Determination and haemolytic activity of saponins in hairy root culture of *Platycodon grandiforum* A.DC.

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Summary

The sum of saponins in the hairy root lines (6, 17) of *Platycodon grandiforum* A.DC. was compared. Hairy root line 6 showed a higher total saponin content (6.92%) than the line 17 (6.01%). According to the Chinese Pharmacopoeia standards the content of saponins in *Platycodi Radix* should be not less than 2%. Our results seem to indicate that the hairy root culture of *Platycodon grandiforum* A.DC. is a good source of saponins. The Haemolytic

Index of the hairy root line 6 was 1600. Digitonin was used as a reference. Moreover, the haemolytic activity of TLC subfractions of saponins varied.

Key words: *Platycodon grandiforum A.DC., hairy roots, saponins, Haemolytic Index*

INTRODUCTION

The root of *Platycodon grandiforum* A.DC. (*Campanulaceae*) is a well-known traditional Chinese medicine used as an expectorant for pulmonary diseases and a remedy for respiratory disorders. The extensive phytochemical and pharmacological studies on the root of *P. grandiforum* proved oleanane-type saponins to be the main bioactive principles [1], with the various bioactivities such as antitumor [2, 3], anti-inflammatory [4, 5], hepatoprotective [6], anti-atherosclerotic [7] and antinociceptive [8] effects. Besides, the saponins from the *P. grandiforum* was found recently to exhibit adjuvant potentials [9-11].

New generations of vaccines, particularly those based on purified recombinant proteins, synthetic peptides and plasmid DNA, are likely to be less reactogenic and immunogenic than traditional vaccines. The majority of these vaccines require association with adjuvants capable of increasing the potency or stimulating the appropriate immune response. Saponins are natural products that are the promising sources of adjuvants. However, they show undesirable hemolytic effect [11].

In this study we report the evaluation of the hemolytic activity of saponins from the hairy root culture of *P. grandiflorum*. Moreover, the sum of saponins in the hairy roots lines (6, 17) of *P. grandiforum* A.DC. was compared.

MATERIALS AND METHODS

Establishment of hairy root lines

For hairy root induction in *P. grandiflorum*, leaf and stem tissues of micropropagated plants were infected with *Agrobacterium rhizogenes* ATCC 15834. The explants were cultured on phytohormone-free half-strength Murashige-Skoog (MS) [12] solid medium containing 4% sucrose in a dark for four days and then transferred to the same medium added with Claforan (500 mg/l). Explants developed new roots from the wounded tissues after 15 days of culture in the light. The hairy roots were individually excised and placed on the same medium with antibiotic mentioned above.

Two hairy root lines (6, 17) showing rapid growth were maintained on the hormone-free Woody Plant Medium (WPM) [13] with sucrose (40 g/l) which was proved to be the best for optimal growth. The culture was incubated in the dark at 25ºC±2ºC on a rotary shaker (100 rpm) and subcultured every six weeks. Transformation was confirmed by polymerase chain reaction (PCR) experiment. After 46 passages hairy roots were harvested, air-dried and used for next studies.

Determination of saponin content

Dried, pulverized roots (2 g) were put in a Soxhlet apparatus, soaked in methanol (150 ml) and allowed to macerate for 17 hours, next refluxed for 6 hours, put aside overnight and finally filtered. The filtrate was evaporated in vacuum to afford a dark brown residue, which was dissolved in methanol (50 ml). The methanol solution was poured into water (1/1, v/v) and then methanol was removed in vacuum. The aqueous residue was extracted 10 times with diethyl ether (4/1, v/v) and 5 times with butanol saturated with water (4/1, v/v), successively. The butanol layers were combined and evaporated in vacuum. The residue was dissolved in methanol (10 ml). The methanol solution was poured into ether (35 ml). The resulted precipitates of the crude saponins were collected by filtration, dried at 105^0C to constant weight and the content of total saponins was calculated.

Mean value represented the replicate of five determinations. Excel was used to calculate the standard deviation (SD) and the relative standard deviation (RSD).

Determination of haemolytic activity

The haemolytic activity of total saponins from the hairy root culture of *P. grandiflorum* (line 6) was expressed as Haemolytic index (HI), which is defined as the number of ox blood (2%, v/v) that can be haemolysed by 1 g of crude saponins or plant material. Haemolytic index was evaluated by the method of Mazurek [14] using digitonin (HI=88000) as a reference.

Roots (1.5 g) were extracted two times with methanol (150 ml) in a water bath for 1 hour. The obtained extracts were combined, filtered and evaporated to dryness. The residue was soaked in PBS (100 ml) and serial dilutions with ox blood (2%, v/v) were performed. The same series of dilutions were done with reference. The haemolytic index of saponins was calculated according to following equation:

$$\boldsymbol{H} = HIstd \cdot \frac{a}{b};$$

where *HIstd* is the haemolytic index of standard saponin, *a* and *b* are the minimal amounts (mg) of tested plant material and standard saponin, respectively, at which full haemolysis occurred.

Furthermore, the haemolytic activity of TLC subfractions of the crude saponins isolated from the hairy root line 6 was tested. For this, a solution of saponins (0.25 mg/ml) was spotted on TLC plates (Merck, Kieselgel 60) and developed with $CHCl_3$-MeOH-H_2O (63:32:5, v/v). Developed plates were covered with a layer of gelatine-blood solution. After one hour, whitening spots were observed indicating the presence of saponins. To make a comparison, the other visualization sprayer was used. After spraying with 10% H_2SO_4 the plates were heated for 5 min at 105˚C.

RESULTS AND DISCUSSION

This is the first report presenting determination and haemolytic activity of saponins in hairy root culture of *P. grandiforum.* In our studies the total saponins content in the hairy root lines (6, 17) of *P. grandiforum* was compared. The hairy root line 6 was higher in saponins content than the line 17. Whereas in the line 6 it was 6.92%±0.078% (SD), in the line 17 it was 6.01%±0.084% (SD). The RSD was 1.13% and 1.40%, respectively.

Those concentrations of the total saponins were higher than those found by the other authors [15, 16] in the roots of plants. Zandecka-Dziubak et al. [15] concluded that the plants with white flowers had more saponins (4.53%) as compared to the plants with blue flowers (3.73%). The concentration of total saponins in hairy root culture of *P. grandiflorum* qualified in our studies was approximately more than 3 times higher than those found by Hosoda et al. [16] in roots of plants growing wild. However, authors [15, 16] used the others determination methods.

According to Chinese Pharmacopoeia standards [17] the content of saponins in *Platycodi Radix* should exceed 2%. Our results seem to indicate that the hairy root culture of *P. grandiflorum* is a good source of saponins.

Saponins are natural products that are the promising sources of adjuvants. However, they show undesirable haemolytic effect. The aim of this study was to evaluate the haemolytic activity of saponins from the hairy root culture of *P. grandiflorum*. For this purpose, the haemolytic index (HI) of total saponins from the hairy root line 6 was defined. Digitonin (HI=88000) was used as a reference. The HI value for the tested saponins was 1600. It implicates weak haemolytic activity of hairy roots of *P. grandiflorum*.

Results showed in recent publications [9-11] implicate higher haemolytic activity for Quil A than platycodigenin-type saponins isolated from the roots of *P. grandiflorum*. Quil A, isolated from the bark of *Quillaja saponaria,* is the most widely used saponin-based adjuvant. Moreover, authors [9-11] confirmed a promising adjuvant potential of saponins from *P. grandiflorum* roots. These results and our observations suggest that it's worth to investigate the adjuvant activity of saponins from *P. grandiflorum* hairy roots.

During the course of our studies different haemolytic activity of TLC subfractions of saponins was found. Whereas only five whitening spots (Rf 0.1, 0.31, 0.57, 0.76 and 0.96) were seen on plates covered with a layer of gelatine-blood solution, much more spots were observed when 10% H_2SO_4 as a visualization sprayer was used.

CONCLUSIONS

1. The hairy root culture of *P. grandiforum* is a good source of saponins.
2. The sum of saponins in the hairy root lines [6, 17] of *P. grandiforum* varies. The hairy root line 6 showed a higher total saponin content (6.92%±0.078%) than the line 17 (6.01%±0.084%).

3. A relatively low value of the haemolytic index (HI=1600) of *P. grandiflorum* hairy roots implicates the weak haemolytic activity of this plant material.

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OCENA KULTUR KORZENI TRANSFORMOWANYCH ROZWARU WIELKOKWIATOWEGO (*PLATYCODON GRANDIFORUM* A.DC.) POD WZGLĘDEM ZAWARTOŚCI SAPONIN I AKTYWNOŚCI HEMOLITYCZNEJ

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S t r e s z c z e n i e

Porównano zawartość sumy saponin w dwóch klonach 6 i 17 korzeni transformowanych rozwaru wielkokwiatowego (*Platycodon grandiforum* A.DC.). Klon 6 zawierał więcej (6,92%) saponin niż klon 17 (6,01%). Suma saponin w surowcu zgodnie z wymaganiami Farmakopei Chińskiej nie powinna być mniejsza niż 2%. Na podstawie uzyskanych rezultatów można stwierdzić, że kultura *in vitro* korzeni włośnikowatych *Platycodon grandiforum* A.DC. jest wydajnym źródłem saponin. Dla klonu 6 określono również wskaźnik hemolityczny. Wynosił on 1600 względem digitoniny jako saponiny wzorcowej. Wykazano również różną siłę hemolityczną poszczególnych podfrakcji saponin rozdzielonych metodą TLC.

Słowa kluczowe: *Platycodon grandiforum* A.DC, *rozwar wielkokwiatowy, korzenie transformowane, saponiny, wskaźnik hemolityczny*