Evaluations of Hemostatic Activity of Ethanol Extract of *Euonymus fortunei* Aerial Parts

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Authors' contributions

This work was carried out in collaboration among all authors. Author XLO designed the proposal and protocol, performed the experiments and wrote the first draft of manuscript. Authors LYS and XYQ performed the experiments. Author LY managed the experiments and revised the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

**Aim:** To investigate the hemostatic effect of ethanol extracts of *Euonymus fortunei* and their different polar fractions, and to analyze the changes in intracellular calcium ion concentration in platelet cells.

**Methodology:** Bleeding time and clotting time was assessed by the slide method and the tail-breaking method, respectively. Four coagulation indexes, including prothrombin time, activated partial thromboplastin time, thrombin time, and the content of fibrinogen, as well as platelet aggregation effect were also determined. The concentration of calcium ions in platelet cells treated by n-butanol fraction with good active hemostatic activity was also examined.

**Results:** Compared with the control group, the n-butanol fraction of *E. fortunei* can not only significantly shorten the bleeding time and coagulation time, but also obviously shorten activated partial thromboplastin time. However, no significant difference was found on the content of fibrinogen. Platelet aggregation experiments show that the n-butanol fraction can increase the
The increase in cytoplasmic Ca\(^{2+}\) concentration caused by agonists will lead to GPCRs' activation, thereby promoting or inhibiting platelet aggregation [11]. Activating GPCR in platelets may be an important way for the prevention and treatment of hemostasis during the process of hemostasis [12]. Therefore, measuring intracellular Ca\(^{2+}\) signals may be used for initially screening agonists targeting GPCRs. To evaluate the hemostatic effect of *E. fortunei*, we measure the bleeding time (BT), coagulation time (CT), blood coagulation function and platelet aggregation effect of different fractions, and then monitor the concentration of intracellular Ca\(^{2+}\) of platelets treated by the active fraction to elucidate the possible hemostasis mechanism of *E. fortunei*.

### 2. MATERIALS AND METHODS

#### 2.1 Apparatus

Four coagulation indexes were performed using a C2000-4 coagulometer (Beijing Precil Instrument Co. Ltd, China). Platelet aggregation experiments were carried out using LBY-NJ4 platelet aggregation analyzer (Beijing Precil Instrument Co. Ltd, China). The calcium fluorescence was measured by an Eclipse TE2000 optical inverted microscope (Nikon, Japan).

#### 2.2 Reagent

PT, APTT, TT, and Fib determination kits were obtained from Shanghai Sunbiotech Biotechnology Co., Ltd. (Shanghai, China).
Adenosine diphosphate (ADP) was acquired from Ark Pharm. Inc., USA. Vacuum anticoagulation tube (2 mL, containing 0.109 mol·L⁻¹ sodium citrate antibody Coagulant, BD Co., USA) was used to store venous blood samples. Fluo-3/AM, phosphate-buffered saline (PBS), trypan blue, and Tyrode stain were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

*E. fortunei* was collected from Jinxiu Yao Autonomous County of Guangxi Zhuang Autonomous Region, China in August 2013. The samples were identified as the aerial part of *E. fortunei* (Turcz.) Hand.-Mazz by Professor Shaoqiang Tang, the School of Life Sciences of Guangxi Normal University, Guilin, China.

### 2.3 Experimental Animals

Kunming mice (20.2 ± 1.3g) and SD rats (220–250 g) were purchased from the Experimental Animal Center of Youjiang Medical University for Nationalities. The experiments were carried out in accordance with internationally accepted guidelines on laboratory animal use and the protocols were approved by the Experimental Animal Ethics Committee of Youjiang Medical University for Nationalities of Guangxi, China (Approval No. 02/2017).

### 2.4 Preparation and Extraction of Samples

*E. fortunei* were air-dried and cut into 1 to 2 cm-thick slices and extracted with 95% ethanol. The extracts were spin-dried under reduced pressure to obtain the residue. The crude (i.e. total fraction, TF) was dissolved as much as possible in the water and successively extracted with petroleum ether, ethyl acetate, and n-butanol, and then spin-dried to remove the solvent under reduced pressure to obtain petroleum ether fraction (PEF), ethyl acetate fraction (EAF), n-butanol fraction (NBF), and water fraction (WF). All samples were stored at 4°C before testing.

### 2.5 Measurement of BT and CT

BT and CT were assessed by the method in reference [13]. Seventy mice were randomly divided into seven groups (i.e. control group, positive group, and five sample groups). Each group contained ten mice was given a dose of 1.5 g·kg⁻¹ once a day for 7 days. The tails were cut off 0.5 cm from the tip on the 7th day 2 h after oral administration. The blood is sucked out with filter paper every 15 s. The bleeding time was recorded until no blood was founded on the filter paper.

Meanwhile, a glass capillary tube with an inner diameter of 1 mm and a length of 15 cm was inserted into the inner canthus of the left eye of the mouse. The glass capillary tube was taken out and placed flat on the table until filled with the blood from the venous plexus. The ends of capillary tube were broken every 30 s about 0.3 cm and slowly pulled left and right to observe whether there are clotting filaments at the break. The clotting time was recorded from the blood flows into the capillary until the clotting filaments appear.

### 2.6 Measurement of Four Coagulation Indexes

Four coagulation indexes (APTT, PT, TT, and Fib) were performed according to the methods described in previous studies [14]. Seventy mice were randomly divided into 7 groups, and were irrigated with 0.9% physiological saline solution (control group), 2 mg·mL⁻¹ Yunnan Baiyao (positive group), the total fraction (TF) and four different polarity fractions (PEF, EAF, NBF, and WF) of *E. fortunei* (both 10 mg·mL⁻¹). All mice were oral administered daily by gavage for seven consecutive days per day. Two hours after the last oral gavage, blood samples were collected into 2 mL vacuum blood-collection tubes from the orbit, and then centrifuged at 3000 rpm for 15 min to obtain plasma. The PT, APTT, TT, and the content of Fib were determined according to the instructions of the coagulometer manufacturer and test kits. Each sample was measured three times.

### 2.7 Measurement of Platelet Aggregation

Platelet aggregation assay were assessed refer to the literature method with minor modifications [15]. SD rats were anesthetized with 1% pentobarbital. Blood samples collected from the abdominal aorta were mixed with sodium citrate (1:9), and centrifuged at 1000 r·min⁻¹ for 15 min at room temperature to prepare platelet-rich plasma (PRP). Followed by centrifuged at 3000 r·min⁻¹ for 15 min, platelet-poor plasma (PPP) was obtained. 200 μL of PPP was added to the colorimetric tube (preliminarily put a pellet of the stirring rod), and placed the measuring tube in the constant temperature preheating hole.
for preheating. PPP was used as the blank and adjust the reference point to "0". Then 200 μL of PRP and 10 μL of drug solution were added to the tube and incubated at 37°C for 5 min. 11 μL 0.04 mmol·L⁻¹ ADP were added with a 25 μL microinjector, and immediately recorded the maximum platelet aggregation rate in 5 min. 200 μL PRP + 10 μL aspirin were used as the positive control group, and 200 μL PRP + 10 μL normal saline were used as the control group. All groups were determined three times in parallel.

2.8 Statistical Analyses

Results are expressed as mean±SD. Statistical analyses were performed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Unpaired or two-tailed paired t-tests were used to evaluate the significance of differences between two groups. Statistical significance was determined at $P < 0.05$.

3. RESULTS

3.1 Effects of Different Fractions on Bleeding Time and Clotting Time

The results of $E. fortunei$ extracts on BT and CT were shown in Table 1. When the mice were treated with alcohol extract and four fractions with a dose of 1.5 g·kg⁻¹, BT and CT were shortened compared with the control group. CTs were significantly shortened by all samples in comparison to the control group, and the difference was statistically significant ($P<0.05$). Compared with the positive group, the difference between EAF and NBF is statistically significant ($P<0.01$). At the same time, compared with the control group, TF and NBF can shorten the BT, and the BT of the NBF is the shortest (90.9 ± 22.5 s). Therefore, the results indicate that the $n$-butanol fraction exhibited the best coagulation effect, and may be the active fraction for hemostasis.

3.2 Effects of Different Fractions on Coagulation Indexes

To explore the possible mechanism of hemostasis of $E. fortunei$ extract, four coagulation indexes of total extract and fractions were further determined and analyzed. As shown in Table 2, the extracts and different fractions can shorten APTT to different extents, but no significant effect was founded on the content of Fib. Among them, NBF significantly shortens APTT and reduces the content of Fib. The positive group on the content of Fib increased significantly compared with the control group, while no significant differences were found on TT, PT, and APTT. The result indicated that the coagulation of Yunnan Baiyao, a well-known hemostatic Chinese patent medicine in China, may be related to the increase in the content of Fib. The experimental results suggested that the hemostatic activity of $E. fortunei$ may enhance the ability of plasma fibrinogen to transform into fibrin, increase the endogenous pathway coagulation factors, thereby increase the coagulation activity, but no significant difference was observed on the content of fibrin.

3.3 Effect of $n$-Butanol Fraction on Platelet Aggregation

In vitro platelet aggregation assay was measured to evaluate the effect of NBF on platelet aggregation (Fig. 1). NBF showed weaker anti-platelet activity compared with the positive group, whereas NBF exhibited stronger anti-platelet aggregation effect compared with the control group with the maximum aggregate on rate in 5 min at 68±10%. The result indicates that NBF has important chemical compositions to inhibit platelet aggregation.
Table 1. Comparison of different fractions from *E. fortunei* on bleeding time and coagulation time

| Group | Coagulation time /s | Bleeding time /s |
|-------|--------------------|------------------|
| Control group | 97.8 ± 21.5 | 130.2 ± 33.8 |
| Positive group | 80.2 ± 21.3 | 98.7 ± 15.7 |
| TF | 85.0 ± 21.2 | 101.3 ± 22.0 |
| PEF | 75.1 ± 17.0 | 116.4 ± 26.7 |
| EAF | 64.5 ± 25.9 | 128.0 ± 21.8 |
| NBF | 73.1 ± 18.3 | 90.9 ± 22.5 |
| WF | 80.8 ± 22.5 | 86.4 ± 14.6 |

\*P<0.05, \**P<0.01, compared with the control group. The abbreviations used are: TF, total fraction; PEF, petroleum ether fraction; EAF, ethyl acetate fraction; NBF, n-butanol fraction; and WF, water fraction.

Table 2. Effects of different extracts from *E. fortunei* on the mice plasma coagulation parameters

| Group    | PT/s     | APTT/s   | TT/s     | Fib/g·L⁻¹ |
|----------|----------|----------|----------|-----------|
| Control group | 8.16 ± 0.46 | 26.83 ± 3.47 | 16.52 ± 1.39 | 3.07 ± 0.35 |
| Positive group | 8.71 ± 0.40 | 26.03 ± 2.61 | 16.59 ± 1.35 | 3.92 ± 0.77 |
| TF | 8.46 ± 0.48 | 23.87 ± 3.16 | 15.49 ± 1.54 | 3.04 ± 0.61 |
| PEF | 9.22 ± 0.62 | 24.19 ± 2.87 | 15.99 ± 1.79 | 3.24 ± 0.96 |
| EAF | 9.02 ± 0.44 | 23.74 ± 2.98 | 15.55 ± 1.48 | 3.00 ± 0.63 |
| NBF | 9.10 ± 0.26 | 22.54 ± 1.79 | 15.98 ± 0.51 | 2.94 ± 0.31 |
| WF | 9.03 ± 0.57 | 21.98 ± 3.24 | 15.75 ± 1.85 | 3.29 ± 0.80 |

\*P<0.05, \**P<0.01, compared with the control group. The abbreviations used are: PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; Fib, fibrinogen.

Fig. 1. Effect of *n*-butanol fraction (NBF) on the maximum platelet aggregation rate in 5 min

Fig. 2. Effect of *n*-butanol fraction on Ca²⁺ fluorescence intensity in platelets
3.4 Effect of n-Butanol on the Intracellular 
Ca\(^{2+}\) in Platelets

Fig. 2 showed the release of intracellular Ca\(^{2+}\) in platelet cells treated by NBF incubated for 24 h. Compared with control group, the platelet cells treated by NBF showed a stronger green fluorescence. With the increase of NBF concentration, the green fluorescence intensity in the cell gradually increased, indicating that the intracellular Ca\(^{2+}\) release amount increased significantly. Quantitative analysis showed that NBF with concentration at 20 and 40 μg·mL\(^{-1}\) acted on platelet cells for 24 h, the relative fluorescence intensities were 121±13 and 225±8, respectively. Obviously, treatment of platelet cells with NBF caused intracellular Ca\(^{2+}\) concentration elevation in a concentration-dependent manner.

4. DISCUSSION

In this study, the effective hemostatic fractions of E. fortunei were screened by measuring the bleeding time (BT), coagulation time (CT), blood coagulation indexes, and platelet aggregation effect. Tail vein bleeding and capillary tube method in mice are presently the most commonly used bleeding and clotting time for assessment [17,18]. The values of BT and CT are influenced by different reasons. For example, BT can be affected by the function of capillary, shrinkage ability of tissue, count and function of tissue factor and platelet, fibrinolytic system function, while CT is mainly influenced by activities of various coagulation factors and anticoagulation factors [19]. The results showed that ethanol extract of E. fortunei has an excellent hemostatic effect. Meanwhile, the n-butanol fraction exhibits stronger hemostatic activity than the total fraction, indicating that the n-butanol fraction may be the best hemostatic part, and its hemostatic effect may be related to platelet aggregation and clotting factors. According to our previous research, flavonoids such as kaempferol-3,7-dirhamnopyranoside and kaempferol-3-(4″-O-acetyl)-O-α-L-rhamnopyranoside-7-O-α-L-rhamnopyranoside, are the main components in the n-butanol fraction of E. fortunei [8, 20]. In addition to usually regarded as polyphenolic compounds with antioxidant activity, kaempferol-3,7-dirhamnopyranoside can also have a hypoglycemic effect [21], and may also be used for cardiovascular treat cardiovascular disorders, exerts endothelium-dependent and independent vasorelaxation in thoracic aorta of normotensive and hypertensive rats [22]. So far as we know, it isn’t reports on the hemostatic effect of these compounds. Flavonoids may be responsible for hemostasis, so it is worth for us to further explore.

The fluorescence intensities of Ca\(^{2+}\) in platelet cells suggested that extracts of E. fortunei can trigger changes in calcium ion concentration. The calcium flux in platelets is considered to be an important second messenger for platelet activation. High concentrations of Ca\(^{2+}\) activate trigger fibrinogen binding to integrin αIIbβ3 and finally make fibers protein is converted to fibrin. Due to all agonists including ADP, thrombin, and thromboxane A2 stimulate platelet aggregation by corresponding GPCRs, which are P2Y1 (activated by ADP), PAR1 (protease-activated receptor-1, activated by thrombin), PAR4 (activated by thrombin), and TP (activated by thromboxane A2). Our results also confirmed that the compositions of n-butanol fraction as agonists induced the hemostatic effect by activated GPCR, which causes the increase of intracellular Ca\(^{2+}\) concentration and the activation of the downstream Ca\(^{2+}\) signal transduction pathway [23]. Although we have not conducted a detailed investigation of which receptor or multiple receptors are involved in calcium signaling, however, the involvement of P2Y12 and IP receptors was excluded in this study [10].

5. CONCLUSION

In conclusion, the results of this study show that the n-butanol fraction of E. fortunei can significantly shorten bleeding time and coagulation time caused by trauma in mice, and shorten activated partial thromboplastin, which may be an effective fraction of E. fortunei for hemostasis. Moreover, the n-butanol fraction can induce the increase of calcium ion concentration in platelets and further activate platelets. E. fortunei could be a potential medicinal plant used for hemostatic treatment.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All
experiments were carried out under the approval of the Experimental Animal Ethics Committee of Youjiang Medical University for Nationalities (Approval No. 02/2017).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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