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In vivo evaluation of *Polygonum bistorta* extract effect on blood coagulation factors

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ABSTRACT

Polygonum bistorta contains phytochemical compounds that can affect coagulation. It is used as hemorrhoid for bleeding control in traditional medicine. For its in vivo evaluation, 35 mice were randomly divided into 5 groups (n = 7) as well as the negative and positive control. On the 13th day of treatment, blood samples were collected for bleeding time (BT), clotting time (CT) and the number of platelets and on the 14th day, for prothrombin time (PT), activated partial thromboplastin time (aPTT). The results were shown although *P. bistorta* extract had a significant reducing effect on all coagulation tests in a dose-dependent manner, the greatest reduction in coagulation time (16.5 times) in the CT test and the best significant dosage is 1000 mg/kg/day shows that the *P. bistorta* extract has a greater effect on the common pathway than intrinsic and extrinsic pathways.

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1. Introduction

The hemostatic system includes a complicated network of enzymes, platelets, coagulation and anticoagulation factors (Borissoff et al., 2011) that are categorized as an either primary or secondary processes (Donat et al., 2020). During primary hemostasis upon vessel injury, the interaction of blood platelets, collagen, and adhesive plasma proteins including von Willbrand (VWF), leads to platelet activation and platelet aggregation to the damaged site (Favaloro and Lippi, 2019; Donat et al., 2020). In secondary hemostasis, cascading activation of zymogen coagulation factors in extrinsic and intrinsic pathways leads to the activation of fibrin from fibrinogen, formation of fibrin network and blood coagulation (Rodrigues et al., 2019). Normal hemostasis depends on a well-regulated system of procoagulant and and anticoagulant pathways, and the instability of these processes causes a lack of hemostatic control, with the possibility of excessive bleeding or thrombosis (Negrier et al., 2019).

Although there are different treatment methods, the herbs have long been used in traditional medicine to treat bleeding disorders. According to the WHO, up to 80% of the world's population still uses herbal medicines to treat their diseases (Ekor, 2014; Zizka et al., 2015; Kasole et al., 2019). Many herbs have been reported to be effective in treating of bleeding disorders because of their effective compounds including phenolic compounds (Fazeli-Nasab et al., 2019). *Polygonum bistorta* is from Polygonacea family and is a rich source of secondary metabolites such as phenolic compounds (Jamshidi-Kia et al., 2018) that can be used in therapeutic disease. *P. bistrorta* has been utilized as a treatment for a variety of conditions in traditional Indian, Chinese, Japanese, Pakistan, Singapore, African, European and America medicine (Intisar et al., 2013; Karuppiah Pillai, 2021). *P. bistorta*

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is a very great hemostyptic, so it was traditionally used in bleeding disorders including menorrhagia, bleeding hemorrhoids, hematuria, epistaxis, hemoptysis and dysentery (Mehar and Tabarak, 2013).
Due to some coagulant compounds in this plant, traditional use in bleeding reduction and previous *in vitro* results, the aim was to investigate the effects of hydroalcoholic extract of the roots of *P. bistorta* on the coagulation pathwaies in mice.

2. Experimental

2.1. Extraction

Root of *P. bistorta* was provided from spicery in Sabzevar, Iran. The collected plant was authenticated by an expert botanist and the voucher specimen was kept in the herbarium of Hakim Sabzevari University (HSUH) with HSUH0210 number. The roots were washed under running water, shade dried, powdered and mixed with 70% ethanol (180 g with 470 mL) and placed on a shaker for 48 hours at 1000 rpm. The extract was filtered and concentrated at 55 °C by rotary evaporation. The yield of the extract was then placed in a drying oven at 40 °C to drive off the ethanol and water excess. The dried extract (28 g) was kept at 4 °C and used for further study.

2.2. Experimental design and animal grouping

Forty-nine 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 prior to the experiment. Based on statistical analysis with G-POWER software, instruction of Hakim Sabzevari University's animal ethics committee and lethal dose of the *P. bistorta* (LD50: 1000 mg/kg) (Khushtar et al., 2018b), five groups (n = 7) were designed for dosage of 150, 200, 300, 500 and 1000 mg/kg/day of *P. bistorta* extract. Negative and positive control groups (n = 7) were administered orally with 0.3 mL distilled water and 1200 mg/kg/day tranexamic acid, respectively. The present research was performed in accordance with the Guidelines in the Care and Use of Animals and was approved by the Hakim Sabzevari University's Animal Ethics Committee (IR.HSU.REC.1399.001).

2.3. Measurement of total phenolic content (TPC) and total flavonoid content (TFC)

The TPC of the plant extract was measured using a spectrophotometric method with some modifications (Stankovic, 2011). The reaction mixture was prepared by mixing 0.5 mL of methanolic solution of extract (1 mg/mL), 0.5 mL of 10% Folin-Ciocalteu's reagent in water, and 2 mL of $NaHCO_3$ (10%). Blank was also concomitantly prepared. The samples were incubated in a dark space for 2h at room temperature. The absorbance was determined using spectrophotometer at 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was drawn. Based on the measured absorbance, the TPC was calculated using a calibration curve. The TPC was expressed in terms of gallic acid equivalent (mg of GAE/g of dry extract) (Stankovic, 2011).
The TFC of *P. bistorta* extract was also determined using the aluminum chloride method (Stankovic, 2011) with some modification. The sample contained 1 mL of methanol solution of the extract (1 mg/mL) and 1 mL of $AlCl_3$ (2%) solution dissolved in methanol. The samples were incubated for 30 min at room temperature. The absorbance was determined using spectrophotometer at 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and the calibration curve was drawn. Based on the measured absorbance, the TFC was calculated using rutin calibration curve. The TFC of *P. bistorta* extract was expressed in terms of rutin equivalent (mg of RuE/g of dry extract) (Hosseinpoor Mohsen Abadi et al., 2016).

2.4. GC-MS analysis

To identify individual components, a hexane solution (50:50) of *P. bistorta* extract was subjected to analysis on an Agilent GC-MS system (Agilent GC 6890A equipped with an Agilent 5973 mass detector) using ZB-5ms capillary column (30.0 m × 0.25 mm i.d.; 0.25 µm film thickness; from Zebron). The employed oven temperature programming was as follows. Accordingly, its initial temperature was adjusted at 50 °C for 5 minutes, then raised to 150 °C by a ramp of 5 °C/ minute. The oven was set at this temperature for 10 minutes. Finally, the open temperature again raised to the final temperature of 260 °C using a ramp of 5 °C/ minute and held at this pressure for 20 minutes. The injector temperature was 260 °C. Helium was used as a carrier gas with a flow rate of 1.0 mL/ minute. Samples were injected at splitless mode. The either adjusted operational parameters for the mass detector there as follows. The ionization voltage: 70 eV, ion source temperature: 200 °C over a mass range of 50-500 amu. The peak area was determined using MSD ChemStation from Agilent Technology. A library search was carried out for all the peaks using the NIST Mass Spectral Library software (2005). The homologous saturated hydrocarbon standards (C_8-C_{20} and C_{21}-C_{40}) were analyzed using the same column and conditions to calculate the retention indices (RI) of compounds (Mahdavi et al., 2018; Mahdavi et al., 2021). The detection of compounds was based on a comparison of the measured retention indices and mass spectral patterns with those available in the literature. All the peaks with a match quality of ≥ 90% were considered and their names were specified.

2.5. Coagulation tests

Various concentrations of *P. bistorta* extract were administered in mice for 14 days. Following anesthetizing of mice using a Ketamine (100 mg/kg) and Xylazine (12.5 mg/kg) (Alfasan, The Netherland)

on the 13th day, clotting time (CT), bleeding time (BT) and platelet count tests were carried out. On the 14th day, blood samples are also taken from heart of the anesthetized mice, were collected into 3.2% sodium citrate (1 mL of citrate: 9 mL of blood) and centrifuged at 2500 rpm for 15 minutes. Prothrombin time (PT) and activated partial thromboplastin time (APTT) tests were carried out according to the manufacturer's instructions (Thermo Fisher).

2.5.1. 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 Dejana method with some modification (Dejana et al., 1979) on the 13th day. Bleeding time was assessed by amputating 2 mm of the tail tip and issuing blood was carefully blotted every 15 s 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 (s) 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.

2.5.2. Clotting time (CT)

The CT test shows the ability of the intrinsic pathway to initiate clot formation and the common pathway of blood clotting (Klotoé et al., 2012). Lee and White method (Lee and White, 1913) was used to perform this experiment. On the 13th day, the 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 (Huang et al., 2010). The normal time for CT test is 2 to 6 minutes.

2.5.3. Platelet counts test

A 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 13th 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 scopes and their mean was recorded (Brahimi et al., 2009).

2.5.4. 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 14 and blood samples are 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, 100 µL citrated plasma and 100 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.

2.5.5. 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, 100 µL of prewarmed aPTT reagent (Thermo Fisher) was mixed with 100 µL of citrated plasma and incubated for 3 min at 37 °C. The clotting time was recorded after adding 100 µL of prewarmed $CaCl_2$ solution (1 mM) to the mixture.

2.6. Statistical analysis

Graphpad Prism Software (version 9) was used to measure the analysis data. The significance between the two groups was evaluated using an analysis of variance (One-Way ANOVA) followed by the Shapiro-Wilk test. All results are presented as the means ± SEM by the Tukey test and are statistically significant at a p value < 0.05.

3. Result and Discussion

3.1. Analysis of extract compounds

To verify the presence of phenol and flavonoid in the *P. bistorta* extract, total phenol (TPC) and flavonoid (TFC) in extract was tested. TPC of the extract was 187 mg GAE/g of sample in dry weight (mg/g) and TFC of the extract was also 25 mg RuE/g of sample in dry weight (mg/g).

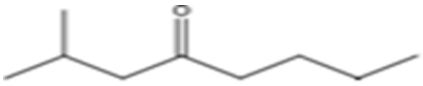
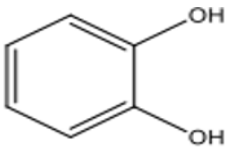
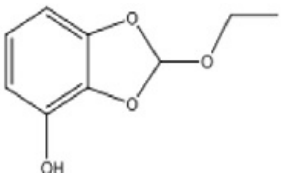
Qualitative determination of the different biologically active compounds from *P. bistorta* extract using GC/MS technique revealed the presence of three main compounds: 2-Ethoxy-1,3-benzodioxol-4-ol, pyrocatechol and 2-methtyl-4-octanone having 48, 40 and 12%, respectively (Table 1).

3.2. Coagulation tests

The BT result indicates a non-significant prolonged BT in 150, 200 and 300 mg/kg doses, although the *P. bistorta* hydroalcoholic extract-treated groups with 500 and 1000 mg/kg doses was significantly reduced BT up to 6.5-7.5-fold less than that of negative control, respectively (p < 0.05) (Table 2) (Fig 1A). The same results were obtained for CT and in a dose-dependent manner, clotting time inversely reduce with dose increase (Fig 1B). Interestingly dosage of 1000 mg/kg was significantly reduce CT about 16.5 fold less than of negative control and 4 times less than of dosage of 500 mg/kg (Table 2).

Analysis of PT data shows that prothrombin time in the treated groups with *P. bistorta* extract has reduced in a dose-dependent manner from 16.8 to 10.9 s (Table 2). In comparison with the control (13.6), administration with 150 and 1000 mg/kg dose result in a significant increase and decrease, respectively (p < 0.05) (Fig 1C). The aPTT results was same of the PT and in a dose-dependent manner, coagulation time was decrease

Table 1
Gas Chromatography mass spectroscopy (GC-MS) analysis of the hydroalcoholic extract of *P. bistorta*. [a]

No	Name	RT (min)	Structure
1	2-Methtyl-4-octanone	11.95	
2	Pyrocatechol	13.92	
3	2-Ethoxy-1,3-benzodioxol-4-ol	19.42	

[a]RT: Retention time

from 43.8 to 13.9 s for 150 to 1000 mg/kg (Table 2). In comparison with the control (17.5), a 2.5-fold significant prolonged aPPT for a dosage of 150 and a 0.8-fold significant decrease for a dosage of 1000 mg/kg was observed ($p < 0.05$) (Fig 1D). On the contrary of BT, CT, PT and aPTT, the number of platelets is reduced with an increase in concentration. Although in comparison with control, platelet numbers in 150 and 200 mg/kg treatment groups were significantly increase up to 4.6 and 3.7 fold ($p < 0.05$) respectively, platelet numbers in 500 and 1000 mg/kg treatment groups were decrease to half and even more ($p < 0.05$) (Table 2).

3.3. Interpretation of the results

Many plants have been reported to be effective in treating bleeding disorders because of their effective compounds including phenolic and flavonoid compounds (Fazeli-Nasab et al., 2019). The *P. bistorta* can also probably be an effective plant in the blood coagulation system because of its chemical components such as tannins, ellagic acid, gallic acid, and so on (Hazra, 2019). Considering the compounds in the *P. bistorta* and previous *in vitro* effective results on coagulation indices (Mazinani, 2020), an animal study was performed to inquire effect of its hydroalcoholic extract in mice. Results of PT, aPTT, CT, and, BT showed that the coagulation time reduces by increasing the *P. bistorta* extract concentration. The highest coagulation time was in 150 mg/kg dose, and the lowest was seen in 1000 mg/kg dose.

Phenol and flavonoid compounds in the *P. bistorta* extract are possible factors in the coagulation time decrease. In line with other (Intisar et al., 2013; Khushtar et al., 2018a), presence of phenol and flavonoids in the *P. bistorta* extract was verified by TPC and TFC measurement as well as GC-MS. These compounds can effect on intrinsic and extrinsic pathways and result in decrease of coagulation time in aPTT and PT test, respectively. Tannins that have a phenolic ring in their structures and are among the polyphenolic compounds, when mixed with blood, immediately form a clot due to the coagulation of albumin (Partovi and Zabihi, 2014). Tannins as phenol compounds can be hydrolysed to gallic acid, catechol and ellagic acid (Kögel-Knabner and Amelung, 2014). Ellagic acid can activate the coagulation factor XII in the beginning of the intrinsic pathway (Lu et al., 1994) and result in decrease of coagulation time in aPTT. Moreover, flavonoids and tannins can increase migration and accumulation of platelets to the damaged area and result in bleeding stop (Partovi and Zabihi, 2014). Since the CT test is depending on platelet aggregation, decrease of clotting time in CT indicate effect of flavonoids and tannins. Highest decrease in coagulation time (16.5-fold) in the CT test indicate that the *P. bistorta* extract has a greater effect on the common pathways then intrinsic and extrinsic.

The results showed that although coagulation time was decreased in a dose-dependent manner, the best significant dosage is 1000 mg/kg. This is consistent with previous reports that have shown the effect of tannic acid as a tannin derivative is dose-dependent (Deng et al., 2019).

Contrary to other tests, the number of platelets decreased with increasing doses. It seems that gallic acid inhibits ATP production by inhibition of protein kinase C. Since the presence of ATP is necessary for the production of platelets (Chang et al., 2012), gallic acid indirectly reduces the number of platelets.

4. Concluding remarks

The present experiment shows that of the *P. bistorta*

Table 2
Distractive statistics *P. bistrorta* hydroalcoholic extract in the mice. The data are the means ±SD of three individual experiments.

Dose (mg/kg)	BT (s)	CT (s)	PT (s)	aPTT (s)	PLT (number)
150	236.3 ± 83.6	162.4 ± 23.4	16.8 ± 0.3	43.8 ± 3.5	33.3 ± 4.4
200	230.6 ± 95.2	124.6 ± 19.6	14.9 ± 0.4	32 ± 1.1	24.3 ± 3.6
300	227.5 ± 117.1	104.8 ± 24.7	13.9 ± 0.3	27.5 ± 1.1	10.2 ± 1.6
500	18 ± 1.1	26.4 ± 3.5	12.4 ± 0.6	14.2 ± 0.3	3.6 ± 0.5
1000	15 ± 8.2	7.5 ± 0.2	10.9 ± 0.3	13.9 ± 0.2	1.8 ± 0.2
Control -	115.7 ± 14.4	122.8 ± 11.7	13.6 ± 0.4	17.5 ± 0.7	7.2 ± 0.9
Control +	45 ± 27.6	54 ± 9.6	-	-	36.7 ± 5.5

BT: Bleeding time; CT: Clotting time; PT: Prothrombin time; aPTT: Active partial thromboplastin time; PLT: Platelet count.

hydroalcoholic extract is a broad-spectrum medicinal product that has different effects on primary and secondary homeostasis. According to our result, although treatment with low dosage of *P. bistorta* extract seems to be effective in the primary homeostasis process (platelate decrease), high dosage have positive effects on the secondary hemostasis especially common pathway (highest decrease,16.5-fold, in the CT test). Therefore considering the results, high and low dosage of the *P. bistorta* extract are recommended for bleeding disorders and thrombocytopenia, respectively. However, further study is needed for toxicity evaluation and discovery the exact mechanism of action of the active component.

Conflict of interest

The authors declare that there is no conflict of interest.

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Ethical considerations

The protocols were approved by the Hakim Sabzevari University's Animal Ethics Committee (IR.HSU.REC.1399.001).

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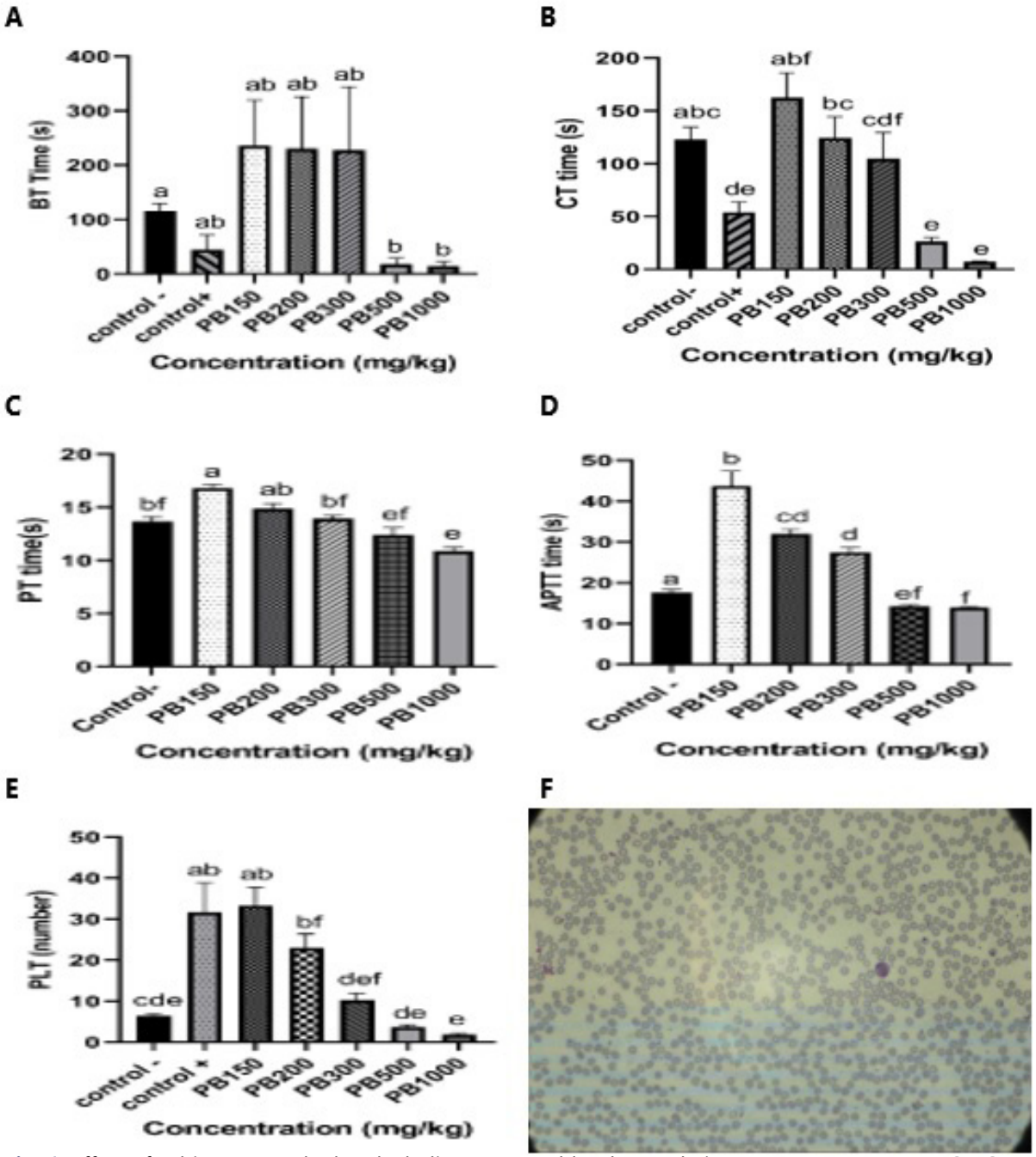

Fig. 1. Effect of P. bistorta (PB) hydro alcoholic extract on blood coagulation parameters. **A**) BT, **B**) CT, **C**) PT, **D**) aPTT and **E**) PLT. **F**) Microscopy picture of platelets.

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