Antihaemostatic effect of combination of *Allium sativum* L. ethanol extract and warfarin in *Wistar* rats

Efecto antihemostático del extracto alcohólico de *Allium sativum* L. combinado con warfarina en ratas *Wistar*

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ABSTRACT

**Background:** concomitant use of herbal products with commonly prescribed conventional medications has been associated with higher risks of adverse effects. *Allium sativum* L. (garlic) has long been used both for flavoring and for medicinal purposes in many cultures but many serious concerns over surgery or contraindications with anticlotting medications such as warfarin are expressed in the medical arena.

**Objectives:** to evaluate the antihaemostatic effect resulting from the combination of *Allium sativum* and warfarin in *Wistar* rats.

**Methods:** 22.5 % *A. sativum* ethanol extract was obtained from fresh *A. sativum* cloves and the phytochemical screening was done. Twelve *Wistar* rats were divided into four groups, one control and three experimental. A single oral warfarin dose (3 mg/kg) was administered to one experimental group. The other two groups received orally *A. sativum* ethanol extract (10 mg/kg) once daily for 11 days, and a single dose of warfarin (3 mg/kg) was added to one of them the last day of the study. The clotting time and clot retraction were determined to assess the hemostatic functions on the study groups. The mean differences were calculated and *P* value less than 0.05 were considered significant.

**Results:** fats and oils, terpenoids, reducing sugars, and tannins were present in the *A. sativum* ethanol extract. The clotting time was significantly delayed in the experimental groups which received *A. sativum* and the combination of *A. sativum* and warfarin. The clot retraction was poor in the group treated with the combination of the drugs studied.
Conclusions: *a. sativum* oils may be responsible for the antihaemostatic effects found which can be potentiated by its additive interaction with warfarin increasing the risk of bleeding complications when both drugs are used concomitantly.

**Key words:** *allium sativum*, warfarin, antihemostatic, additive interaction, concomitant.

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**RESUMEN**

**Introducción:** el uso concomitante de productos herbarios con medicamentos convencionales ha sido asociado con un elevado riesgo de reacciones adversas. Muchas culturas han utilizado *Allium sativum* L. (ajo) con propósitos saborizantes y medicinales, pero en el campo de la salud existen serias preocupaciones sobre su efecto durante la cirugía o contraindicaciones con drogas anticoagulantes como la warfarina.

**Objetivos:** evaluar el efecto antihemostático de la combinación de *Allium sativum* y la warfarina en ratas Wistar.

**Método:** fueron usados bulbos frescos de *A. sativum* para obtener el extracto alcohólico al 22,5 %, y su estudio fitoquímico fue realizado. Doce ratas Wistar fueron divididas en cuatro grupos, uno control y tres experimentales. Un grupo experimental recibió warfarina (3 mg/kg) en dosis única vía oral. Los otros dos grupos recibieron extracto alcohólico de *A. sativum* (10 mg/kg) vía oral una vez al día por 11 días. A uno de estos dos grupos se le administró además 3 mg/kg de warfarina en dosis única vía oral, el último día del estudio. El tiempo de coagulación y la retracción del coágulo fueron determinados. Las diferencias entre las medias fueron calculadas considerándose significativa el valor de *P* menor que 0,05.

**Resultados:** aceites, grasas, terpenoides, azúcares reducidos y taninos, fueron encontrados en el extracto alcohólico de *A. sativum*. El tiempo de coagulación fue mayor en los grupos experimentales que recibieron *A. sativum* y la combinación de *A. sativum* y warfarina. La retracción del coágulo fue pobre en el grupo tratado con la combinación de drogas.

**Conclusiones:** los aceites presentes en el extracto de *A. sativum* pueden ser responsables de su efecto antihemostático, el cual pueden ser potenciado por su interacción aditiva con la warfarina, lo que incrementa el riesgo de complicaciones hemorrágicas cuando ambas drogas se administran relacionadas.

**Palabras clave:** *Allium sativum*, warfarina, antihemostático, interacción aditiva, concomitante.

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**INTRODUCTION**

The use of herbs as medicine is the oldest form of healthcare known and has been used in all cultures throughout history.\(^1\) Herbal medicines form the basis of therapeutic use in developing countries but in recent years it has been seen an increase in the use of herbal medications in the developed world as well.\(^2,3\) It is estimated that up to four billion people (80 % of the world’s population) living in
the developing world rely on herbal medicinal products as a primary source of healthcare and traditional medical practice. In Africa up to 90% of the population use medicinal plants to treat diseases of varying etiology, in fact this practice is part of the African tradition.

Several reasons explain the increasing use of traditional medicine. It is more affordable, more closely related to the patient’s ideology and allays concerns about the adverse effects of chemical medicines because they are widely perceived as natural and safe drugs which is not necessarily true, especially when herbs are taken with prescribed drugs, over-the-counter medications, or other herbs, that is very common.

Based on current public perception, many patients do not consider the herbal self-medications a risk and they unlikely associate plants with drug interactions, therefore the healthcare professional is not informed about their use. Beyond this, healthcare professionals usually do not request from their patients information about consumption of herbal medicines. Hence there is lack of awareness of the potential for herbal products to cause undesirable clinical outcomes.

Vasospasm, platelet plug and clotting are the major mechanisms of haemostasis being clotting the most potent. These mechanisms are dependent one from the other and their interaction is a complex process responsible for blood loss prevention. Haemostatic dysfunction, however, arises from any alteration of this complex system, leading to pathological thrombosis or vascular occlusion by thrombus fragments. Haemostatic dysfunction can result in increased risk of haemorrhage or thrombosis.

Warfarin is an oral anticoagulant known to inhibit vitamin K dependent synthesis of clotting factors II, VII, IX and X, normally used to prevent the progression or recurrence of acute deep vein thrombosis or pulmonary embolism, venous thromboembolism in patients undergoing orthopedic or gynecological surgery, and systemic embolization in patients with acute myocardial infarction, prosthetic heart valves, or chronic atrial fibrillation.

The list of drugs and other factors that may affect the action of warfarin is prodigious. Many herbal medicines have confirmed to alter the metabolism of warfarin by acting on cytochrome P450 enzymes. Other potentially interacting complementary medicines include those containing natural coumarins and those with possible effects on platelets and clotting factors thus leading to bleeding complications.

Allium vegetables, such as garlic and onions, are commonly consumed across the world and are sources of a variety of nutrients and phytochemicals. *Allium sativum* L. (*A. sativum*) species, which belongs to Alliaceae family, is a plant native to Central Asia, used commonly for its pungent odor and taste being the bulb the most commonly used part of the plant. It has been used throughout history for both culinary and medicinal purposes.

*A. sativum* has a higher concentration of sulfur compounds (oil- and water-soluble) than any other *Allium* species which are responsible for both its pungent odor and many of its medicinal effects. *In-vitro* studies have reported antimicrobial, antithrombotic, anticancer, antiplatelet aggregation and antioxidant activities of *A. sativum*. Additionally, *in vivo* studies in both animal and human clinical trials demonstrated enormous benefits of the plant in hyperlipidemia, cardiovascular disorders and arteriosclerosis.
A. sativum may be more effective in preventing health problems and as a complementary medicine than as a therapeutic option. Long-term supplementation is required to obtain the preventive benefits of A. sativum, which makes necessary to consider its toxicity and interactions. Due to the narrow therapeutic index of warfarin, its interaction with A. sativum can result in potentially fatal bleeding complications.

The above background motivated this study, aimed to evaluate the antihaemostatic effect resulting from the combination of A. sativum and warfarin since herbal drug interaction is a serious problem that leads to dangerous side effects.

METHODS

The antihaemostatic effect resulting from the combination of A. sativum and warfarin was assessed determining the clotting time and clot retraction in Wistar rats.

Plant material

Fresh A sativum (garlic) bulbs were purchased from the local market of Mbarara, Uganda and identified by Dr. Olet Eunice, a botanist at Mbarara University of Science and Technology; and given a Voucher number Musubika Barbra 001.

The bulb consists of about daughter bulbs (clove) arranged roughly in a circle around a central axis. Each daughter bulb has a tough, white or reddish skin around a fleshy tubular leaf, investing a more or less rounded elongated cone of leaf primordia and vegetative apex.

Preparation of A. sativum ethanol extract

One kilogram of A. sativum cloves were peeled to obtain the fresh cloves which were crashed using a blender. The mashed A. sativum was weighed and 300 g were placed into 2L beaker and 1500 mL of 95% absolute ethanol was poured into the beaker which was then closed tightly to prevent loss of the solvent. The cold maceration of A. sativum was allowed to take place for over 72 hours for extraction of A. sativum components. The resultant supernatant was decanted using a filter cloth. The filtrate was concentrated using a rotary evaporator in the Nature Chemotherapeutic Laboratory to obtain a viscous solid which was then dried in an oven between 40-50°C for a period of two weeks. A sticky solid extract was obtained at a percentage yield of 22.5% w/w.18

Phytochemical screening

Phytochemical analysis was performed on 22.5% ethanol extract using some chemical reactions (tests) to identify, predominantly by color change or precipitated formations, the presence of secondary metabolites:

- Saponins (foam).
- Tannins (ferric chloride FeCl₃).
- Terpenoids (Libermann-Buchard).
Cardiac glycosides (Keller-killiani).
Flavonoids (Shinoda).
Reducing sugars (Fehling).
Fats and oils (sudan III) and volatile oils peptides (ammonia gas).

Animals

Twelve Wistar rats from 150 to 200 g of weight were divided into four groups of three rats each. The rats were obtained from the Animal house of MUST and were kept for two months under controlled conditions, 23 ± 0.5 °C, relative humidity around 50 %, in a 12h:12h alternate light-dark cycle, food and water ad libitum. The Guide for the care and use of laboratory animals was strictly followed.

The feeding of the animals which received treatment with warfarin was restricted for a limited period of time to avoid decrease rate of absorption of the drug because presence of food in the gastrointestinal tract is considered as a factor to reduce warfarin absorption; the free access to water was not restricted.

Pain, suffering or distress was minimized both in duration and magnitude to the greatest possible extent without jeopardizing the aim of the experiment. The method used for animal´s euthanasia was overdose of anesthesia (three times the anesthetic dose of sodium pentobarbital) as described by the American Veterinary Medical Association Guidelines for the Euthanasia of Animals.

Administration of drug extract and warfarin to the experimental animals

The experiment was carried out following the method designed by Blumenthal and collaborators (1998) for human beings modified by the researchers in order to achieve the objectives of the study using rats as biomodel. The modifications are listed below:

For A. sativum administration

Type of formulation from enteric coated tablets to a liquid solution.
Dose from 2000mg twice a day for two weeks to 10mg/kg once a day for 11 days.

For warfarin administration

A single dose from 25 mg to 3 mg/kg prepared from the tablet dissolved in distilled water.
Group 1 (N): received neither the extract nor warfarin, this group served as the normal control.
Group 2 (G): was administered 10 mg/kg A. sativum extract once a day for 11 days orally.
Group 3 (W): was administered 3 mg/kg warfarin in single dose orally only after an overnight fast.
Group 4 (GW): was administered 10mg/kg A. sativum extract once a day for 10 days orally and, on the day 11,3 mg/kg of warfarin in a single dose orally after an overnight fast.
The 11th day of the study and after 12 hours of administering warfarin to the respective experimental groups, the blood was collected by cardiac puncture under anesthesia with sodium pentobarbital at 30mg/kg through intraperitoneal injection. The blood sample was used to determine the clotting time and clot retraction and the animals were humanely euthanized after the blood collection.

Antihemostatic effect

The clotting time was determined to assess the intrinsic pathway of coagulation. To evaluate the platelet function the clot retraction time was performed.

Clotting time

During the blood sample collection, the stopwatch was started as soon as the blood entered the syringe. Blood samples (2 mL) collected using a syringe were delivered into the test tubes and immediately placed in a water bath at 37°C. The test tubes were gently tilted every 30 sec to horizontal level until the blood clot was not flowing out even when tilting the tubes to 90 degrees. The stopwatch was stopped at this point and the time was recorded. This was considered to be the clotting time. The results obtained were then compared to the normal values of clotting time in rats (1.88 - 2.27 min).

Clot retraction time

The clot retraction test was determined in the whole blood sample using the following procedure:

After clotting of the blood sample the test tubes were kept undisturbed for its subsequent retraction in the water bath at 37°C. The tube was inspected at 1, 2, 4 and 24 hours.

The clot retraction was evaluated qualitatively:

- Poor: If clot retraction has occurred at 2-4 hours.
- Fair: If clot retraction occurs after 4 hours but within 24 hours.
- Good: If no retraction occurs even at 24 hours.

Statistical analysis

Statistical analysis of the data was carried out using SPSS (version 16.0) software. The mean differences were calculated by one-way ANOVA test and P value less than 0.05 was considered significant.
RESULTS

Phytochemistry

The phytochemical screening of the *A. sativum* ethanol extract found a positive reaction to fats and oils, terpenoids, tannins, and reducing sugars. Other secondary metabolites were present in trace quantities *(table 1).*

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fats and oils</td>
<td>++</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>++</td>
</tr>
<tr>
<td>Tannins</td>
<td>++</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>++</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
</tbody>
</table>

*Table 1. Phytochemical constituents of *A. sativum* ethanol extract*

Key: ++ present, + trace quantities.

Clotting Time

The clotting time obtained per rat and the mean calculated in each group are shown in *table 2.* The groups of rats which received *A. sativum*, warfarin or the combination of both exhibited clotting times beyond the normal range. The highest value (6.73) was found in group 4 which received the combination of warfarin and *A. sativum* ethanol extract. The higher value between the groups which received either warfarin or *A. sativum* extract alone was obtained in the group administered with warfarin (5.27).

The statistical analysis results obtained from the comparison between group 4 which received the combination of *A. sativum* and warfarin with each of the other three groups of study according to the clotting time are shown in *table 3.* The mean obtained in group 4 was significantly higher (P < 0.05) when comparing it with both group 1 (control) and group 2 (received warfarin).
The results of the clot retraction time are represented in table 4. The combination of *A. sativum* and warfarin demonstrated to interfere with the clot retraction, found poor in this experimental group. The *A. sativum* ethanol extract alone also delayed to some extend the clot retraction.

### Clot retraction

The screened *A. sativum* extract contained fats and oils, terpenoids, reducing sugars, and tannins; the same components reported by Olusanmi and collaborators. These phytochemicals are known to have medicinal importance. For example, fats and oils have cardiovascular effects like hypolipidemic, anticoagulant/antiplatelet and procirculatory effects, terpenoids are known to have antioxidant and cardiotonic properties.

### Table 2. Clotting time in the study groups

<table>
<thead>
<tr>
<th>Study group</th>
<th>Clotting time (min)</th>
<th>Mean (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1W1</td>
<td>7.23</td>
<td></td>
</tr>
<tr>
<td>G2W2</td>
<td>6.13</td>
<td></td>
</tr>
<tr>
<td>G3W3</td>
<td>6.83</td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>4.72</td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>5.83</td>
<td></td>
</tr>
<tr>
<td>W3</td>
<td>5.25</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>3.82</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>3.40</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>3.08</td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>2.42</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>2.08</td>
<td></td>
</tr>
<tr>
<td>N3</td>
<td>2.51</td>
<td></td>
</tr>
</tbody>
</table>


### Table 4. Clot retraction in the study groups

<table>
<thead>
<tr>
<th>Study Groups</th>
<th>Clot retraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>GW</td>
<td>Poor</td>
</tr>
<tr>
<td>W</td>
<td>Good</td>
</tr>
<tr>
<td>G</td>
<td>Fair</td>
</tr>
<tr>
<td>N</td>
<td>Good</td>
</tr>
</tbody>
</table>


### DISCUSSION

The screened *A. sativum* extract contained fats and oils, terpenoids, reducing sugars, and tannins; the same components reported by Olusanmi and collaborators. These phytochemicals are known to have medicinal importance. For example, fats and oils have cardiovascular effects like hypolipidemic, anticoagulant/antiplatelet and procirculatory effects, terpenoids are known to have antioxidant and cardiotonic properties.
An increase of the clotting time is an indicator of the coagulation function defect (secondary hemostasis) and the inefficient clot retraction indicates platelet function impairment or reduced number in the blood (primary hemostasis).

The significant rise (with mean values = 6.7) in the clotting time of the group of rats which received a combination between *A. sativum* extract and warfarin in comparison with those groups that received any of the drugs of the study, means that one or more clotting factors were either low in number or functionally affected. This shows the interaction that exists between warfarin and *A. sativum* and this is in line with previous studies and cases, and it emphasizes the antihemostatic activity of *A. sativum* already demonstrated in studies conducted by Harshita Chandhary and collaborators among others. The results obtained suggest the existence of additive interaction between *A. sativum* and warfarin whose anticoagulant effects can be potentiated by the first one with proven antiplatelet activity. These is in agreement with researches previously conducted to evaluate the interaction between drugs or herbs that possess antiplatelet activity with warfarin in an additive way resulting in increased bleeding time, prothrombin time and clotting time, hence leading to bleeding complications.

To complete the hemostatic mechanisms, the clot already formed is then retracted aided by the action of platelets. Contractile components found in the platelet structure make this process of retraction possible. The poor clot retraction exhibited by the group that received *A. sativum* and warfarin implies that one or both drugs impaired the function of the platelets in the sample. It is known the action of warfarin in the coagulation cascade, so the *A. sativum* extract shall be considered the one responsible for the antiplatelet function demonstrated in this group of rats.

The good quality of the retracted clot observed in groups 1 and 3 was due to the ability of the activated platelets to interact with the fibrin within the clot and the contraction of the platelet cytoskeleton needed for the retraction.

The presence of oil components found through the phytochemical screening may be responsible for the anticoagulant/antiplatelet pharmacological action of *A. sativum*. Researchers like Gebreselema and Mebrahtu in 2013, stated that *A. sativum* constituents can reduce fibrin formation and also help to reduce the fibrin existing in the blood even better than aspirin. These researches also asserted that Ajoene, an oil-soluble sulfur compound found in *A. sativum*, seems to be responsible for its anti-clotting effect.

An Egyptian study examined the effects of concomitant intake of aspirin and *A. sativum* preparations on the coagulation profile in cardiac patients and a significant difference was evident in those who received both *A. sativum* and aspirin when compared with those who received aspirin alone. In addition, there was a significant association between *A. sativum* intake with aspirin and the incidence of gastrointestinal bleeding.

While several in vitro studies suggest that *A. sativum* or its individual components may decrease platelet aggregation, recent clinical trials suggest otherwise. For example, a small randomized, double-blind, placebo-controlled, crossover research study was performed involving 14 healthy volunteers and the use of *A. sativum* macerate preparation containing ajoene and dithiins (oil components). Four hours after consuming one large dose of *A. sativum* oil, the study showed that there was little or no effect in platelet aggregation.
Taking into account the results of this study can be concluded that A. sativum has antihaemostatic effects in regard to clotting mechanisms and further clot retraction which may be attributed to the presence of A. sativum oils. The antihaemostatic effects of the plant can be potentiated by an additive interaction with warfarin when both drugs are used concomitantly increasing the risk of bleeding complications.

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