**Journal of Medicinal Plants and By-products (2024) 1: 107-112**
https://doi.org/10.22034/jmpb.2023.128871

## Original Article

# Evaluation of Coagulation Parameters in Mice Treated with *Terminalia bellirica* (Gaertn.) Roxb. Extract

**Ihktiyar Mohammad Safi[1,2], Jafar Vatandoost[1*], Fatemeh Sadeghifar[1*] and Toktam Hajjar[1]**

[1]*Department of Biology, Hakim Sabzevari University, Sabzevar, Iran*
[2]*Department of Biology, Sayed Jamaluddin Afghani University, Kunar, Afghanistan*

**Article History**

Received: 08 August 2022
Accepted: 05 February 2023


**Keywords**
Coagulation disorders
*Terminalia bellirica* (Gaertn.) Roxb.
Homeostasis
Herbal medicine
Hydroalcoholic extraction

***Corresponding author**
j.vatan@hsu.ac.ir

**ABSTRACT**

Coagulation disorders and bleeding are the main problems among people with hemophilia. Although its common treatment is replacement therapy, the effects of herbs on bleeding treatment has been proven. In this study, the coagulation effect of the *Terminalia bellirica* (Gaertn.) Roxb. hydro-alcoholic extract was evaluated. 32 mice were randomly divided into four groups (n = 8) and received single doses of 2000, 1500, 1000 or 500 mg/kg/day. Blood samples were taken from the animals 14-16 days after treatment, and coagulation indices were examined including PT, aPTT, BT, CT, and platelets. The hydro-alcoholic extract of *T. bellirica* significantly reduces bleeding time in the BT test (10-fold) and increases platelet numbers (four-fold), showing the effect of this extract on initial homeostasis. A significant reduction of coagulation time in the CT test (five-fold) also indicates the effect of this plant extract on platelet aggregation. Moreover, the results showed the effectiveness of *T. bellirica* hydroalcoholic extract in the extrinsic coagulation pathway because all concentrations reduced the coagulation time in the PT test. Furthermore, given the ineffectiveness of the *T. bellirica* extract on the aPTT test, this extract probably had no effect on intrinsic coagulation factors. Considering the results, *T. bellirica* extract seems to have a coagulation effect on primary homeostasis and extrinsic pathway of secondary homeostasis.

## INTRODUCTION

Homeostasis is a complex process where different components of the blood coagulation system are activated to respond to injured vessels and bleeding control [1]. The basic response of a hemostatic system to vascular damage is associated with the reaction between the blood vessel wall, circulating platelets, and coagulation system factors [2], all of which are performed in three steps. After vascular wall damage, primary homeostasis starts with vasoconstriction and platelet adhesion in a single layer on the endothelial fibrils. Then more platelet aggregation occurs to form a platelet block that prevents blood flow. The coagulation system activity which leads to the formation of fibrin filaments is called secondary homeostasis. These fibrin filaments are located between the platelets, and strengthen the platelet block. The development of fibrinolysis or third homeostasis calls for the activation of fibrin-bound plasminogen, which causes clot dissolved. This degradation is modulated by fibrinolysis inhibitors which are either activated by thrombin or released from platelets. Finally, these processes, together with active platelets and endothelial cell membranes, provide conditions for activation of coagulation factors and fibrin production or degradation [3].

Disruption of any of these three homeostasis stages prevents clot formation and bleeding. Although there are different treatment options such as gene therapy, replacement therapy and so on, the herbs have long been used in traditional medicine to treat bleeding disorders. Many plants have been reported to be effective in treating bleeding disorders because of their effective compounds including phenolic and flavonoid compounds [4]. *Terminalia bellirica* can also probably be an effective plant in the blood coagulation system because of its chemical components such as tannins, ellagic acid, gallic acid, anthraquinone, mannitol, glucose, and so on [5].

Considering the coagulant compounds in *T. bellirica* and the previous in vitro results on coagulation indices [6] which were contradicted with a cheminformatics' study [7], our aim was to identify the actual effect of hydroalcoholic extract of the *T. bellirica* in vivo.

## MATERIALS AND METHODS

### Extraction

The dried fruit of *T. bellirica* was powdered, mixed in 95% ethanol and placed on a shaker for 72 hours at 100 rpm after identifying, cleaning and removing its core. The extract was filtered and concentrated at 55°C with 64 rpm by rotary evaporation for 60 min. Then the extract was placed in a drying oven at 40°C for 12 hours to dry off the ethanol and water remaining. The dried extract was kept in 4 °C for further study.

### Animal Survey

Animal evaluations were performed in accordance with the Guidelines in the Care and Use of Animals and were approved by the Hakim Sabzevari University's Animal Ethics Committee (IR.HSU.REC.1399.023). Since dosages above 2000 mg/kg/day were not used in any report, a pre-test step was carried out for the 2000 dosage. To this end, a dosage of 2000 mg/kg hydroalcoholic extract was injected daily into 3 mice for 3 days. Given the absence of suspicious clinical and behavioral symptoms in mice, four groups (n = 8) were designed for dosages of 500, 1000, 1500 and 2000 mg/kg/day. Forty male NMRI mice (25-30 g, 6–8 weeks old) were purchased from the Animal Center, Royan Karaj, IRAN. The mice were housed under normal laboratory conditions (21 ± 2 °C, 12/12-h light/dark cycle) with free access to standard rodent chow and water. The animals were adapted for 2 weeks before the experiment. The control group (n=8) was gavaged with normal saline.

### Coagulation Tests

Various concentrations of *T. bellirica* extract were administered in mice for 16 days. On day 14, clotting time (CT), bleeding time (BT) and platelet count tests and on day 16, prothrombin time (PT) and activated partial thromboplastin time (APTT) tests were carried out.

### Bleeding Time (BT)

The bleeding time, or the time needed for a superficial wound to clot, is used to assess primary hemostasis. Bleeding time was measured based on IVY method [8] on the 14$^{th}$ day. Bleeding time was assessed by amputating 2 mm of the tail tip and issuing blood was carefully blotted every 15 second (Sec) using the rough side of a filter paper. When no further blood appeared on the filter paper, the number of bloodstains on the filter paper was counted, and bleeding time (Sec) was calculated by multiplying the total number of blood stains by 15. The normal range for a BT test is between 2 to 7 minutes.

### Prothrombin Time Test (PT)

The PT test examines the function of the extrinsic coagulation pathway and its normal range is 10-14 seconds. The mice were anesthetized using a Ketamine-Xylazine (KX) on day 16 and blood samples were taken from the heart of the mice. Blood samples were mixed with 3.2% sodium citrate (1 mL of citrate: 9 mL of blood) and plasma was separated at 2500 rpm centrifuge for 15 minutes. For the PT assay, 50 µl citrated plasma and 50 µl of warmed thromboplastin solution (Thermo Fisher) were mixed, incubated for 7 seconds at 37 °C and bleeding time (formation of the first white fibrin filaments) was recorded.

### Activated Partial Thromboplastin Time (aPTT)

APTT measures the coagulation time of plasma after activation of coagulation factors without the addition of tissue thromboplastin and thus shows the efficiency of the intrinsic blood coagulation pathway. The normal aPTT time is 30 to 45 seconds. For the aPPT assay, 50 µl of prewarmed aPTT reagent (Thermo Fisher) was mixed with 50 µl of citrated plasma and incubated for 3 min at 37 °C. The clotting time was recorded after adding 50 µl of prewarmed CaCl2 solution (1 mM) to the mixture.

#### Clotting Time (CT)

The CT test shows the ability of the intrinsic pathway to initiate clot formation and the common pathway of blood clotting. Lee and White method [9] was used to perform this experiment. On the 14$^{th}$ day, tail tip was punctured with a scalpel and a drop

of blood from the supraorbital vein was collected on a glass slide. The clotting time was recorded between blood collection and fibrin formation [10]. The normal time for CT test is 2 to 6 minutes.

### Platelet Counts Test

Platelet counts test that measures the number of platelets in blood is a useful aid in the assessment of primary hemostasis. Platelet count was performed manually. On the 14th day, each tail tip was punctured and a drop of blood was collected and smeared on a glass slide. Dried blood smear was incubated with methanol for 3 minutes and stained with Gimsa dye for 15 min. After washing and drying in room temperature, platelets were counted from 10 scope and their mean was recorded [11].

### Statistical Analysis

Analysis of Variance (ANOVA) was used to analyze the difference between the means of more than two groups followed by the Tukey multiple. Statistical significance was accepted for $P< 0.05$.

## RESULTS

### Bleeding Time (BT)

Although the most reduction was seen in 1500 dose group, there was a significant ($P< 0.05$) decrease in BT of all groups treated with *T. bellirica* extract compared with the control. The mean value of BT in control and treated groups with 500, 1000, 1500 and 2000 mg/kg/day, was 105±3, 42±2, 30±1, 10 ±0.6 and 15±.08 seconds, respectively (Fig. 1).

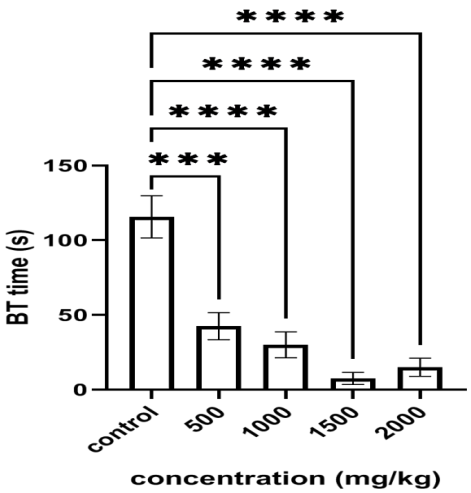


**Fig. 1** The effect of various dosage of *T. bellirica* hydroalcoholic extract on BT test. The data are the means ± SD of three individual experiments and significance of the data was shown with star.

### Activated Partial Thromboplastin Time (aPPT) Test

The results of aPPT test of treated mice with the *T. bellirica* hydroalcoholic extract indicate that although the aPPT was reduced in all four doses compared to the control, but the mean time in only doses of 500 mg/kg/day significantly differ from the control ($P< 0.05$) (Fig. 2). The mean aPPT time in control mice was 16.6±0.5 seconds, while it was 14±0.3, 15±0.4, 15±0.3, and 15±0.5 seconds in 500, 1000, 1500, and 2000 mg/kg/day dosage, respectively.

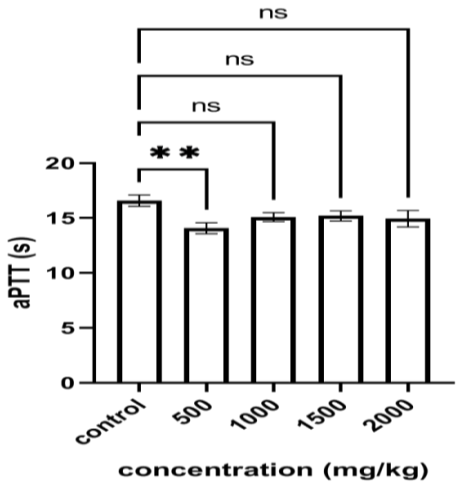


**Fig. 2** The effect of various doses of *T. bellirica* hydroalcoholic extract on aPPT test. The data are the means ± SD of three individual experiments and significance of the data was shown with star.

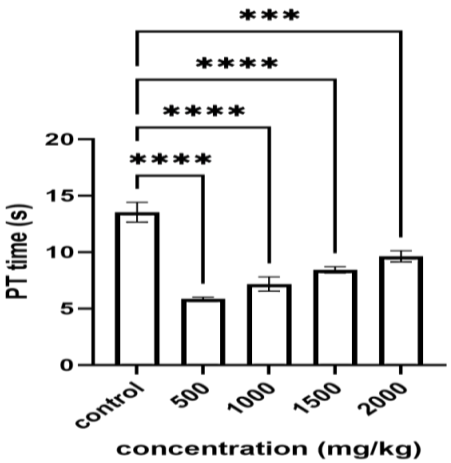


**Fig. 3** The effect of various doses of *T. bellirica* hydroalcoholic extract on PT test. The data are the means ± SD of three individual experiments and significance of the data was shown with star.

### Prothrombin Time (PT) Test

Although prolonged PT was observed in a dose-dependent manner from 6, 7, 8, and 9 seconds, in 500, 1000, 1500, and 2000 mg/kg/day dosage,

respectively (Fig. 3), the findings indicate that the PT in all four groups was significantly less than control ($P< 0.05$).

## Clotting Time (CT) Test

Although the most reduction was seen in 2000 dosage group, there was a significant ($P<0.05$) decrease in CT of the 1000, 1500 and 2000 mg/kg/day compared with the control except 500 dosage group (Fig. 4). The mean value of CT in control and treated groups with 500, 1000, 1500 and 2000 mg/kg/day, were 140±3, 112±3, 53±1, 68±2 and 27±0.5 seconds, respectively.

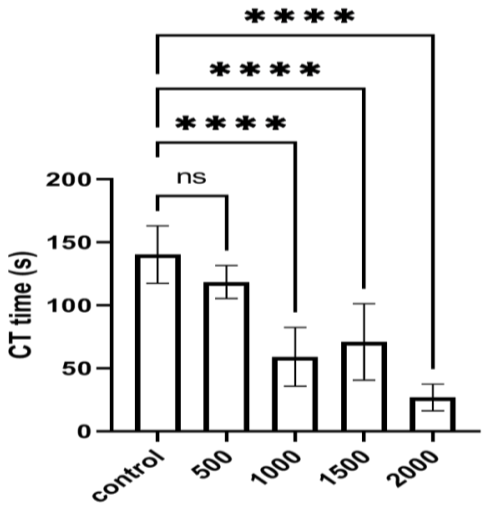


**Fig. 4** The effect of various doses of *T. bellirica* hydroalcoholic extract on CT test. The data are the means ± SD of three individual experiments and significance of the data was shown with star.

## Platelet Count Test

The results of platelet count test indicate that the platelet-count has increased in all four doses in a dose-dependent manner (Fig. 5). The mean platelet count in control mice was 6, while the mean at doses of 500, 1000, 1500 and 2000 were 9, 16, 19 and 23, respectively.

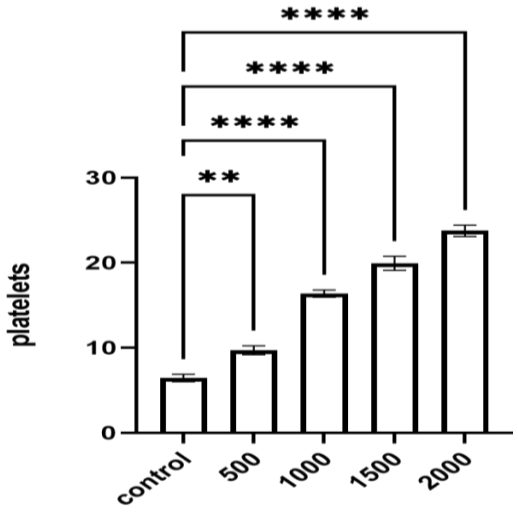


**Fig. 5** The effect of various doses of *T. bellirica* hydroalcoholic extract on platelet count. The data are the means ± SD of three individual experiments and significance of the data was shown with star.

## Analysis of Extract Compounds

Qualitative determination of the different biologically active compounds from *T. bellirica* hydroalcoholic extract using GC/MS technique revealed the presence of 14 different compounds, in which Pyrogallol with 83.5%, was the main ingredient of *T. bellirica* (Table1).

**Table 1** Some GC – MS identified phytochemical components of *T. bellirica* hydroalcoholic extract

| No | RT (min) | Area % | Name | Quality | CAS Number |
|---|---|---|---|---|---|
| 1 | 17.222 | 83.50 | Pyrogallol | 95 | 000087-66-1 |
| 2 | 44.073 | 3.00 | Cholest-5-en-3-ol, 23-ethyl-, (3b,23S) | 99 | 113845-28-6 |
| 3 | 16.433 | 2.90 | 5-Ethoxy-4-phenyl-2-isopropylpheno | 83 | 000000-00-0 |
| 4 | 10.539 | 2.29 | 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl | 49 | 028564-83-2 |
| 5 | 14.026 | 1.87 | 5-Hydroxymethylfurfural | 90 | 000067-47-0 |
| 6 | 26.655 | 1.61 | Palmitic acid | 99 | 000057-10-3 |
| 7 | 26.987 | 1.47 | Ethyl palmitate | 99 | 000628-97-7 |
| 8 | 29.576 | 0.71 | Ethyl Oleate | 99 | 000111-62-6 |
| 9 | 29.296 | 0.70 | 9-Octadecenoic acid, (E) | 95 | 000112-79-8 |
| 10 | 34.199 | 0.45 | 3,3'-Dimethylbiphenyl | 42 | 000612-75-9 |
| 11 | 41.422 | 0.43 | D alpha.-Tocopherol | 95 | 000000-00-0 |
| 12 | 45.24 | 0.42 | 2-Ethylacridine | 53 | 000000-00-0 |
| 13 | 38.589 | 0.31 | 3-(3-Bromophenyl)-7-chloro-10-hydroxy-3,4-dihydro-1,9(2H,10H)-acridinedione | 83 | 000000-00-0 |
| 14 | 29.451 | 0.30 | Ethyl linolenate | 99 | 000544-35-4 |

## DISCUSSION

Hemophilia is a sex-dependent bleeding disorders with more prevalent among men. Although research and development of drugs for this disease is costly, finding an effective herbal medicine can greatly reduce the costs of research and treatment and provide more access to medicine for patients. Nowadays, herbs are used to treat some diseases and one of those plants is *T. bellirica* that introduced as a blood coagulant in various traditional medicine books. Moreover, the effect of *T. bellirica* in progressive wounds, chronic wounds and wound healing have been reported [12]. Considering previous *in vitro* effective results on coagulation indices [6], in this study effect of hydroalcoholic extract of the *T. bellirica* on coagulation in mice was examined.

There were no signs of side effects generally following injection of *T. bellirica* extract. No significant changes of blood pressure, liver and mice renal function were observed. The hydroalcoholic extract of *T. bellirica* significantly reduced bleeding time in the BT test up to 10-fold. Since this test shows the number and function of platelet inversely, reduction in BT shows the effect of this extract on increasing platelet number and therefore effect on primary hemostasis. The results of platelet number confirm BT result as the treatment of hydroalcoholic extract leads increasing the number of platelets up to four-fold. It was shown that the alkaloids, flavonoids, and tannins [13] in *T. bellirica* can reduce BT. Moreover, carbohydrates, phenolic, flavonoid, and alkaloid compounds are effective in increasing platelet number [14].

In addition to the effect on platelet number, a significant reduction in coagulation time in CT test (five-fold) shows the effect of *T. bellirica* extract on platelet accumulation. Hence, it confirms the effect of this plant on the primary hemostasis of blood coagulation. Tannins [13], phenolic, flavonoid [15] and alkaloid [16] compounds are effective in reducing CT by increasing platelet aggregation. It seems that the hydro-alcoholic extract of *T. bellirica* in the concentration of 2000 is the optimal concentration for affecting on blood coagulation process through primary homeostasis.

Our findings show the effect of hydroalcoholic extract of *T. bellirica* on the external coagulation pathway as all concentrations reduced the coagulation time in the PT test. Tannin [13] and flavonoid [15] compounds in the extract can be a possible reason for reducing PT time. Moreover, because of the ineffectiveness of *T. bellirica* extract on aPTT test, the extract probably has no effect on intrinsic coagulation factors. The slight decrease in bleeding time in aPTT is probably due to the low levels of Ethyl palmitate in the extract, which have been shown to be effective in reducing the levels of factors VIII and IX in the intrinsic pathway [17]. It seems that phenolic compounds such as pyrogallol with about 83% are among the effective compounds in blood coagulation in *T. bellirica*.

Although the number of examined samples was limited, based on the results regarding *T. bellirica* extract and the significant effect in reducing the coagulation indices of this species, it seems that the extract of the plant has a coagulation effect in primary homeostasis and external secondary homeostasis.

## CONCLUSION

Plant extracts contain a variety of compounds whose function is due to one or more specific compounds, some of which have coagulant and some anticoagulant effects. On the other hand, those that have coagulation effects can affect one or more pathways or show different effects in each pathway. This confirms the effects of the extract on the external pathway of blood coagulation and primary homeostasis.

### Conflict of Interest

The authors declare that there is no conflict of interest.

## ACKNOWLEDGMENT

The authors are grateful for the financial support provided by Hakim Sabzevari University.

## REFERENCES

1. McPherson R., Pincus M. Clinical diagnosis and management by laboratory methods. Elsevier Health Sciences; 2021.
2. Hoffbrand V., Steensma D.P. Hoffbrand's Essential Haematology. 2019.
3. David G., Ludlam C. Fast facts: bleeding disorders. 2004.
4. Fazeli-nasab B., Moshtaghi N., Forouzandeh M. Effect of solvent extraction on phenol, flavonoids and antioxidant activity of some Iranian native herbs.

scientific journal of ilam university of medical sciences. 2019;27:14-26.
5. Hazra K. Phytochemical investigation of Terminalia bellirica fruit inside. Asian Journal of Pharmaceutical and Clinical Research. 2019;12:191-4.
6. Mazinani J. The effect of some plant extracts on the blood coagulation system: Hakim Sabzevari University; 2020.
7. Alikhani Pour M., Sardari S., Eslamifar A., Azhar A., Rezvani M., Nazari M. Cheminformatics-based anticoagulant study of traditionally used medicinal plants. Iranian Biomedical Journal. 2017;21:400.
8. Dejana E., Callioni A., Quintana A., de Gaetano G. Bleeding time in laboratory animals. II-A comparison of different assay conditions in rats. Thrombosis Res. 1979;15:191-7.
9. Lee R.I., White P.D. A clinical study of the coagulation time of blood. The American Journal of the Medical Sciences. 2013;145:495.
10. Huang L., Lin C., Li A., Wei B., Teng J., Li L. Pro-coagulant activity of phenolic acids isolated from Blumea riparia. Natural product communications. 2010;5:19-24.
11. Brahimi M., Osmani S., Arabi A., Enta-Soltan B., Taghezout Z., Elkahili B.S., *et al*. The estimation of platelet count from a blood smear on the basis of the red cell: platelet ratio. Turkish J. Hematology. 2009;26.
12. Singh M.P., Gupta A., Sisodia S.S. Wound healing activity of *Terminalia bellerica* Roxb. and gallic acid in experimentally induced diabetic animals. J. Complementary and Integrative Medicine. 2020;17.
13. Fauzi A., Lamma S., Ruslin M. Total tannin levels analysis of brown algae (sargassum sp. and padina sp.) to prevent blood loss in surgery. J Dentomaxillofacial Sci. 2018;3:37-40.
14. Huang J., Zhang J., Xu Z-w. Effect of astragalus polysaccharide on the proliferation of bone marrow mesenchymal stem cells and the expression of stem cell factor in vitro. Fudan University J. Medical Sci. 2011;38:343-8.
15. Pignatelli P., De Biase L., Lenti L., Tocci G., Brunelli A., Cangemi R., *et al*. Tumor necrosis factor-α as trigger of platelet activation in patients with heart failure. Blood. 2005;106:1992-4.
16. Doss A., Parivuguna V., Vijayasanthi M., Surendran S. Antibacterial evaluation and phytochemical analysis of Medicago sativa L. against some microbial pathogens. Indian J. Sci. Techno. 2011;4:550-2.
17. Noha S., El-Demerdash E., Abdel-Rahman H.M., Algandaby M.M., Al-Abbasi F.A., Abdel-Naim A.B. Anti-inflammatory activity of methyl palmitate and ethyl palmitate in different experimental rat models. Toxicology and Applied Pharmacology. 2012;264:84-93.