INTERACTION OF POLYPHENOLS EXTRACT FROM POLYGONUM MULTIFLORUM THUNB. ROOTS WITH GELATIN AND TOXICITY OF EXTRACT IN MICE

Le Pham Tan Quoc

ABSTRACT
The roots of Polygonum multiflorum Thunb. (Vietnamese name: Ha-thu-o-do, HTOD) are used in processed form or the raw state in traditional Vietnamese medicine for many diseases and in extract form in the food industry. Some studies pointed out that HTOD extract had toxicity in humans. However, the toxicity of this herb plant currently remains unclear. In addition, this material contained a large amount of bioactive compounds, especially phenolic compounds. They have a strong antioxidant capacity and they can also interact with many different substrates such as protein, enzyme, lipid and carbohydrate. In this study, the received extracts from HTOD had the polyphenols concentrations of 415, 277, 208 and 166 (mg GAE.L⁻¹), respectively. Besides, we only evaluated the gelatin-polyphenols interaction and the toxicity of HTOD extract in Swiss mice. The results show a strong gelatin-polyphenols interaction and no acute or subacute toxicity in mice. The polyphenols extract of HTOD at the concentration tested in this study is safe to use in food.

Keywords: Interaction; gelatin; polyphenols; root; toxicity

INTRODUCTION
Polygonum multiflorum Thunb. is a wild herbal plant distributed throughout the mountainous regions of North Vietnam (Cao Bang, Lang Son, Lai Chau, Hoa Binh province, etc.). It is known as Ha-thu-o-do (HTOD) in Vietnamese. In addition, HTOD is found in many other Asian countries such as China, Korea, Japan, etc. Ethnomedical uses of HTOD have been recorded for many centuries, and it contains more than 100 chemical bioactive compounds, for example, tannins, anthraquinones, stilbenes, flavonoids, phospholipids (Lin et al., 2015), saponins, and alkaloids (Quoc and Muoi, 2018). Thus, it can be used as a traditional spice in Chinese food (Li and Gao, 2015) or drugs to prevent some diseases, such as certain forms of cancer (Way et al., 2014), and for its anti-aging effects, tonic tension (Lim et al., 2014), and antioxidant activity (Wang et al., 2008).

Nowadays, polyphenols extract from HTOD is used in food processing, for example, it serves as an antioxidant during the storage of minced red tilapia (Le and Nguyen, 2018) or combine with edible film (alginate) to store fresh-cut papaya (Quoc and Muoi, 2016). Moreover, HTOD has also been used in wine processing (Hoang and Thuat, 2015).

There are many methods to extract phenolic compounds from HTOD with various solvents (water, acetone, methanol, ethanol, etc.), such as the decoction method (Li et al., 2007), microwave-assisted extraction (Quoc and Muoi, 2015), ultrasound-assisted extraction (Wu et al., 2012), pectinase-assisted extraction (Quoc and Muoi, 2017), etc. The total polyphenols content, antioxidant capacity, and type of phenolic compounds in all methods are significantly different. Thus, these results can strongly affect the protein-polyphenols interaction and toxicity of polyphenols extract in mice.

Recently, many studies reported that HTOD can have hepatotoxic effects (Huang, Zhang and Sun, 2011; Wu et al., 2012); other studies noticed that HTOD is good for the liver (Huang et al., 2007; Bhadauria, 2010). The results of the above-described studies appear to be contradictory. Until now, there have been no studies on the interaction of gelatin with polyphenols extract from HTOD. Therefore, the main aim of this research was to investigate the gelatin-polyphenols interaction and toxicity of polyphenols extract in mice.

Scientific hypothesis
The objective of this study was to determine the capacity for interaction between polyphenols extracts of Polygonum multiflorum Thunb. roots and protein (gelatin). This interaction results in precipitation of the protein. An additional objective of the study was to determine the effect of the toxicity of various polyphenols extract...
concentrations in mice. We are expecting an insignificant effect of the extract on acute and subacute toxicity in mice.

MATERIAL AND METHODOLOGY

Extract preparation

*Polygonum multiflorum* Thunb. roots were harvested from Cao Bang province (Vietnam). The roots were then cleaned with tap water, sliced, and dried at 60 °C until the moisture level was less than 12%. The slices were then ground into a fine powder (diameter less than 0.5 mm) and vacuum-packed. Polyphenols from the dried powder of *Polygonum multiflorum* Thunb. roots were extracted in a microwave system with an acetone concentration of 57.33%, solid/solvent ratio of 1/39.98 (w/v), extraction time of 289 sec, and microwave power of 127 W. The crude extract was filtered through Whatman paper (Quoc and Muoi, 2015). The filtered extract was evaporated at 45 °C until the solvent was completely removed and the extract was used for the preparation of 415, 277, 208 and 166 mg GAE.L⁻¹ solution in distilled water.

Chemicals and reagents

Folin-Ciocalteu reagent and gallic acid were purchased from Merck (Germany). All organic solvents and other chemicals were of analytical reagent grade.

Determination of total polyphenols content (TPC)

The TPC in the extracts was slightly modified and determined by the Folin-Ciocalteu colorimetric method (Siddiqua et al., 2010). The results were based on a standard curve obtained with gallic acid. TPC was expressed as kg of gallic acid equivalents per gram of dry weight (mg GAE.g⁻¹ DW) or per gram of solution volume (mg GAE.L⁻¹).

Interaction of polyphenols extract with gelatin

Reactions took place in 10 mL volumetric flasks. Polyphenols were dissolved in water to 415, 277, 208 and 166 (mg GAE.L⁻¹). Gelatin solutions in water, at concentrations varying from 30 to 120 mg.ml⁻¹ were prepared. Polyphenols and gelatin solutions were mixed quantitatively in flasks with shaking. After standing for 24 h at 25 °C, the mixture was centrifuged (3000 rpm, 20 min), and the suspended substances (reaction products) were removed. The supernatant was analyzed at 280 nm in a UV spectrophotometer. Assuming that a polyphenols solution with a fixed concentration has an initial absorbance (A₀), and after interaction with gelatin the absorbance decreases to A, RA can be defined as RA = (A₀−A)/A₀ (Bi, He and Haslam, 1995).

Animals

Male and female Swiss mice (approximately 22 g) were obtained from the Pasteur Institute (Ho Chi Minh city, Vietnam). All mice were maintained in plastic cages under standard environmental conditions at 28 ±2 °C with a relative humidity of 75 ±10%. The mice were fed on a standard chow diet and given water ad libitum. The mice were used for experimentation after 7 days’ acclimatization. All experiments were performed during the daytime. The experimental procedure was strictly in compliance with the “Declaration of Helsinki” in 1964.

Acute toxicity

Both male and female healthy mice were fasted overnight and only allowed to access to water ad libitum. They were randomly divided into five groups (10 animals per group). The mice of the first group (control group) were fed with water only. All groups were given 0.2 mL of the extract on the first day by oral gavage. The mice of groups 2 – 5 were treated with acetone extracts of *Polygonum multiflorum* Thunb. root at doses of 138, 276, 414, and 552 mg dry extract.kg⁻¹ of body weight per day. The dosages were equivalent to 25, 50, 75, and 100 times the upper dosage for humans recommended in the study of Le and Nguyen (2018) (415 mg GAE.L⁻¹, approximately 607 mg dry extract.L⁻¹ or 5.52 mg dry extract.kg⁻¹ of body weight). The dosage was set at a high level to uncover any potential toxicity in order to investigate the hepatic risk. The general behavior, hazardous symptoms, and mortality of the mice were monitored for a period of 3 days after treatment. The LD₅₀, clinical biochemistry analysis, gross morphology, and histology of the liver were also evaluated in this test.

Subacute toxicity

Both male and female healthy mice were also randomly divided into four groups (10 animals per group). The mice of the first two groups (control groups) were fed with water only. The mice of groups 3 and 4 were treated with the acetone extracts of *Polygonum multiflorum* Thunb. root for 3 and 6 weeks. Treated groups were given 0.2 mL extract at a concentration of 415 mg GAE.L⁻¹ (approximately 607 mg dry extract.L⁻¹ or 5.52 mg dry extract.kg⁻¹ of body weight, the dosage for humans recommended in the study of Le and Nguyen (2018)). The body weight of the mice was recorded weekly, and signs of abnormalities in the mice were recorded during the treatment period. The clinical biochemistry, gross morphology, and histology of the liver/kidney were also evaluated every 3 weeks.

Histopathologic examination, biochemical analysis, and hematological parameters

The mice were dissected to collect the livers/kidneys for histopathologic examination. Biochemical analysis was performed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), Urea/BUN (Blood Urea Nitrogen), and creatinine. In addition, a total leukocyte count and total hemocyte count were also performed.

Statistical analysis

The experimental data were analyzed by one-way analysis of variance (ANOVA) and significant differences between the means from triplicate analyses at p <0.05 were determined by Fisher’s least significant difference (LSD) procedure using Statgraphics software (Centurion XV). The values obtained were expressed as mean ± standard deviation (SD).
RESULTS AND DISCUSSION

Research on the gelatin-polyphenols interaction

Polyphenols concentrations of 415, 277, 208, and 166 (mg GAE/L) react with gelatin concentrations of 30, 60, 90 and 120 mg/L (gelatin/polyphenols ratio=1/1, v/v). The results show that relative absorbance (RA) increases with an increase in the polyphenols concentration, and these results were significantly different ($p<0.05$).

The minimum and maximum RA are 0.246 and 0.718, meaning that the interaction between gelatin and polyphenols is strong and significantly affects the RA. This is concordant with a study by He, Lv and Yao (2007), who noted that polyphenols in tea extract interact with gelatin at a low gelatin concentration of 20 mg/L and a polyphenols concentration of 50 mg/L (22% of the gelatin was precipitated). The precipitate increased to 84% with an increase in the gelatin concentration (160 mg/L) and the polyphenols concentration (150 mg/L).

Gelatin was chosen in this study because gelatin is proline-rich, and has an open, random-coil conformation and a molecular weight of 100 kDa, thus it has a high affinity for polyphenols (He, Lv and Yao, 2007; Frazier et al., 2010). Phenolic compounds can form strong hydrogen bonds easily with the protein’s carboxyl group. They must be small enough to penetrate the inter-fibrillar regions of protein molecules but large enough to crosslink peptide chains at many points on the protein molecule (Mulaudzi et al., 2012). The protein-polyphenols interaction depends on many factors, such as pH, temperature, the type of protein, and the structure of polyphenols (Ozdal, Capanoglu and Altay, 2013).

This interaction has both advantages and disadvantages. On the one hand, it can protect the polyphenols’s activity, and prevent oxidation of the surrounding environment (Jakobek, 2015). On the other hand, it decreases protein quality (Yuksel, Avci and Erdem, 2010), for example, by affecting protein solubility (Rawel et al., 2002) and by decreasing the in vitro digestion properties of proteins (Petzke et al., 2005).

Acute toxicity of polyphenols extract

This extract was concentrated and was administered to each treatment group at single doses of 25, 50, 75, and 100 times the upper dosage for humans as recommended by Le and Nguyen (2018) (approximately 138, 276, 414, and 552 mg dry extract·kg$^{-1}$ of body weight), respectively, by oral gavage. The control groups were treated with the same volume of distilled water (0.2 mL).

After a one-hour exposure to the extract, drowsiness and exhaustion were observed in all mice in the extract-treated group. No death or obvious clinical signs were found in any groups throughout the study. None of the extract-treated rats showed signs of toxicity in their skin, fur, eyes, sleep, salivation, diarrhea, and behavior after 72 hours.

Gross morphology and histology of the liver did not show any unusual signs (Figure 2 and Figure 3). There was no difference in parenchymal tissue, portal space and central vein structures in all experimental liver sections. Hepatocytes in all experimental groups had a polygonal shape with the nucleus in the middle of the cell and were well organized in plates. The structure of the portal space was normal without inflammation, and there were no degenerative lesions in the surrounding hepatocytes. Hence, polyphenols extract from Polygonum multiflorum Thunb. root did not cause acute toxicity in mice, and the LD$_{50}$ could not be estimated at the studied concentrations.

Subacute toxicity of polyphenols extract

Using a polyphenols concentration of 415 mg GAE.L$^{-1}$ (5.52 mg dry extract·kg$^{-1}$ of body weight), we evaluated semi-chronic toxicity over 6 weeks. After 6 weeks, all groups gained the same amount of weight, and no statistically significant differences in clinical biochemistry analysis and hematological parameters were noted between the control and treated groups at the third and sixth weeks ($p<0.05$) (Table 2). Gross morphology and histology of the liver/kidney did not show any injuries or unusual signs. In addition, the glomerulus had a normal structure with wide Bowman’s capsules, and the renal tubes were lined by a simple cuboidal epithelium with a uniform appearance. Furthermore, no damage to the structure of hepatocytes and nephrocytes was observed (Figure 4 and Figure 5). Therefore, there was no subacute toxicity at this polyphenols concentration.

This result shows that the extract concentration employed in this study is safe for human health. Our results also differ from those of Wu et al. (2012), who noticed that both acetone and water extract from fresh Polygonum multiflorum Thunb. roots were toxic and had dose-dependent hepatotoxicity. On the contrary, many studies reported that extract from this material is good for the liver (Huang et al., 2007; Bhadauria, 2010), thus the toxicity of the extract depends on many factors, such as the extraction method, the composition of the material, solvent, etc. Moreover, toxicity was also related to the following factors: improper drug compatibility, dose, mode of administration, physical condition of the patients, and processing methods. The above-described studies appear to be contradictory. However, Polygonum multiflorum Thunb. roots have still been used in different ways for a long time, such as in Heshouwu tea, Heshouwu wine, Heshouwu soup, etc. in Chinese daily meals (Li and Gao, 2015).
Figure 1 The RA value of the interaction between polyphenols and gelatin.

Figure 2 Gross morphology of the liver in acute toxicity. Note: a, b, c and d show the gross morphology of the liver at 138, 276, 414, and 552 mg dry extract per kg of body weight, respectively.

Figure 3 Histology of the liver in acute toxicity. Note: a, b, c and d show liver histology at 138, 276, 414, and 552 mg dry extract per kg of body weight, respectively.

Figure 4 Gross morphology of the kidney and liver in subacute toxicity for 6 weeks.
Figure 5 Histology of the kidney and liver in subacute toxicity for 6 weeks.

Table 1 Clinical biochemistry analysis and hematological parameters of mice in acute toxicity.

| Criteria                          | Control sample | Dry extract.kg⁻¹ of body weight ratio (mg.kg⁻¹) |
|-----------------------------------|----------------|-----------------------------------------------|
|                                   | 138            | 276                                          | 414 | 552          |
| Urea/BUN (mmol.L⁻¹)               | 10.51±1.28b    | 8.8±0.75b                                     | 9.03±1.38b | 9.6±2.22ab | 9.47±1.17ab |
| Creatinine (µmol.L⁻¹)             | 36.53±4.25b    | 33.6±1.64a                                    | 32.6±4.22a | 33.15±1.38a | 33.17±1.61a |
| AST (SGOT) (U.L⁻¹)                | 147.1±44.45b   | 111.43±25.82a                                | 173.5±58.03b | 144.1±52.91ab | 149.1±47.5b |
| ALT (SGPT) (U.L⁻¹)                | 83.57±29.96b   | 77.94±11.59ab                                | 61±7.54a     | 82.5±19.05b | 75.3±12.21ab |
| Total leukocyte count (K.µL⁻¹)    | 1.97±0.25a     | 2.43±0.91b                                   | 2.03±0.15ab  | 1.97±0.35a | 1.86±0.24a  |
| Total hemocyte count (M.µL⁻¹)     | 9.33±0.49b     | 8.92±0.57a                                   | 9.2±0.2ab    | 8.85±0.44a | 8.94±0.23ab |

Note: Different lowercase letters in the same row denote significant differences (p<0.05).

Table 2 Clinical biochemistry analysis and hematological parameters of mice in semi-chronic toxicity.

| Criteria                          | 3 weeks                     | 6 weeks                     |
|-----------------------------------|-----------------------------|-----------------------------|
|                                   | Control samples             | Experimental samples        | Control samples | Experimental samples |
| Change in weight (%)              | 6.2↑                        | 5.7↑                        | 8↑              | 7.4↑               |
| Urea/BUN (mmol.L⁻¹)               | 6.84±0.72a                  | 7.36±1.35a                  | 8.09±0.71A      | 7.2±1.07A          |
| Creatinine (µmol.L⁻¹)             | 37.3±3.51a                  | 33.1±5.21a                  | 34.1±3.65A      | 31±2.91A           |
| AST (SGOT) (U.L⁻¹)                | 105.38±9.93a                | 105.84±23.7a                | 116.23±28.46A   | 119.33±22.92A      |
| ALT (SGPT) (U.L⁻¹)                | 62.99±9.09a                 | 63.67±13.23a                | 70.87±33.46A    | 51.27±8.53A        |
| Total leukocyte count (K.µL⁻¹)    | 2.24±1.01a                  | 2.75±0.61a                  | 2.28±0.75A      | 2.7±1.08A          |
| Total hemocyte count (M.µL⁻¹)     | 8.17±1.42a                  | 8.28±0.93a                  | 6.21±1.49A      | 7.04±1.68A         |

Note: Different lowercase letters in the same row for 3 weeks denote a significant difference (p<0.05). Different uppercase letters in the same row for 6 weeks denote a significant difference (p<0.05).
CONCLUSION
In summary, polyphenols acetone extract from Polygonum multiflorum Thunb. roots can interact strongly with gelatin. At the same time, polyphenols extract at the studied concentrations was not acutely or subacutely toxic in mice.

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Contact address:
*Le Pham Tan Quoc, Industrial University of Ho Chi Minh City, Institute of Biotechnology and Food Technology, 12 Nguyen Van Bao street, Ward 4, Go Vap district, Ho Chi Minh City, Vietnam, Tel.: +84906413493, E-mail: lephamtanquoc@iuh.edu.vn
ORCID: [https://orcid.org/0000-0002-2309-5423](https://orcid.org/0000-0002-2309-5423)

Corresponding author: *