanolic and aqueous extracts were prepared using rectified sprit and reverse osmosis (RO) water; their phytochemical analysis was conducted using the prepared stock standards. The collected sample was activated in its matured state 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 rinsed with purified water and packed in an air-sealed packet at -4°C until the further procedure. UV spectroscopic and FTIR analysis on crude samples was conducted assess the qualitative analysis of Allium sativum. Phytochemical screening and proximate analysis was conducted as described by safowra [26], Trease and Millit [27].

In this study, maceration, decoction, and soxhlet extractions were conducted 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.

2.2. Collection of Blood Samples
Plasma samples were taken from healthy people according to a methodology that had been 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 (e.g., thrombosis). 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 this study. Adult participants gave their informed consent to participate in the study.

Blood samples were collected in tubes containing 0.105 mol/L (i.e., 3.2%) in a ratio of nine volumes of whole blood to one volume of trisodium citrate an anti-coagulant. The prothrombin time test was performed according to the procedure described in the literature [29-31]. For appropriate coagulation investigations, the proper blood-to-anticoagulant ratio should be used. According to NCCLS standards [32], the value of blood specimens hematocrits (HCT) should be about 55 percent for Prothrombin time screening i.e. 9 parts freshly collected whole blood and one part anti-coagulant should be used for each sample [33, 34].

2.3. Statistical Analysis

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

3. Results

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 transition of valance electron in sulfonyl group [35, 36]. We used an 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.

3.1 Phytochemical analysis

Allium Sativum are rich in nutrients such as protein, carbohydrate, minerals, dietary fiber and vitamins. These vitamins to play a significant role in preventing tissue damage because of free radicals, hence it is believed that Garlic is good source of antioxidants and anti-coagulant properties as shown in Table 1. Phytochemicals such as saponin, flavonoid, tannin, reducing sugar, steroid, and terpenoid were found in both aqueous and ethanolic extract of A. sativa.

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 determine the organic and dry weight of the sample and estimate the nutrient and constituent ratio in the provided sample using proximate determination. To fulfil the criteria, this technique estimates moisture, protein, lipid, fat, and carbohydrates in the sample and compares them to standard statistics. This analysis employs a variety of methodologies and adaptations, but in present study, we used the preliminary method. Three samples of data were collected and statistically analyzed to assess the outcomes. The data was provided in Mean Standard Error of Mean (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).

3.3 Prothrombin Time and Anticoagulation Investigation

To investigate the anti-coagulant effects of Allium sativum extracts, a 5 ppm dose of garlic extract obtained using various extraction methods was used. The primary active dose with an anti-coagulant therapeutical effect on humane blood plasma is garlic extract, extracted with maceration.
in an aqueous and soxhlet extractor. In a healthy person, the usual prothrombin time is 12-14 seconds. In a double-blind trial, we investigated the effect and efficiency of our Garlic crude extract using a positive controlled EDTA 5 mg combination and a negative control. To compare and verify the null hypothesis with garlic anti-coagulant action on humane blood plasma, we used the laboratory anti-coagulant chemical compound Ethylene diamine tetraacetic acid (EDTA) as a positive active control. Table 3 compares the anticoagulant effects of *A. sativum* L. (1ppm extract on humane whole blood to the other two controls groups).

Table 4 shows the prothrombin time extrinsic route coagulation time in seconds, with two independent groups serving as positive and negative controls to verify anti-coagulant capabilities. To test and verify the null hypothesis with garlic anti-coagulant activity on humane blood plasma, we included laboratory anti-coagulant chemical product Double Oxalate (combination of ammonium and potassium oxalate in the ratio of 3:2) as positive active dose.

4. Discussion

This study was carried on humane blood plasma that was extracted and isolated by centrifugation. Prothrombin time was determined to the method and protocol described by Quick A.J and WHO expert committee on biological standardization[46]. To investigate the anticoagulant properties of *A. Sativum* extract we used as primary active dose to check anticoagulant effect on human blood plasma. Normal value of prothrombin time in healthy person range 12-14 sec [46, 47]. In our study we preliminary investigated the effect of crude extract of garlic and efficiency with positive controlled EDTA 5 mg mixture and also along with negative control in double blind trail. However, any studies have reported the antithrombotic activity of raw garlic to be more effective than boiled product [48]. Another study has reported the in vitro effectiveness of aqueous extract of raw garlic to be more effective in comparison to the extract of boiled garlic against platelet aggregation [49].

The garlic extract that we prepared by the method of soxhlet extraction showed promising and better result than aqueous extract. The variance between all groups and validate our data significant
we set significant p-value at P<0.05 and counter verified the groups significant and authentically rejected null hypothesis. Many studies have reported that depending on the type of solvent, extraction method and the plant used for extraction, the effectiveness of bioactive compounds in an extract can vary greatly [50].

In the second experiment, we examined the efficiency of garlic extract from both aqueous and ethanolic soxhlet extractions at 5 ppm against double oxalate as a positive control, and found that garlic extract at both 1 and 5 ppm concentrations surpassed the positive control group. However, when we compare the data from garlic extracts in aqueous solution with ethanolic extracts obtained using soxhlet extraction, we find that the ethanolic extract yields a better results. The standard deviation graph given in Figure 3 depicts the mean standard error among the random groups selected in the present study.

The presence of sulfonyl functional group in the testing sample suggests that Allicin or Ajoene are both organosulfonic compounds with active features of garlic therapeutical activity on humane 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\(^{-1}\) in the near infrared area [31, 37]. The broad and prominent peak at 220 nm show strong presence of Allicin in extracted garlic extract shown in fig [42, 43].

The phytochemical screening of the 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] in both aqueous and ethanolic extraction. Phytochemicals such as saponin, flavonoid, tannin, reducing sugar, steroid, and terpenoid were found in both aqueous and ethanolic Allium sativa extracts, according to the findings. The findings of this study on the Phytochemistry of Garlic corroborated those of Deresse [38], who discovered that garlic extracts had activity
against both gramme negative (E. coli, Salmonella sp., Citrobacter Enterobacter, Pseudomonas Klebsiella) and gramme positive (S. aureus, S. pneumonia, streptococcus, and Bacillus anthrax) bacteria due to the presence of phytochemicals such as saponin and tannins. This study also discovered that A. sativum has a low fat content, and low fat diets are known to lower cholesterol levels. Low fat products have shown to reduce hypertension. Increased garlic consumption has shown to reduce hypertension [39, 51].

**Conclusion**

The study was conducted to evaluate anticoagulant properties of aqueous and ethanolic extract of garlic on human blood. Among all the fractions of garlic extract tested in this study, ethanolic extract fraction had significant anticoagulant activity. The PT prolongation literally specified the inhibition of the extrinsic coagulation cascade. The phytochemical constituents of garlic can decrease fibrin formation. It was however not possible to determine which constituents in the extracts exactly were accountable for the activity. These herbal products are the symbol of safety in contrast to the synthetic drugs that are regarded as unsafe to human being and environment. Although herbs had been priced for their medicinal, flavoring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. It is time to promote them globally.

**Conflict of interest**

There is no conflict among authors.

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Figure 1 FTIR Spectrum of Allium Sativum L. Extract at 1-ppm Concentration in Ethanol
96%
Figure 2 standards 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*
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
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
Table 3: Anticoagulant properties of Allium sativum L. 1-ppm extract on humane 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 prothrombin time investigation on humane plasma compared with three random groups samples

| Aq. Ext. by Maceration 5 ppm | Thermal Soxhlet Ext. 5 ppm | EtOH Ext. 5 ppm | Controlled group as negative | Double Oxalate as positive control 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