



## Evaluation of the activity of crude alkaloids extracts of *Zingiber officinale* Roscoe, *Thymus vulgaris* L. and *Acacia arabica* L. as coagulant agent in lab mice.

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### Abstract:

As Alkaloids known for their pharmaceutical importance; this research included the extraction of crude alkaloids of three plants (*Zingiber officinale* Roscoe., *Thymus vulgaris* L. and *Acacia arabica* L.) and evaluate their activity as coagulant agent by using three degraded concentrations of each plant extract and tested them on lab mice through the observation of the variations in bleeding time (BT), clotting time (CT) and platelet count. The results revealed differences in the percentage of alkaloids in the plants under the study; *Z. officinale* extract was the higher one followed by *T. vulgaris* and *A. arabica* respectively. *Z. officinale* extract was also the most effective plant as coagulant factor than other two plants as it decreased both BT and CT and increased platelets count more than what *T. vulgaris* and *A. arabica* affected on blood characters mentioned before. This makes crude alkaloids extract of *Z. officinale* dry rhizomes is the best plant product therapeutically and commercially.

**Keyword:** alkaloids, coagulant agent, bleeding time, clotting time, platelets count.

### تقييم فعالية المستخلص الخام للمركبات القلويدية لنباتات

*Zingiber officinale* Roscoe, *Thymus vulgaris* L. and *Acacia arabica* L.

### كعامل مسبب لتخثر الدم في الفئران المختبرية

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### الخلاصة

لكون ان القلويدات عرفت بأهميتها العلاجية فقد تضمن هذا البحث تضمن استخلاص المركبات القلويدية الخام في ثلاث نباتات هي *Zingiber officinale* Roscoe، و *Thymus vulgaris* L. و *Acacia arabica* L. وتقييم فعاليتها على انها مواد مسببة لتخثر الدم بإستخدام ثلاث تراكيز متدرجة لكل مستخلص نباتي واختبار هذه التراكيز في الفئران المختبرية من خلال ملاحظة التغيرات في زمن النزف وزمن التخثر وعدد الصفائح الدموية. أظهرت النتائج اختلافات في نسبة القلويدات للنباتات قيد الدراسة فقد كان مستخلص الزنجبيل هو الاعلى متبوعا بالزعرور والاكاسيا على التوالي. كذلك كان مستخلص الزنجبيل الاكثر فعالية في تخثر الدم حيث قلل كل من زمن النزف وزمن التخثر وزاد من عدد الصفائح الدموية اكثر مما سببه كل من الزعرور والاكاسيا على صفات الدم المذكورة آنفاً. هذا مما يجعل المستخلص القلويدي الخام للرايزومات الجافة للزنجبيل هو المنتج النباتي الافضل علاجياً واقتصادياً.

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## Introduction

Alkaloids rank among the most efficient and therapeutically significant plant substances [1]. 5,500 alkaloids were known and they comprise the largest single class of secondary plant substances which contain one or more Nitrogen atoms, usually in combination as part of a cyclic structure [2]. They are usually organic bases and form salts with acids and when soluble gives alkaline solutions. Pure, isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agents for their analgesic, anti spasmodic and bactericidal effects [3]. From the plants that have been used medicinally for a long time ago till now *Zingiber officinale* Roscoe, *Thymus vulgaris* L. and *Acacia Arabica* L.

*Zingiber officinale* Roscoe which belongs to the family zingiberacea is used worldwide for different purposes such as a cooking spice, condiment and herbal remedy (e.g. treatment of bleeding disorders and rheumatism) [4]. *Thymus vulgaris* L. which belongs to the family lamiaceae is also one of the plants that have many uses. It adds a distinctive aromatic flavoring to sauces, stews, stuffing, meats, and poultry; it possesses antispasmodic, antiseptic, expectorant, carminative and anti-oxidative properties [5, 6]. *Acacia arabica* L. belongs to the family fabaceae; Acacia seeds were often used for food and a variety of other products. It was used as an effective medicine for diarrhea [7].

## Materials and methods

### 1- Chemicals and Apparatus

#### a- Chemicals

Table 1- Chemicals used in this study are listed.

Chemicals	Origin
Ethanol absolute	Scharlau / Spain
Sulphuric acid	Merk / Germany
Chloroform	Scharlau / Spain
Methanol	FLUKA / Switzerland
Ammonium hydroxide	BDH / England
Tannic acid	BDH / England
HgCl <sub>2</sub>	BDH / England
KI	BDH / England

#### b- Apparatus

Table 2- Apparatus used in this study listed.

Apparatus	Company
Oven	Mermmet/ Germany
Electric Balance	Adam / England
Refrigerator	Ishtar / Iraq
Rotary Evaporator	Heidolph / Germany
Apparatus	Company
Electric Mill	El-arabi / Egypt
pH-meter	Milwaukee / USA
Non heparinized capillaries	Vitrex / Denmark
Electric shaker	BAIRD & TATLOCK London Limited / England
Automated platelets count device	Cell-DYN Emerald / Abotte USA
Electric Blender	Moulinex / France
Anticoagulant tubes	DMD- DISPO /Syria

### Collection of plant samples

Plant samples included *Zingiber officinale* Roscoe dry rhizomes Figure 1, dry leaves of *Thymus vulgaris* L. (not Iraqi species) Figure 2- and dry gum of *Acacia arabica* L. Figure 3- were brought from the local herbarium market in Baghdad city, then rhizomes, dry gum and also the leaves were

grinded in an electric mill so to get their powder in forms that easily deal with in the extraction steps; these powdery samples stored in dry and clean conditions until use.



**Figure 1-** *Zingiber officinale* Roscoe dry rhizomes ([www.en.wikipedia.org](http://www.en.wikipedia.org))



**Figure 2-** *Thymus vulgaris* dry leaves, ([www.gernot-katzers-spice-pages.com](http://www.gernot-katzers-spice-pages.com))



**Figure 3-** *Acacia arabica* dry gum, ([ww.tradeindia.com](http://ww.tradeindia.com))

#### **Preparation of crude alkaloids extract**

The extract was prepared according to the method of [8]. A quantity of 100g plant powder was homogenized in electrical blender with 350 ml of (4:1) ethanol: D.W. then filtered through muslin. Then through a filter paper in Boukner funnel, after that it has been acidified by drops of (2% sulphuric acid) until the pH became between 1 and 2, then the solution was extracted with chloroform 3 times in the separating funnel until we got two layers; the upper one was neglected and the lower one was used. Drops of concentrated ammonium hydroxide were added to this layer until the PH became between 9 and 10. Then the solution was again extracted in the separation funnel with (1:3) chloroform: methanol twice and one time with chloroform alone. After that the solution was separated

into two layers; the upper layer (solvent) was neglected and the lower layer was evaporated in a rotary evaporator at 40°C for (1-2) hours then dried in the oven until it turned into powder and kept in refrigerator until use.

#### **Preparation of different concentrations of plant extracts:**

Alkaloid extracts were prepared by dissolving certain weight of each plant extract according to the concentration in Distilled Water. Different concentrations (1, 5 and 10) mg / ml of plant extracts were prepared according to the following equation:

$$\text{Concentration mg/ml} = (\text{Weight/ Volume}) \times 1000$$

#### **Alkaloid indicators**

##### **1- Mayer reagent**

This reagent [9] was used for the detection of alkaloids. The stock solution (1) was prepared by dissolving 13.5g HgCl<sub>2</sub> in 60 ml H<sub>2</sub>O, stock solution (2) was prepared by dissolving 5g KI in 10 ml water, then combined with stock (1) and (2) and diluted with H<sub>2</sub>O up to 100 ml, then 1-2 ml of Mayer reagent were added to 5 ml of alcohol extract. A creamy or white precipitate indicates the presence of alkaloids.

##### **2- Tannic acid reagent**

This acid was used to precipitate alkaloids [10]. 1% tannic acid was prepared, and then 1-2ml of reagent was added to 5ml of the extract. The white turbidity was appeared indicating the presence of alkaloids.

#### **Hematological Test in Vivo**

In this study 30 male lab mice *Mus musculus* of Balb c. strain were used; their average weight was (28±2) gm; they were divided into 10 groups kept in clean lab cages (3 mice in each cage) in the animal house of the Biotechnology Research Center in Al- Nahrain University under optimal conditions of light and ventilation; fed on standard food and adequate amount of water. The groups were labeled with the plant name, its active group i.e. (alkaloids) and the degraded concentrations (1, 5, and 10) mg/ml of alkaloid extract.

Intra gastric dose of (0.4ml) of the replicates for each concentration of the three active groups of the plants under study were given once daily for seven days continuously [11].

#### **Clotting Time Measurement in vitro**

Blood was drawn into a capillary tube. The time of appearance of the drop of the blood on the cut tail was noted. The capillary glass tube is then kept between the palms of both hands for 30 second to keep it at body temperature. After 30 second the tube was taken out and small portion of the capillary tube was broken at regular intervals of 10 seconds, until a thread of clotted blood appears between the two pieces of capillary glass tube. The time interval between the appearance of the drop of the blood and the thread of the blood clot was the clotting time of the blood sample of the mouse expressed in minutes [12,13].

#### **Bleeding Time Measurement in vivo**

Mouse tail was cut with a scalpel 1-2 cm proximal from the end and bleeding time was calculated from the time of starting of bleeding till bleeding stopped. Spots were made with the bleeding tail on a blotting paper every 15 seconds till bleeding stopped and bleeding time was calculated accordingly. Or the time taken between the appearances of blood to the cessation of bleeding is taken as the bleeding time expressed in minutes [12, 13].

#### **Platelets Count**

Platelets count was measured by putting the blood samples of the mice under the test in anticoagulant tubes and measured them in automated platelet count device; this method was recommended because it is more accurate than the classic manual methods [14].

#### **Statistical Analysis**

The Statistical Analysis System [15] was used to study the effect of different factors in the study parameters. The significant differences between the means in this study were determined by using Least Significant Difference (LSD) test.

#### **Results and Discussion**

The dry rhizomes of *Zingiber officinale* Roscoe, dry leaves of *Thymus vulgaris* L. and dry exudates (gum) of *Acacia arabica* L. were extracted for detection of alkaloids. The yield of extraction was determined in table 3.

**Table 3-** Alkaloids yielded from plant parts expressed as %

Plant name	Alkaloid extract yield (%)
<i>Zingiber officinale</i> Roscoe Dry rhizomes	5.86
<i>Thymus vulgaris</i> L. Dry leaves	1.52
<i>Acacia arabica</i> L. Dry gum	1.32

The relative proportion between the amounts of plant used for extraction and the crude products was variable depending on several factors such as methods of extraction, solvent used in extraction process, and the plant parts or species [16]. Results in table 3- showed that alkaloids extract yielded from *Z. officinale* was higher than that obtained by [17] who mentioned that alkaloids percentage was (1.46%) while [11] reported that no alkaloids in the crude extraction of *Z. officinale* were detected. These differences could be due to place of sampling (i.e. different herbarium markets in which they differ in the means of preserving the samples). In this study as far as in our knowledge, in the crude extraction of *T. vulgaris* most researchers emphasized on terpenes and phenol extracts only without reporting the amount or percentage of alkaloids in the plant. [18] and [19] both mentioned the presence of alkaloids in leaves of *A. arabica* while [20] reported negative results of alkaloids. Results showed that the yield of alkaloids obtained from *Z. officinale* was the highest (5.86 %) than alkaloids yielded from *T. vulgaris* and *A. arabica* which were (1.52%), (1.32%) respectively. Hemostasis which is the arrest of blood loss from severed blood vessels and the maintenance of the blood fluidity involves coagulation and fibrinolysis. A wound or cut on blood vessels causes vasoconstriction and thrombin activation which is then accompanied adhesion and platelet activation, fibrin formation from circulating fibrinogen and coagulation inactivation mechanism [21]. The present study was carried out to determine the potentials of *Zingiber officinale* dry rhizomes, *Thymus vulgaris* dry leaves and *Acacia arabica* dry gum on the hemostatic mechanism, with primary interest on how it affects bleeding time, clotting time and platelets count. Bleeding time evaluates the vascular and platelet responses with hemostasis [22, 23], whereas the clotting time measures the intrinsic clotting factors (I, II, V, VIII, IX, X, XI and XII). Clotting time test is a qualitative measurements of factors involved in the intrinsic pathway [24]. Therefore the deficiency in these factors will affect the results. After the intra gastric administration of the mice under the test with three concentrations (1, 5, 10 mg/ml) of *Z. officinale* crude alkaloid extract for 7 days continuously. Results of the bleeding time (BT), clotting time (CT) and platelets count tests showed variations compared with the control group as the extract decreases the bleeding time and clotting time while increases the platelets count at significant difference of ( $P < 0.05$ ). This agreed with [25] who mentioned that there is an inverse relationship between bleeding time and platelets count.

Bleeding time is affected by many factors including vasoconstrictive effect of blood vessels, the formation of hemostatic plug and platelet activity. In general, anticoagulants and aspirin have been reported to increase BT in animals and humans while coagulants have opposite effect [26].

In table 4- and figure 4&5- the results showed that both (1mg/ml) and (5mg/ml) of crude alkaloid extract decreased the BT to (1.18) compared with control (1.88) and also decreased the CT to (2.10) and (2.30) respectively compared with control (2.52) while the third concentration of the extract (10 mg/ml) was the most effective because it decreased the BT to (0.94) and the CT to (1.23) compared with the control value mentioned above. However [27] who used crude extract of fresh rhizomes juice of *Z. officinale* to investigate BT and CT on Albino Wister rats reported an increase in BT compared to control and mentioned that CT didn't changed due to the extract. These differences might be due to plant part used and also the type of lab animals.

Cessation of bleeding indicates the formation of hemostatic plugs, which are in turn dependent on an adequate number of platelets and on the ability of the platelets to adhere to the sub endothelium and to form aggregates [28]. The platelets count also significantly changed but adversely with BT and CT, the normal value (control) was ( $359 \times 10^9/L$ ) while the platelets count of the concentrations (1mg/ml; 5mg/ml; 10mg/ml) was ( $1393 \times 10^9/L$ ) ( $728 \times 10^9/L$ ) ( $1563 \times 10^9/L$ ) respectively. As mentioned above (1

mg/ml) concentration was more efficient than (5 mg/ml) this might happened due to negative response to the extract that has been given to the mice under investigation which might increases the blood fluidity rather than decreasing it [29]. In general for the three parameters (10 mg/ml) of crude alkaloid extract of *Z. officinale* was the most effective concentration.

Results in table 5- and Figure 6&7- crude alkaloids extract of *T. vulgaris* dry leaves also revealed significant differences at ( $p < 0.05$ ). The concentration (1mg/ml) and (10 mg/ml) decreased the BT to (1.65) and (1.28) respectively compared with the control (1.88) and also decreased the CT to (2.12) and (1.68) respectively compared with control (2.52) while the BT of the (5 mg/ml) concentration was (1.02) and CT was (1.27), The results recorded in this study are in consonance with the reports of [30] on the haemostatic activities of the leaf extract of *Aspilia africana* which arrested bleeding from fresh wounds by reducing both bleeding and clotting times. While the platelets count of the experiment concentrations (1mg/ml) and (10 mg/ml) increased significantly to (668\*10<sup>9</sup>/L) and (836\*10<sup>9</sup>/L) respectively compared with control. However the platelets count of the concentration (5 mg/ml) was higher than the other concentrations which was (1296\*10<sup>9</sup>/L) compared with control. In general for the three parameters (5 mg/ml) of crude alkaloid extract of *T. vulgaris* was the most effective concentration.

*Thymus vulgaris* generally showed decrease in both BT and CT while platelets count increased significantly; but it has not been studied separately before just as a component of mixture of different plants extracts for hemostatic activity evaluation for example the Ankaferd Blood Stopper (ABS) in which *T. vulgaris* represents one of its component; ABS was found to be effective in shortening the duration of bleeding and decreasing the amount of bleeding [31, 32].

In table 6- and Figure 8 & 9 results for crude alkaloid extract of *A. arabica* showed significant differences at ( $p < 0.05$ ). The concentrations (1 mg/ml), (5 mg/ml) and (10 mg/ml) decreased the BT compared with control to (1.34), (1.27) and (1.20) respectively; as well as decreased the CT compared with control to (2.24), (2.04) and (1.39) respectively. These results obtained in this work reflecting that there was an increase in one or more of the clotting factors involved in the intrinsic pathway. Plasma fibrinogen which was not measured in this study has been known to facilitate the rate of fibrin polymer formation which ultimately leads to more effective clot formation [33] while [26] showed marked effect of gum arabic on the coagulation system of rats that it prolongs the BT and CT. In this study platelets count increased significantly compared with the control to (868\*10<sup>9</sup>/L), (981\*10<sup>9</sup>/L) and (1196\*10<sup>9</sup>/L) respectively. In general for the three parameters (10 mg/ml) of crude alkaloid extract of *A. arabica* was the most effective concentration.

## Conclusions

Crude alkaloids activity of the three plants under study revealed that crude alkaloids of *Z. officinale* dry rhizomes was the most effective as the yield percentage was higher than crude alkaloids of *T. vulgaris* dry leaves and *A.arabica* dry gum respectively as well as it was efficient at the concentration 1mg/ml while the efficient concentration for *T. vulgaris* was 5 mg/ml and for *A. arabica* was 10 mg/ml which confirms the results obtained in this study as alkaloids yield percentage of each plant under the study was compatible with their most effective concentrations. This makes crude alkaloids extract of *Z. officinale* dry rhizomes is the best plant product therapeutically and commercially.

## Recommendations

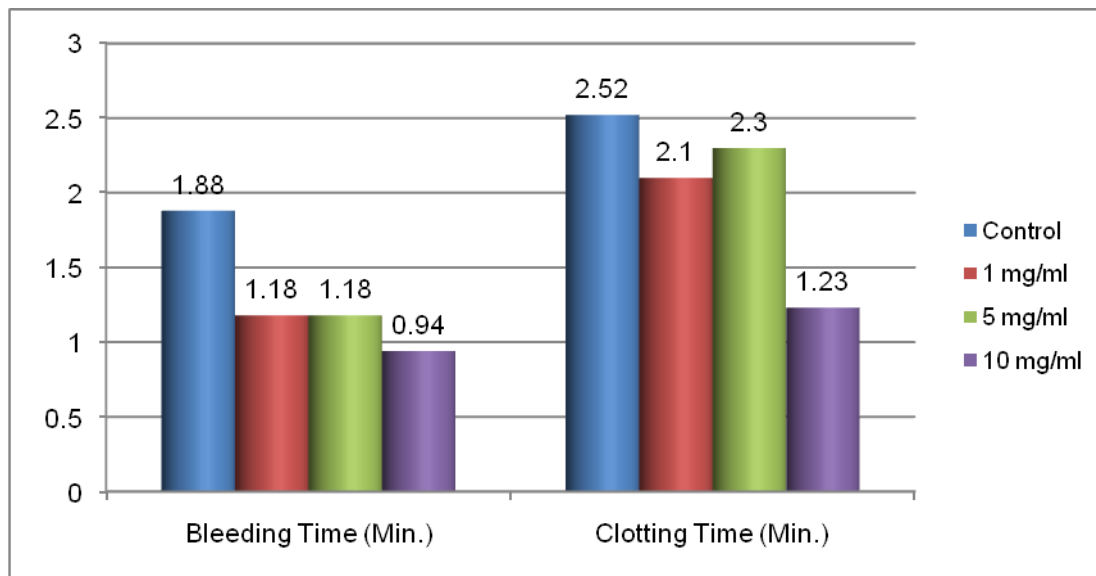
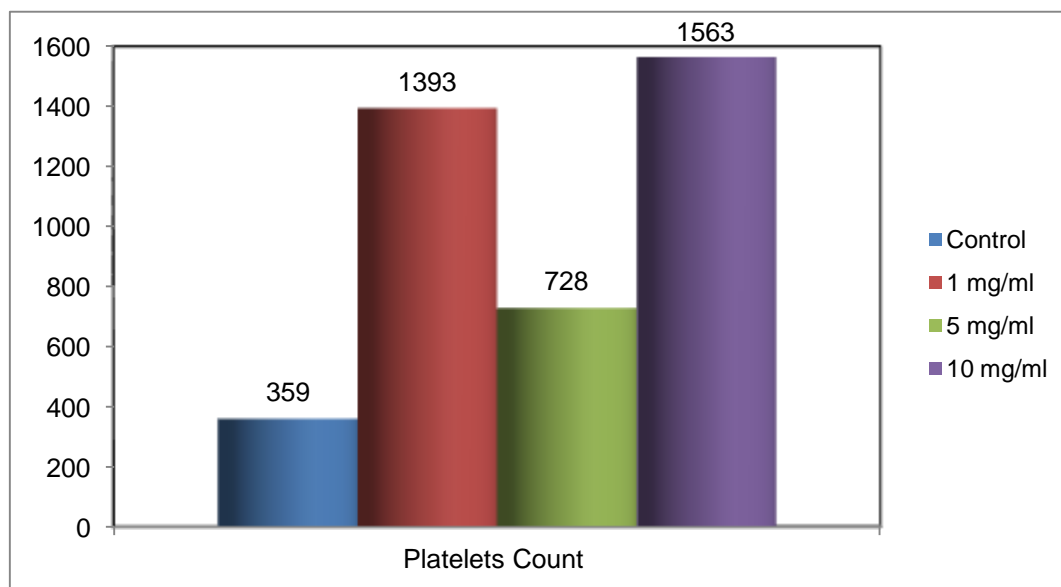
From all obtained and analyzed results the following points can be recommended:

- 1- Further study of chemical separation and purification of the active components in *Zingiber officinale* dry rhizomes, *Thymus vulgaris* dry leaves and *Acacia arabica* dry gum.
- 2- Intra venous injection of plant extracts instead of intra gastric administration recommended to be studied.
- 3- Study the histopathological effects of *Z. officinale* dry rhizomes, *T. vulgaris* dry leaves and *A. arabica* dry gum extracts.
- 4- Study the biochemical and enzymatic activity of the factors present in *Z. officinale* dry rhizomes, *T. vulgaris* dry leaves and *A. arabica* dry gum.
- 5- Conducting a study about the toxicological, therapeutic and immunological effects of the active groups tested in *Z. officinale* dry rhizomes, *T. vulgaris* dry leaves and *A. arabica* dry gum extracts.

**Table 4-** *Zingiber officinale* crude alkaloids extract effect on the tested blood characters by using different concentrations

Blood Characters	Conc. of <i>Zingiber officinale</i> crude alkaloid extract				LSD Value
	Control	1 mg/ ml	5 mg/ ml	10 mg/ ml	
Bleeding time (Min.)	1.88 ± 0.21	1.18 ± 0.03	1.18 ± 0.04	0.94 ± 0.19	0.472 *
Clotting time (Min.)	2.52 ± 0.04	2.10 ± 0.03	2.30 ± 0.08	1.23 ± 0.09	0.206 *
Platelet count (x 10 <sup>9</sup> /L)	359.67±12.44	1393.33±98.34	728.67±140.52	1563.00±162.12	385.37 *

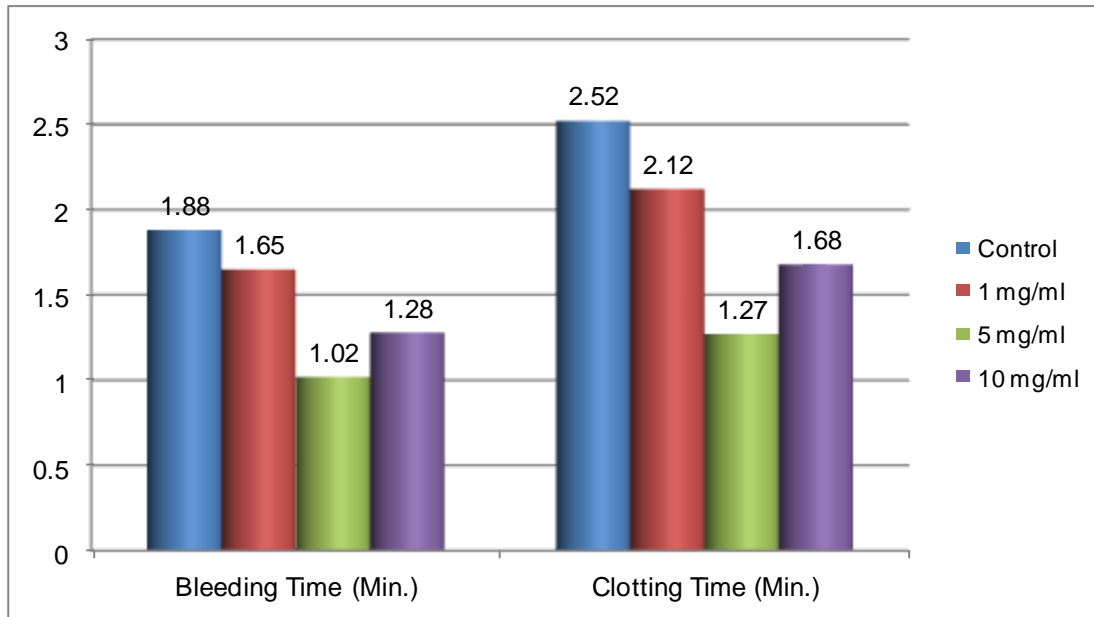
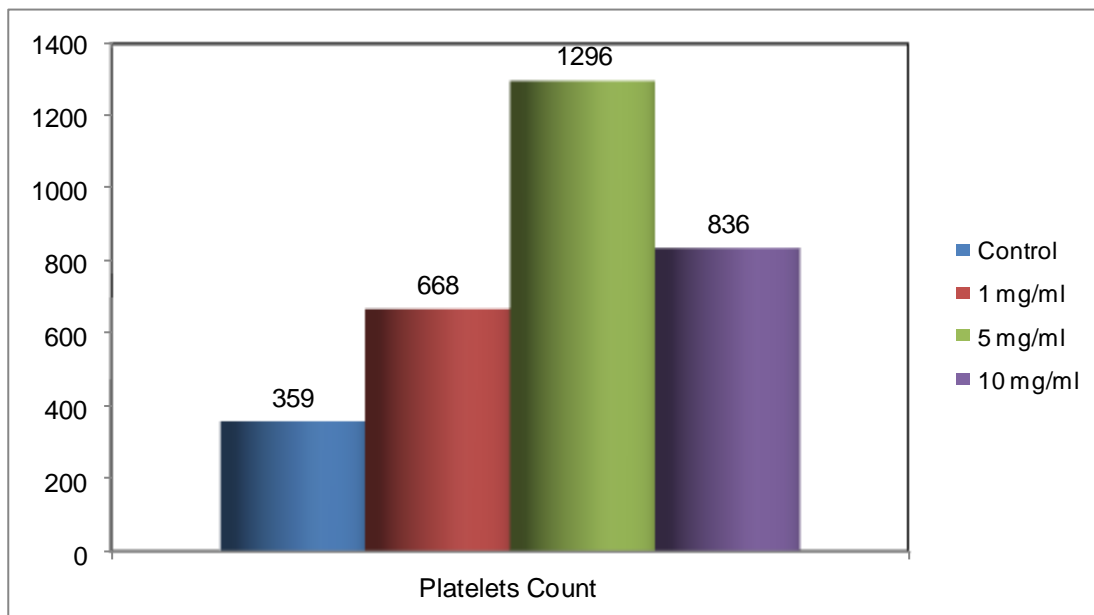
\* (P<0.05).

**Figure 4-** the effect of *Zingiber officinale* crude alkaloids extract of three concentrations on bleeding time and clotting time.**Figure 5-** the effect of *Zingiber officinale* crude alkaloids extract of three concentrations on platelets count.

**Table 5-** *Thymus vulgaris* crude alkaloids extract effect on the tested blood characters by using different concentrations

Blood Characters	Conc. of <i>Thymus vulgaris</i> crude alkaloid extract				LSD Value
	Control	1 mg/ ml	5 mg/ ml	10 mg/ ml	
Bleeding time(Min.)	1.88 ± 0.21	1.65 ± 0.17	1.02 ± 0.23	1.28 ± 0.01	0.586 *
Clotting time(Min.)	2.52 ± 0.04	2.12 ± 0.01	1.27 ± 0.04	1.68 ± 0.16	0.277 *
Platelet count (x10 <sup>9</sup> / L)	359.67±12.44	668.67±20.53	1296.67±55.23	836.33±125.79	227.42 *

\* (P<0.05).

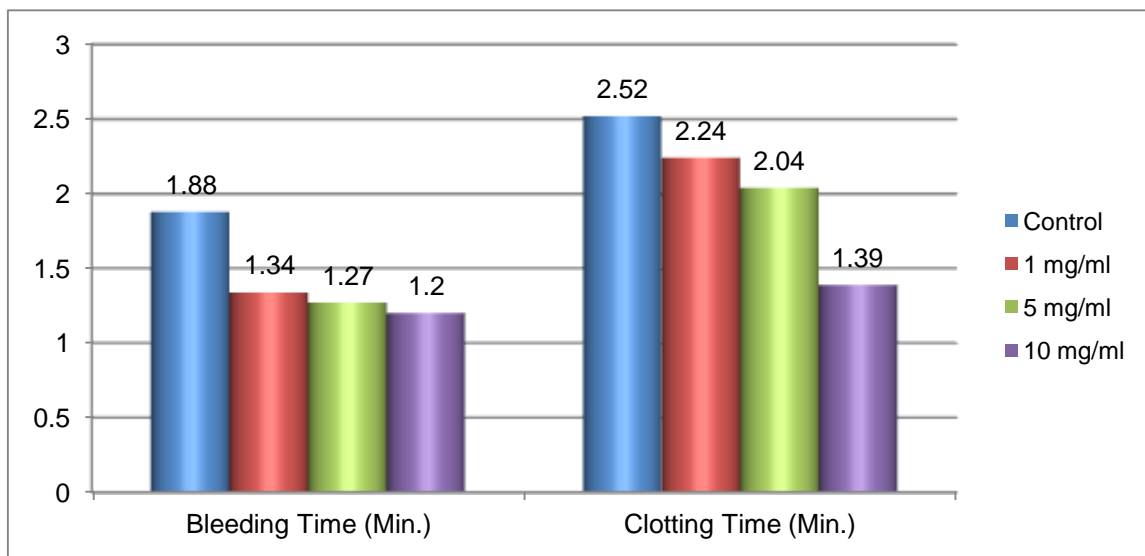
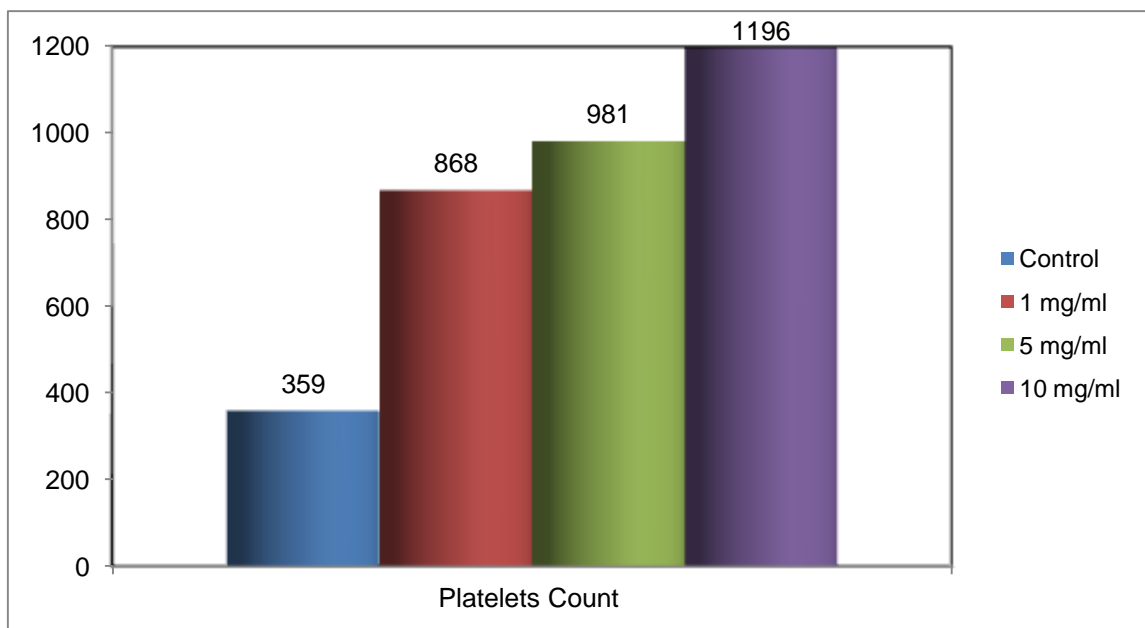
**Figure 6-** the effect of *Thymus vulgaris* crude alkaloids extract of three concentrations on bleeding time and clotting time.**Figure 7-** the effect of *Thymus vulgaris* crude alkaloids extract of three concentrations on platelets count.



**Table 6-** *Acacia arabica* crude alkaloids extract effect on the tested blood characters by using different concentrations

Blood Characters	Conc. of <i>Acacia arabica</i> crude alkaloid extract				LSD Value
	Control	1 mg/ ml	5 mg/ ml	10 mg/ ml	
Bleeding time (Min.)	1.88 ±0.21	1.34 ± 0.05	1.27 ± 0.07	1.20 ± 0.02	0.372 *
Clotting time (Min.)	2.52 ±0.04	2.24 ± 0.03	2.04 ± 0.02	1.39 ± 0.05	0.117 *
Platelet count ( $\times 10^9/L$ )	359.67±12.44	868.00±122.64	981.33±154.00	1196.33±222.72	485.13 *

\* ( $P < 0.05$ ).

**Figure 8-** the effect of *Acacia arabica* crude alkaloids extract of three concentrations on bleeding time and clotting time.**Figure 9-** the effect of *Acacia arabica* crude alkaloids extract of three concentrations on platelets count.

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