

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

AyyadWajeesh Raaof^{1,*}, Zahraa Abdul-Elah Al-Naqqash², Abdul-Latif M. Jawad¹, Salah M. Muhsan³

¹Department of Biology, College of Science, Baghdad University, Baghdad, Iraq

²College of Pharmacy, Al-Mustansyria University, Baghdad, Iraq

³Biotechnology research center, Al.Nahrain University, Baghdad, Iraq

*Corresponding author: ayyadalshahwany@yahoo.com

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Abstract As Alkaloids known for their pharmaceutical importance; this research included the extraction of crude alkaloids of three plants (*Zingiberofficinale* Roscoe, *Thymus vulgaris* L. and *Acaciaarabica* 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), platelet, red blood cells(RBC), packed cell volume(PCV), white blood cells (WBC) and hemoglobin counts(Hb) with Calcium count (Ca) in blood. The results revealed differences in the percentage of alkaloids in the plants under the study; *Zingiber* was the higher one followed by *Thymus* and *Acacia* respectively. *Zingiber* was also the most effective plant as coagulant factor than other two plants as it decreased both BT and CT and increased platelets, RBC, PCV, WBC and Ca count. more than what *T. vulgaris* and *A. arabica* affected on blood characters mentioned before.

Keywords: alkaloids, coagulant agent, bleeding time, clotting time, platelets, blood cells, packed cell volume, white blood cells, hemoglobin and calcium counts

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1. 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 spasmodiatic and bactericidal effects [3]. There are at least five different components involved: blood vessels, platelets, plasma coagulation factors, their inhibitors and fibrinolytic system (Lewis and Bain, 2006; Baklaja et al., 2008); disorders of this system are the leading immediate cause of mortality and morbidity in the modern society. The most prominent of them is thrombosis; the intravascular formation of clots that obstruct blood flow in the vessels (Davies, 2000).

During the last twenty years, the hemostasis system was a subject of intense interest in this field; reviews are available that describe these theoretical studies of blood coagulation and platelet-dependent hemostasis and

thrombosis (Ataullakhanov and Panteleev, 2005; Panteleev et al., 2007; Xu et al., 2011 and Xu et al., 2012).

Platelets are small fragments of cytoplasm derived from megakaryocytes. On average 1.5- 3.5µm in diameter but may be larger in some disease states. They do not contain a nucleus and are bounded by a typical lipid bilayer membrane. Beneath the outer membrane lies the marginal band of microtubules, which maintain the shape of the platelet and depolymerize when aggregation begins (Ruggeri, 1997; Nurden, 1999).

Calcium is the most common mineral in the body and one of the most important. The body needs it to build and fix bones and teeth, help blood clot, and help the heart to work. Almost all of the calcium in the body is stored in bone. So Aim of the study are detecting the presence of alkaloids in *Zingiber officinale* Roscoe, *Thymus vulgaris* L. and *Acacia arabica* L. by extracting them specifically, and determine the yield of alkaloids in each plants under the study. Also check coagulant properties of the extracts and which plant has higher coagulant effect.

2. Materials and Methods

2.1. Collection of Plant Samples

Plant samples included *Zingiberofficinale* Roscoe. Dryrhizomes (e.g. Figure 1), dry leaves of *Thymus vulgaris* L. (e.g. Figure 2) and dry gum of *Acacia arabica* L. (e.g. 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. *Zingiberofficinale* Roscoe dryrhizomes



Figure 2. *Thymus vulgaris* dry leaves



Figure 3. *Acacia arabica* dry gum

2.1.1. Preparation of Crude Alkaloids Extract

The extract was prepared according to the method of [4]. 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.

2.1.2. 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:

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

2.1.3. Alkaloid Indicators

2.1.3.1. Mayer reagent

This reagent [5] was used for the detection of alkaloids. The stock solution (1) was prepared by dissolving 13.5g HgCl_2 in 60 ml H_2O , stock solution (2) was prepared by dissolving 5g KI in 10 ml water, then combined with stock (1) and (2) and diluted with H_2O 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.

2.1.3.2. Tannic acid Reagent

This acid was used to precipitate alkaloids [6]. 1% tannic acid was prepared, and then 1-2 ml of reagent was added to 5ml of the extract. The white turbidity was appeared indicating the presence of alkaloids.

2.2. Hematological Test *in vivo*

In this study 30 male lab mice from Balb c. breed 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 [7].

2.2.1. 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 Table 3. Font Sizes for Papers. 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 [8,9].

2.2.2. 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 [8,9].

2.2.3. Blood Characters Count

Red blood cells (RBC) and white blood cells (WBC) were counted with haemocytometers. Packed cell volume (PCV) was determined using the microhaematocrit method. Haemoglobin (Hb) concentration was measured by the cyanmethaemoglobin method [10]. 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 [11].

2.3. Statistical Analysis

The Statistical Analysis System [12] 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.

3. Results and Discussion

The dry rhizomes of *Z. officinale* Roscoe., dry leaves of *T. vulgaris* L. and dry exudates (gum) of *A. arabica* L. were extracted for detection of alkaloids. The yield of extraction was determined in Table 1.

Table 1. 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 [13]. Results in Table 1 showed that alkaloids extract yielded form *Z. officinale* was higher than that obtained by [14] who mentioned that alkaloids percentage was (1.46%) while [7] 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 terpens and phenol extracts only without reporting the amount or percentage of alkaloids in the plant. [15] and [16] both mentioned the presence of alkaloids in leaves of *A. arabica* while [17] 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 [18]. The present study was carried out to determine the potentials of *Z. officinale* dry rhizomes, *T. 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 [19,20], 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 [21]. 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), platelets count, Red blood cells (RBC), White blood cells (WBC), Packed cell volume (PCV) and Ca concent tests showed variations compared with the control group as the extract decreases the bleeding time and clotting time while increases the platelets count, Red blood cells (RBC), White blood cells (WBC), Packed cell volume (PCV) and Ca concent at significant difference of (P<0.05). This agreed with [22] 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 [23].

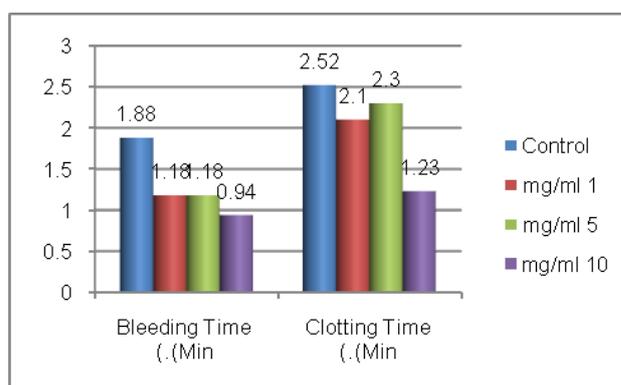


Figure 4. the effect of *Z. officinale* crude alkaloids extract of three concentrations on bleeding time and clotting time

In Table 2 and (e.g. Figure 4, Figure 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 [24] 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.

Table 2. *Z. officinale* crude alkaloids extract effect on the tested blood characters by using different concentrations

Blood Characters	Conc. of <i>Z. officinale</i> crude alkaloid extract				LSD Value
	Control	1 mg/ml	5 mg/ml	10 mg/ml	
Bleeding time (Min.)	1.88	1.18	1.18	0.94	0.472 *
Clotting time (Min.)	2.52	2.10	2.30	1.23	0.206 *
Platelet count (x * 10 ⁹ /L)	359.67	1393.3	728.67	1563.00	385.37 *
RBC (10 ⁶ µl ⁻¹)	6.00	5.18	6.34	8.13	0.542*
Hb (g dl ⁻¹)	9.28	9.78	9.38	12.76	0.776*
PCV %	28.9	28.6	27.2	37.4	2.241*
WBC (10 ³ µl ⁻¹)	3.83	3.84	3.96	5.52	0.61*
Ca ²⁺ (mg dl ⁻¹)	79.7	80.79	84.80	89.94	2.91*

* (P<0.05).

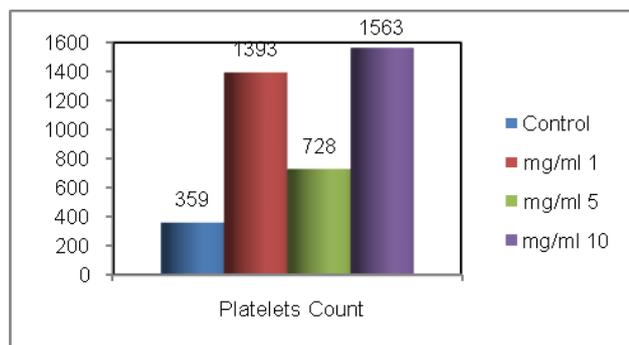


Figure 5. the effect of *Z. officinale* crude alkaloids extract of three concentrations on platelets count

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 [25]. The platelets count also significantly changed but adversely with BT and CT, the normal value (control) was (359) while the platelets count of the concentrations (1mg/ml; 5mg/ml; 10mg/ml) was (1393*10⁹/L) (728*10⁹/L) (1563*10⁹/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 [26]. In general for the three parameters (10 mg/ml) of crude alkaloid extract of *Z. officinale* was the most effective concentration.

Results in Table 3 and (e.g. Figure 6, Figure 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 [27] on the haemostatic activities of the leaf extract of *Aspiliaafricana* 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*10⁹/L) and (836*10⁹/L) respectively compared with control. However the platelets count of the concentration (5 mg/ml) was higher than the other concentrations which was (1296*10⁹/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.

Table 3. *T. 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	1.65	1.02	1.28	0.586 *
Clotting time (Min.)	2.52	2.12	1.27	1.68	0.277 *
Platelet count (x * 10 ⁹ /L)	359.67	668.67	1296.67	836.33	227.42 *
RBC (10 ⁶ µl ⁻¹)	6.00	7.22	7.58	7.89	0.542*
Hb (g dl ⁻¹)	9.28	11.20	12.12	11.20	0.776*
PCV %	28.9	32.42	35.08	32.42	2.241*
WBC (10 ³ µl ⁻¹)	3.83	4.20	4.28	4.30	0.61*
Ca ²⁺ (mg dl ⁻¹)	79.7	81.48	84.48	81.48	2.91*

* (P<0.05).

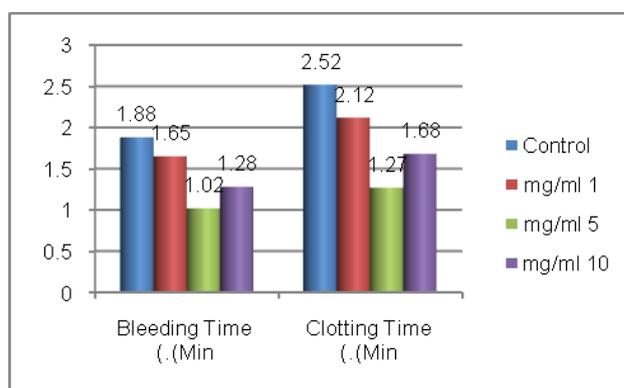


Figure 6. the effect of *T. vulgaris* crude alkaloids extract of three concentrations on bleeding time and clotting time.

T. 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 [28,29].

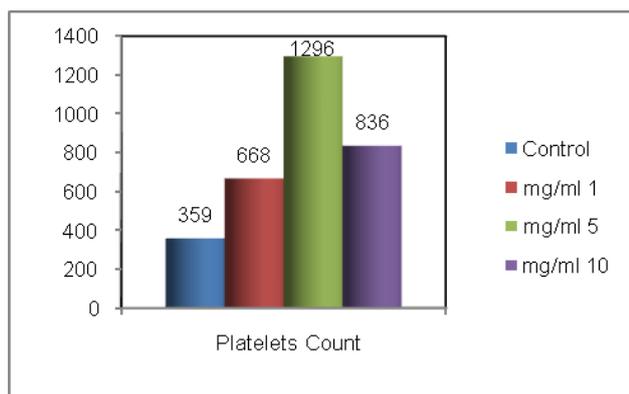


Figure 7. the effect of *T. vulgaris* crude alkaloids extract of three concentrations on platelets count

In Table 4 and (e.g. Figure 8, Figure 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 [30] while [31] showed marked effect of gum *A. 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 ($868 \times 10^9/L$), ($981 \times 10^9/L$) and ($1196 \times 10^9/L$) respectively. In general for the three parameters (10 mg/ml) of crude alkaloid extract of *A. arabica* was the most effective concentration.

Table 4. *A. 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	1.34	1.27	1.20	0.372 *
Clotting time (Min.)	2.52	2.24	2.04	1.39	0.117 *
Platelet count ($x \times 10^9/L$)	359.67	868.00	981.33	1196.33	485.13 *
RBC ($10^6/\mu l^{-1}$)	6.00	7.36	7.66	8.10	0.542*
Hb ($g dl^{-1}$)	9.28	11.52	12.02	12.78	0.776*
PCV %	28.9	34.28	35.28	37.48	2.241*
WBC ($10^3/\mu l^{-1}$)	3.83	4.62	4.63	5.46	0.61*
Ca ⁺² ($mg dl^{-1}$)	79.7	81.44	84.48	83.38	2.91*

* ($P < 0.05$).

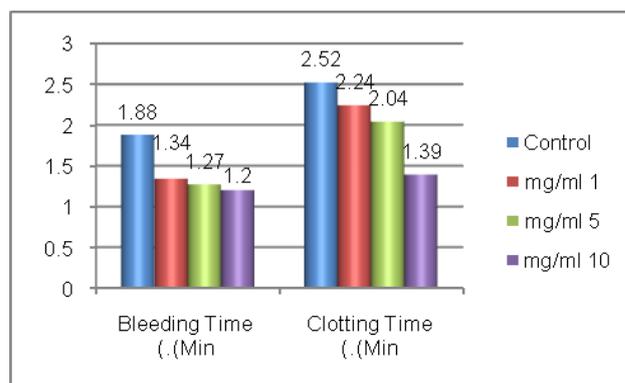


Figure 8. the effect of *A. arabica* crude alkaloids extract of three concentrations on bleeding time and clotting time

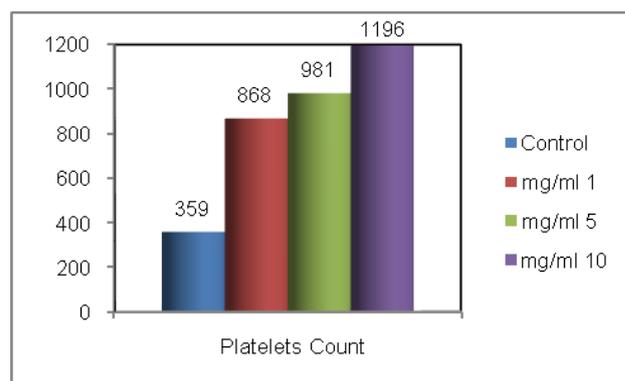


Figure 9. the effect of *A. arabica* crude alkaloids extract of three concentrations on platelets count

The results of the effects of crude alkaloid extract of *Z. officinale*, *T. vulgaris* and *A. arabica* on haematological indices of PCV, Hb, RBC, WBC and Ca count are shown in Table 2, Table 3, and Table 4) Consumption of the different concentration of the extracts caused significant increases in the PCV, Hb, RBC, WBC count, with Ca count in blood. showing the most significant responses to the Clotting time, that is, the RBC and Hb count, PCV, WBC and Ca increased by about 35.5, 37.5, 29.4, 43.75 and 12.84 % for *Z. officinale* crude alkaloids extract respectively, while for *T. vulgaris* the RBC and Hb count, PCV, WBC and Ca increased by about 31.5, 30.6, 21.3, 11.9 and 6.29 % respectively. Also for *A. Arabica* the blood indices increased by about 35, 37.7, 29.6, 42.18 and 5.99% respectively.

4. 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.

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