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Bioguided fractionation and isolation of esculentoside P from *Phytolacca americana* L.

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ABSTRACT

Various solvent extracts from different organs of *Phytolacca americana* L. were tested for their acaricidal activities against female carmine spider mite [*Tetranychus cinnabarinus* (Boisduval)] adults by slide-dip bioassay. The acetone extracts of roots with mortality rates of 95.72% and 100.00% at 2.5 mg/mL were more active than those of leaves and stems and compared with other solvent extracts from other organs after 48 and 72 h of treatment, respectively. The root acetone extracts were fractionated using a silica gel column; after thin-layer chromatography, the concentrated extracts were separated into 15 groups and further analyzed for their acaricidal activities. The ninth fraction exhibited the highest acaricidal activity. Phytochemical evaluation and liquid chromatography/mass spectrometry analysis revealed that esculentoside P was the dominant active phytolaccasaponin in the said fraction. This biological compound is a promising candidate as an acaricide against *T. cinnabarinus*.

1. Introduction

The carmine spider mite *Tetranychus cinnabarinus* (Boisduval) (Acarina: Tetranychidae) is an important economic pest mite worldwide and has significant effects on crop yield and quality (Cakmak et al., 2005). This species is widely distributed in China. Due to its small size, rapid growth, short life cycle, population density, and high rate of inbreeding, the carmine spider mite is more easily to generate resistance to conventional chemical acaricides (Sertkaya et al., 2010). For a long time, research has mainly focused on its chemical control. However, frequent use of a single chemical pesticide not only results in high mortality among the natural enemies of pest mites but also allows them to develop resistance to acaricides (Sachiko et al., 1999). It has also caused stress to crops, which may reduce their production and raise safety issues for human health resulting from crop residues (Hazan et al., 1974). Phytogenic acaricides, which are selective, have low toxicity to mammals, are water soluble, produce non-residual effects to the environment, and often can be biodegraded into non-target organisms, are suitable for application in integrated mite management (Isman, 1995,2001; Sardá et al., 2007). Therefore, the identification of reasonable alternatives to chemical pesticides has been the object of considerable global research interest. The extraction of effective acaricides from plants at home and abroad has become an important direction for research.

The tropical pokeweed *Phytolacca americana* L. is a member of the Phytolaccaceae family. It is an herbaceous perennial indigenous to the Amazon Rainforest and widely distributed in other areas (Gomes et al., 2008). Preparations of *P. americana* have been used extensively as traditional medicines for the treatment of many disorders in South and Central America because of their anti-inflammatory, antimicrobial, anticancer, and stimulant effects, among others (Kubec et al., 2002). Use of *P. americana* preparations is typically based on folklore (i.e., without any scientific evidence of efficacy), and their bioactive compounds are unknown (Blainski et al., 2010).

Compounds from plant extracts provide a potential alternative to existing acaricides – based on promising results to control mites susceptible and resistant to acaricides obtained by researchers using other plant species (Rosado-Aguilar et al., 2010). Many natural products or extracts are known to possess really interesting toxic activities against different agricultural mites (Kim et al., 2005a; Wang et al., 2007; Wei et al., 2011). In a recent communication after an extensive literature research, Li et al. (2009) identified eight plant extracts with acaricidal activity against *T. cinnabarinus*. Jia et al. (2011) investigated the acaricidal activities of eight species of plant extracts against *T. cinnabarinus*. Zhang and Feng (2010)

Abbreviations: LC_{50}, median lethal concentration; LT_{50}, median lethal time; LT_{10}, lethal time (h) at which 10% of the *T. cinnabarinus* showed mortality; LT_{90}, lethal time (h) at which 90% of the *T. cinnabarinus* showed mortality.

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also found that the highest mortality rate (100%) against female *T. cinnabarinus* adults was found in thyme, carvacrol, and eugenol (secondary metabolites of plants). The insecticidal and acaricidal properties of *P. americana* were already proven by Vanichpakorn et al. (2010) and Rosado-Aguilar et al. (2010) in *Plutella xylostella* L. larvae and *Boophilus microplus*, respectively. However, data on the acaricidal activity of *P. americana* against *T. cinnabarinus* are limited.

The objectives of this study were thus to investigate the acaricidal activities of various solvent extracts in different parts of *P. americana* (including leaves, stems, and roots), to isolate and identify compounds with acaricidal activity from *P. americana* roots under laboratory conditions, and to identify the most active components in *P. americana.*

2. Materials and methods

2.1. Plant materials

Plant materials (leaves and stems) of *P. americana* were collected from the nearby experimental farm of Southwest University in Beibei, Chongqing, China, on June 2011. *P. americana* roots (20 kg) were purchased from medicinal material markets in Chongqing, China, and identified by Prof. Li X.Y. (College of Horticulture, Southwest University). A voucher specimen has been deposited in the herbarium of the Southwest University College of Plant Protection.

2.2. Preparation of mites

Female carmine spider mite adults were used to test the acaricidal activity of *P. americana*. A laboratory stock culture of *T. cinnabarinus* had been maintained in the laboratory for at least 13 years with no previous chemical and acaricide exposure from the outdoors. They were reared on cowpea seedlings (*Vigna unguiculata*) in a walk-in growth chamber at 26 ± 1 °C and 75–80% relative humidity under a 14 h light/10 h dark cycle in Beibei.

2.3. Chemical reagents

All chemical reagents used in this study were of analytical grade, including acetone, petroleum ether (60–90 °C), ethyl acetate, and methanol. They were obtained from Cheng Du Kelong Chemical Reagents Co. (Chengdu, China). Tween-80 (experimentally pure) was purchased from Jiangsu Haian Petrochemical Plant (China) and used as an emulsifying agent. The silica gel used for thin-layer chromatography (TLC; HG/T 2354-92, GF254) and column chromatography (200–300 mesh) was purchased from Qingdao Haiyang Chemical Co., Ltd. (Shandong, China).

2.4. Extraction and isolation

The leaves and stems of *P. americana* were washed twice by a fast bath with tap water, oven-dried for 3–5 days at 40 °C, and then finely powdered. The roots of *P. americana* obtained from the medicinal material market were already in powder form. The powdered leaves (200 g), stems (200 g), and roots (200 g) were extracted with solvents of increasing polarity [petroleum ether (1000 mL; Fine), acetone (1000 mL; Fine), and methanol (1000 mL; Fine)] in large wide-mouthed bottles at room temperature (26 ± 1 °C) for 5 days. After being filtered, the extracts were evaporated under reduced pressure, with the residues soaked with their equivalent reagents for another 5 days. This experimental process was repeated three times for a total of approximately 15 days. Different concentrated extracts obtained were stored at 4 °C.

The root acetone extract of *P. americana*, which showed the strongest acaricidal activity, was chromatographed on a silica gel column (200–300 mesh) and successively eluted with stepwise gradients of petroleum ether/ethyl acetate (10:1.5, 9:2.5, 8:3.5, 7:4.5, 6:5.5, 5:6.5, 4:7.5, 3:8.5, and 2:9.5, v/v) and ethyl acetate/methanol (4:1 and 1:1, v/v). Fifty-three initial fractions (100 mL) were collected and based on similar R_f values on TLC (silica gel GF254 for TLC) developed with eluted petroleum ether/ethyl acetate (1:5, v/v) and ethyl acetate/methanol (9:1 and 1:1, v/v). Each fraction was identified by UV light at 254 nm or iodine vapor. After merger of the same R_f values, 15 fractions were finally obtained.

2.5. Bioassays

The FAO-recommended slide-dip method with slight modifications (Ma et al., 2009) was used to evaluate the acaricidal activities not only of different solvent extracts from different organs of *P. americana* against female *T. cinnabarinus* adults but also of fractions obtained from column chromatography. The female adult mites were adhered on the dorsal surface with a fine brush to one end of a glass slide and attached with double-sided adhesive tape. Each slide contained approximately 40 mites and was maintained at 26 ± 1 °C and 65%–80% relative humidity for 4 h, following which the mites were examined under a binocular microscope (4×) to remove any dead and unanimated adults, thereby leaving only the vivid ones as the original number. Each treatment with mites was dipped into each extract solution for 5 s, and the extra solution of the slides with filter paper was then carefully absorbed after treatment. The mites dipped in water with 1% Tween-80 to increase the extract's solubility in aqueous medium served as the control. Treatments were maintained under the same conditions as before.

Mortality was assessed under magnification, and a mite was considered dead if it did not exhibit movement of its legs or abdomen following repeated gentle probing with a fine brush. The data were recorded at 24, 48, and 72 h post-treatment. The study was performed in triplicate for each tested extract and with three slides in each replicate. The percentage mortality in all experimental batches of adult mites had to be corrected by applying Abbott's formula (1925) as recommended by FAO (2004):

$$\text{Corrected mortality} = \frac{\%\,\text{test mortality} - \%\,\text{control mortality}}{100 - \%\,\text{control mortality}} \times 100$$

If the mite mortality rate in the control was high (>10%), the bioassay would be nullified and repeated. None of the bioassays among the controls showed mortality rates greater than 5%.

The crude extracts of different parts of *P. americana* properly sequenced and parallel extracts were mixed with 0.5% acetone and 1% Tween-80 as test solutions, after which the mixtures were diluted with water at the concentration of 2.5 mg/mL. Based on the results, the acetone extract of *P. americana* roots was further evaluated at five concentrations as a series of test solutions to determine the median lethal concentration (LC_{50}) values for the eggs and female mites because it exhibited the highest activity compared with other extracts. The mites treated with water and 1% Tween-80 (v/v) were used as the control. The 15 fractions separated from the root acetone extract of *P. americana* were conducted at the concentration of 2 mg/mL to evaluate their acaricidal activities against *T. cinnabarinus* adults. The ninth fraction was selected for further evaluation to determine the LC_{50} value as it was found to possess the highest mortality (>90%) within 24 h.

The solution of the compound isolated from the ninth fraction was diluted at five concentrations with a certain amount of methanol and Tween-80 to determine the median lethal time (LT_{50}) value. Water mixed with 1% Tween-80 was used as the control. Each treatment was replicated three times. The slides with female adult mites were incubated at 25 °C and 75% relative humidity. The

number of dead mites was counted every 4 h. The total incubation period was 72 h.

2.6. Phytochemical analysis

Phytochemical tests were used to detect the presence of saponins. These tests were based on visual observation of color change after the addition of specific reagents. With the use of the characteristic reaction of saponins, the Liebermann, Liebernmann–Burchard, aromatic aldehyde sulfuric acid/perchloric acid, and foam tests were performed. These can identify whether the most active compound is a saponin, thereby laying the foundation for the next step of identifying the structure of the most active compound.

2.7. Liquid chromatography/mass spectrometry (LC/MS)

The ninth fraction was analyzed by LC/MS using a chromatograph (Waters 2487-ZQ 4000) equipped with a fused silica capillary column (10 m, 2.1 mm, 3.5 μm, coated with Xterra C18). Its relative composition was separated by LC and then detected by MS. Samples were separated from the mobile phase during MS. After ionization, the ions were recorded based on their ratio of mass number m and charge number Z (m/Z); their sizes were arranged from small to large in a spectrum after being treated with both the electric field and the magnetic field. The signal strength values of the ion were detected and recorded as y-coordinates, whereas the m/Z values were recorded as x-coordinates. These components comprised the final mass spectrogram. According to the literature, the dominant component can be obtained after comparisons with library spectra.

2.8. Statistical analysis

Lethal activity was classified as follows: strong, mortality greater than 80%; moderate, mortality between 61% and 80%; weak, mortality between 40% and 60%; little or no activity, mortality less than 40%. The mortality percentages were determined and transformed to arcsine square-root values for one-way ANOVA, followed by Duncan's multiple-range test at $P < 0.05$, using SPSS Version 13. Toxicity regression by probit analysis (Finney, 1971) was performed using SAS 8.01 (Cary, NC, USA) (SAS Institute, 2000). The LC_{50} and LT_{50} values were calculated by the complementary log–log (CLL) model (Preisler and Robertson, 1989) using a special microcomputer program (Tang and Feng, 2002).

Table 1
Shape and yield of different parts of *P. americana*.

Part	Solvent	Extract shape	Yield (%)[a]
Root	Petroleum ether	Yellowish paste	0.45g
	Acetone	Yellowish paste	3.12be
	Methanol	Yellowish paste	11.47b
Stem	Petroleum ether	Yellow-green paste	0.59g
	Acetone	Blackish green paste	1.26f
	Methanol	Tan paste	6.23c
Leaf	Petroleum ether	Puce paste	3.56d
	Acetone	Blackish paste	6.04c
	Methanol	Blackish paste	15.75a

[a] Extract rate = (dry weight of extract/dry weight of test plant) × 100%.

3. Results

3.1. Yield of extracts from all parts of the plant materials

Polar, medium polar, and non-polar compounds were extracted from different parts using different solvents (methanol, acetone, and petroleum ether, respectively). Table 1 shows that the yields increased using the increasing polarity of the solvents, such that methanol extracts > acetone extracts > petroleum ether extracts. The yield of methanol extracts significantly differed from the yields of the two other solvent extracts.

3.2. Comparison of acaricidal activities of the crude extracts of different parts of P. americana using different solvents

Comparisons of the acaricidal activities of the crude extracts of different parts using different solvents revealed that all solvents had significant effects on female *T. cinnabarinus* adults ($P < 0.05$). Contact acaricidal activity was tested with 2.5 mg/mL of the different solvent extracts of *P. americana* leaves, stems, and roots (Fig. 1). They showed different activities at 24, 48, and 72 h. At 24 h, the leaves, stems, and roots of the plant did not show any significant acaricidal activity; however, at 48 and 72 h, the crude extracts of all parts did. Acaricidal activity was recorded in the order of acetone extracts > ethanol extracts > petroleum ether extracts; for the acetone extracts, the activity was specifically observed in the order of roots > leaves > stems. The corrected mortalities of the root extracts were 17.00%, 95.72%, and 100.00% after 24, 48, and 72 h of treatment, respectively.

The LC_{50} values of eggs and female mites were determined for the root acetone extract, which showed the strongest acaricidal activity. After 48 h of treatment, the LC_{50} value in female mites was 2.1637 mg/mL, whereas that in eggs was 6.1003 mg/mL (Table 2).

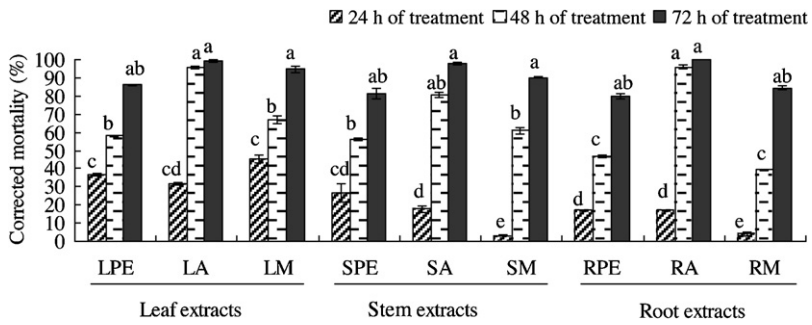

Fig. 1. Corrected mortalities of *P. americana* leaf, stem, and root extracts at 2.5 mg/mL against female *T. cinnabarinus* adults after 24, 48, and 72 h of treatment. Data are expressed as mean ± SE. Tests were replicated three times. The control solution was water mixed with 1% Tween-80. LPE indicates leaf petroleum ether extract; LA: leaf acetone extract; LM: leaf methanol extract; SPE: stem leaf petroleum ether extract; SA: stem acetone extract; SM: stem methanol extract; RPE: root petroleum ether extract; RA: root acetone extract; RM: root methanol extract.

Table 2
Toxicity regression line of *P. americana* roots from acetone extracts against female *T. cinnabarinus* adults (48 h).

Part	Mite stages	Solvent type	Linear regression equation	LC_{50}[a] ± SE_{50} (mg/mL)	95% Confidence interval	χ^2
Root	Adult	Acetone	$Y = -2.1385 + 2.0031x$	2.1637 ± 0.1231	1.8974–2.4614	0.215
	Egg	Acetone	$Y = 3.2351 + 1.4532x$	6.1003 ± 0.7923	4.2310–11.3241	1.212

Each extract solution had five concentrations, and tests were replicated three times. The control solution was composed of water with 1% Tween-80.
[a] $P < 0.05$ (ANOVA, followed by Duncan's multiple-range test).

Table 3
Mortality rates of *T. cinnabarinus* in contact with different fractions of *P. americana* root acetone extract at 2 mg/mL.

Fraction	Yield (%)	Corrected mortality (%)[a]	
		12 h after treatment	24 h after treatment
1	6.43	14.70 ± 3.39cd	42.30 ± 2.83cd
2	2.32	0.01 ± 0.00d	12.88 ± 4.71f
3	1.04	9.49 ± 0.13cd	27.89 ± 2.98def
4	1.47	5.50 ± 3.85cd	22.64 ± 2.93def
5	0.83	6.57 ± 5.05cd	17.93 ± 3.79ef
6	1.03	5.21 ± 1.17cd	22.61 ± 5.94def
7	1.41	24.47 ± 3.93bc	67.47 ± 5.88bc
8	0.53	17.98 ± 3.45cd	46.79 ± 3.73cd
9	2.20	60.84 ± 2.52a	95.75 ± 1.52a
10	1.28	40.63 ± 1.14ab	83.41 ± 2.46ab
11	0.20	44.64 ± 3.56ab	82.63 ± 3.35ab
12	5.80	5.15 ± 1.23cd	31.02 ± 0.00def
13	10.55	19.50 ± 4.13cd	47.53 ± 3.05cd
14	6.96	13.34 ± 1.72cd	45.59 ± 6.20cd
15	21.11	17.93 ± 11.36cd	44.44 ± 2.62cd

Data are expressed as mean ± SE.
[a] Values in the same column with different lowercase letters differ significantly at $P < 0.05$ by Duncan's multiple-range test.

3.3. Comparison of acaricidal activities of different components separated from P. americana root acetone extract by column chromatography

The acaricidal activities of different components separated from the *P. americana* root acetone extract by column chromatography were determined using biological activity tracking, and the results are listed in Table 3. Among the 15 fractions obtained from the crude acetone extract of *P. americana* roots, the ninth fraction exhibited the highest mortality after treatment for 12 and 24 h at the concentration of 2 mg/mL. The corrected mortalities were 60.84% and 95.75%, respectively. The tenth and eleventh fractions also exhibited some bioactivity, and their average corrected mortalities were 83.41% and 82.63% after 24 h of treatment.

3.4. Toxicity evaluation of the ninth fraction

The results of toxicity regression analysis of the ninth fraction with the highest activity within 72 h are detailed in Table 4. At 24, 48, and 72 h, the LC_{50} values of the ninth fraction and the root acetone extract of *P. americana* were 1.2609 and 5.5391 mg/mL, 0.2035 and 2.1637 mg/mL, and 0.1026 and 0.2579 mg/mL, respectively. Based on the 95% confidence intervals of the estimation, toxicity between the ninth fraction and the acetone extract significantly differed. These results indicated that the toxicity of the ninth fraction increased by approximately ten times compared with the acetone extract after 48 h of treatment. In short, the ninth fraction was a relatively pure substance, whereas the acetone extract comprised a complex unique mixture of different phytochemicals such that the active ingredient with acaricidal activity was present in small amounts. The toxicity effects shown in Table 4 could be attributed to different components working synergistically or to only one or two active components working alone in the root extract. From the results, we can preliminarily conclude that the toxicity activity in *P. americana* roots was due to the most active component working alone.

Laboratory tests were carried out to determine the toxicity of the ninth fraction against female *T. cinnabarinus* adults using a CLL model. Pearson's χ^2 analysis and Hosmer–Lemeshow goodness-of-fit statistics indicated that the data fit the CLL model ($P > 0.05$). The LC_{50} and LT_{50} values for the ninth fraction against female *T. cinnabarinus* adults are shown in Tables 5 and 6. The data confirmed that the ninth fraction was toxic to these mites and that acaricidal activity was dependent on time and concentration. The LC_{50} value of the ninth fraction (0.2 mg/mL; log LC_{50}, −0.0798) was approximately 11-fold greater than that of the acetone extract (2.1637) after 48 h of treatment. The LT_{50} value of the ninth fraction for 5.0 mg/mL concentration was 1.44 h, whereas that for 2.5 mg/mL concentration was 19.38 h. The LT_{50} value of the ninth fraction for 1.25 mg/mL concentration was only 40.83 h.

3.5. Phytochemical analysis of the ninth fraction

Phytochemical analysis of the ninth fraction revealed the presence of saponins. In the Liebermann test, the presence of saponins was confirmed by the appearance of a series of color changes after a few drops of concentrated sulfuric acid were added to the acetic anhydride solution of the ninth fraction. In the Liebermann–Burchard test, the presence of triterpenoid saponins was confirmed by the appearance of a characteristic aubergine color after the addition of a few drops of the reagent, which was composed of concentrated sulfuric acid/acetic anhydride (1:20). Vanillin, the most common color-sensitive reagent in the aromatic aldehyde–sulfuric acid/perchloric acid test, is often used as a chromogenic reagent of triterpenoid saponins. After this reagent was sprayed onto the ninth fraction when it appeared in the TLC test, the

Table 4
Toxicity regression analysis of the ninth fraction and the acetone extract against adult *T. cinnabarinus*.

Time	Treatment	Linear regression equation	LC_{50}[a] ± SE_{50} (mg/mL)	95% Confidence interval	χ^2
24 h	The ninth fraction	$Y = -0.2865 + 2.8458x$	1.2609 ± 0.7862	1.1350–1.4006	0.624
	The acetone extract	$Y = -1.9809 + 2.6644x$	5.5391 ± 1.2021	4.9793–6.1714	2.742
48 h	The ninth fraction	$Y = 2.1614 + 5.2920x$	0.2035 ± 0.6603	0.1977–0.4026	0.776
	The acetone extract	$Y = -2.1385 + 2.0031x$	2.1637 ± 0.1231	1.8974–2.4614	0.215
72 h	The ninth fraction	$Y = 2.8939 + 3.6686x$	0.1026 ± 0.1200	0.0681–0.2198	0.092
	The acetone extract	$Y = 1.5370 + 2.6117x$	0.2579 ± 0.1024	0.1022–0.3797	1.566

Each extract solution had five concentrations, and tests were replicated three times. The control solution was composed of water with 1% Tween-80.
[a] $P < 0.05$ (ANOVA, followed by Duncan's multiple-range test).

Table 5
Log LC_{50} and log LC_{90} values of the ninth fraction against adult *T. cinnabarinus* estimated by the CLL model at different time points.

Time (h)	Log LC_{50}		Log LC_{90}	
	Mean value	Standard error	Mean value	Standard error
4	0.5396	0.0442	0.9692	0.0590
8	0.4772	0.0418d	0.9067	0.0555
12	0.4508	0.0411	0.8804	0.0541
16	0.4250	0.0405	0.8546	0.0527
20	0.3996	0.0399	0.8291	0.0515
24	0.3037	0.0376	0.7333	0.0476
28	0.2443	0.0365	0.6739	0.0454
32	0.1558	0.0354	0.5854	0.0421
36	0.1052	0.0350	0.5348	0.0405
40	0.0809	0.0348	0.5105	0.0397
44	−0.0374	0.0349	0.3922	0.0359
48	−0.0798	0.0353	0.3498	0.0346
52	−0.1172	0.0359	0.3124	0.0336
56	−0.1512	0.0362	0.2784	0.0332
60	−0.2020	0.0369	0.2276	0.0320
64	−0.2442	0.0376	0.1854	0.0311
68	−0.2959	0.0390	0.1336	0.0300
72	−0.3405	0.0405	0.0891	0.0294

Pearson's $\chi^2 = 10.35$, $P = 0.83 > 0.05$; Hosmer–Lemeshow value = 3.26, $P = 0.45 > 0.05$.

characteristic purple color of saponins emerged after heating for 15 min. In the foam test, the presence of saponins was determined by the formation of foam after shaking an aqueous solution of the ninth fraction, and its presence persisted for more than 15 min. In conclusion, the active compound can be identified as a kind of saponin in *P. americana* roots. Nevertheless, further studies on the purification and identification of the active acaricidal components in the ninth fraction are needed.

3.6. LC/MS analysis of saponins in the ninth group

From the phytochemical analysis of the ninth fraction, the most active component in the ninth group could be a saponin. Many researchers have characterized the saponins in *P. americana* as oleanane triterpenoid saponins. Oleanolic acid (Fig. 2) is known to have a five-ring structure of triterpenoid saponins. Triterpene glycosides are highly polar, non-volatile, and thermally labile compounds that are consequently difficult to ionize. Consequently, MS has become a useful tool in the structural elucidation of triterpene glycosides.

As the ninth fraction was not a very pure substance, using crystal chromatography at room temperature, we obtained white amorphous powder. After recrystallization, a white amorphous crystal was finally received. Due to its impurity, the identification of the structure could not be confirmed, and LC/MS was thus used. Samples were separated by LC and then set apart from the mobile phase during MS; after ionization, the ion pieces were separated according to different values of the mass-to-charge ratio (m/Z) by an MS quality analyzer. The mass spectrum diagram of the sample was obtained with a detector. LC/MS reflected the advantages of both the LC and the MS. It combined the high separation ability

Table 6
LT_{10}, LT_{50}, and LT_{90} values of the ninth fraction against adult *T. cinnabarinus* estimated by the CLL model at different concentrations.

Time (h)	Concentration (mg/mL)				
	5.0	2.5	1.25	0.625	0.3125
LT_{10}[a] (h)	0.23	6.36	20.06	35.89	30.62
LT_{50} (h)	1.44	19.38	40.83	79.05	62.14
LT_{90}[b] (h)	9.01	59.09	83.10	174.11	126.10

[a] Lethal time at which 10% of the *T. cinnabarinus* showed mortality.
[b] Lethal time at which 90% of the *T. cinnabarinus* showed mortality.

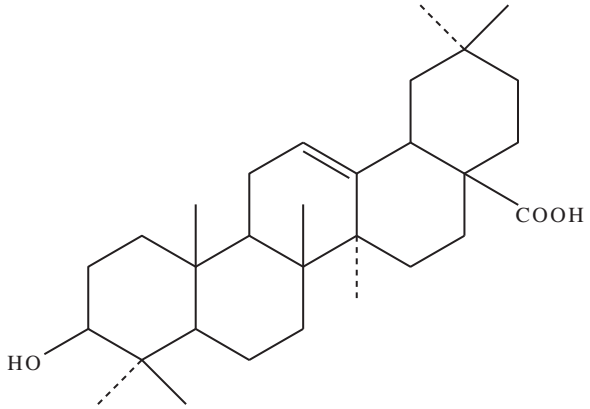

Fig. 2. Structure of oleanolic acid (MW = 456).

of LC with the high selectivity and high sensitivity of MS. Finally, the molecular weight (MW) of the complex sample was obtained by MS. Based on the results, relatively high amounts of triterpenoid saponins were detected through LC/MS determination in the ninth group (Fig. 3). After comparisons with library spectra, esculentoside P (MW = 680), assigned as 3-*O*-*β*-D-glucopyranosyl-2-hydroxyesculentic acid (Fig. 4), was detected in large amounts in the ninth fraction, whereas esculentosides G and H were noted in small amounts.

4. Discussion

This study investigated the acaricidal activities of different parts of *P. americana* against female *T. cinnabarinus* adults. Among the *P. americana* extracts evaluated, the root acetone extract showed the highest acaricidal activity for *T. cinnabarinus* female adults. The best method of studying the acaricidal activity of plants is by collecting different parts of each plant, including roots, stems, leaves, flowers, and fruits, and other locations of plant individuals, because some acaricidal plants might exhibit either localized or distributed acaricidal activity (Zhang et al., 2008). Liu et al. (2010) screened the acaricidal activities of different solvent extracts from different organs of *Euphorbia fischeriana* against *T. cinnabarinus*. Cao et al. (2007) observed that the acaricidal activity of chloroform extract from *Kochia scoparia* seeds was higher than those of the roots, stems, and foliage against *Tetranychus viennensis*.

In addition, different plant species have been reported to significantly differ in their acquisition time and specific requirements (Wu et al., 1998). The chemical nature of constituents varies considerably between species. The same herbal extract may vary depending on the harvest season, plant origin, drying process, and other factors (Damiani et al., 2011). To determine the optimum collection period of *P. americana* for traditional Chinese medicine and to further broaden its medical applications, Li et al. (1997) studied the contents of phytolaccasaponins and polysaccharides in different locations of plant individuals and during different seasons using the sulfuric acid–vanillin colorimetric method and the thick sulfuric acid–phenol colorimetric method. These data indicate that *P. americana* can be investigated in its entirety and that further research would help extend the *P. americana* industrial chain as well as develop and utilize the advantageous resources of traditional Chinese medicine plants. For medical applications, besides the roots, the stems and leaves of *P. americana* also have utilizable values. Moreover, this study showed that terpenoids are present in different amounts in different parts of *P. americana*, thereby possibly explaining why root extracts were more effective than leaf and stem extracts in our research.

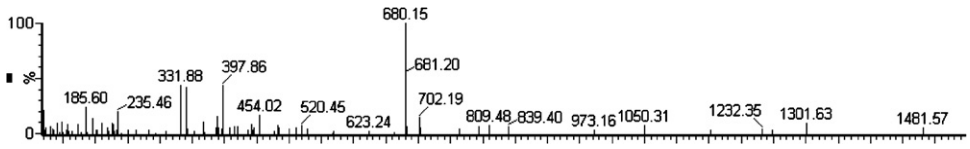

Fig. 3. LC/MS of the white amorphous crystal obtained from the ninth fraction.

P. americana L., as an effective traditional medicine, has been recorded in *Brazilian Pharmacopeia*, *Paraguay Pharmacopeia*, and *Japanese Drug Directory* (Fonnegra and Jimenez, 2007). Recently, many researchers have demonstrated that its extracts exert a broad spectrum of antioxidant, antimicrobial, antifungal, and antiparasitic activities (Ma et al., 2005; Yang et al., 2005; Jiang et al., 2006; He et al., 2007). The present study, however, is the first to report the acaricidal properties of components derived from *P. americana* roots against *T. cinnabarinus*. Many components present in plants from the Phytolaccaceae family are described in the literature as possessing insecticidal activity. Benevides et al. (2001) reported the isolation of the active component di(benzyltrithio) methane, benzyl hydroxymethyl sulfide, and several other antifungal polysulfides from the roots of *P. americana*. Dibenzyl trisulfide, a main lipophilic compound in *P. americana*, has been shown to exhibit interesting biological activities. Methyl benzyl sulfonic anhydride, which is transformed from dibenzyl trisulfide, was found to be more effective than the commercial agents isoniazid and ampicillin in inhibiting the growth of *Bacillus subtilis* and *Pseudomonas fluorescens* (Williams et al., 2003). Although *P. americana* as a phytogenic acaricide has been studied for a long time, the bioactivity of its active components remains largely unexplored.

Natural bioactive compounds are a promising alternative for mite control; they might offer additional advantages, such as low toxicity to mammals, and are more environmentally friendly (Rajakumar and Abdul Rahuman, 2012). Botanically active compounds from *P. americana* against agricultural pests have been reported by various authors and are considered to be underused alternatives to synthetic pesticides. Earlier research has shown that *P. americana* contains a diversity of biologically active compounds, such as essential oil (Petiverina), saponin glycosides, isoarborinol triterpene, isoarborinol acetate, isoarborinol cinnamate, steroids, phenols, alkaloids, flavonoids, and tannins (Adesogan, 1974; Delle Monache et al., 1992). Phenolic compounds found in leaf extracts of *P. americana* are known to affect seed germination, seedling growth, and fungal activity. The allelochemical effects of three *Phytolacca* species from South Korea were also found to be related to phenolic compounds (Kim et al., 2005b). Meanwhile, flavonoids have also been shown to possess a wide range of biological activities, such as anxiolytic properties (Blainski et al., 2010).

In this study, the presence of esculentoside P as the main active phytolaccasaponin in *P. americana* roots could be responsible for its acaricidal value, but its exact mode of action is still poorly understood. *P. americana* is known as a poisonous plant because of its high content of saponins. Macht (1937) reported that the roots from *Phytolacca acinosa* Roxb. are an especially rich source of triterpene saponins and that the important active and poisonous constituent of poke root is an amorphous, bitter, and acrid substance very similar to, if not identical with, saponin, which is probably glucoside. According to the literature, phytolaccasaponins and polysaccharides are the main components in *P. americana* roots (Wang et al., 2008). To the best of our knowledge, saponins produced by plants are often used to stop bacterial and fungal attacks, which qualifies them as natural antibiotics (Okwu and Emenike, 2006). More than a dozen triterpene saponins have been isolated and identified from *Phytolacca* species (Woo and Kang, 1976; Spengel and Schaffner, 1993; Strauss et al., 1995; Treyvaud et al., 2000), and they have been described as displaying important biological activities, such as molluscicidal, anti-inflammatory, antifungal, and antibacterial effects (Escalante et al., 2002; Di Maro et al., 2007). However, studies on the acaricidal activities of phytolaccasaponins of *P. americana* against *T. cinnabarinus* have not been reported to date.

This study suggests that *P. americana* and *P. americana*-derived materials can be used as *T. cinnabarinus* control agents. Applying active components (oleanane triterpenoid saponins) from *P. americana* in acaricide research and development can increase the efficiency of an acaricide and reduce the potential for resistance development in mite populations. To the best of our knowledge, the high economic costs involved are the key constraining factor to the development of natural pesticides. In contrast, the important advantage of the *P. americana* extract over acaricide is that it is a natural product that can be locally grown and obtained at low cost. In general, the extract of a plant has only small concentrations of active compounds but many promising properties (Rates, 2001). However, the purification and identification of the active components of *P. americana* extracts (esculentoside P) as a promising new acaricide should be further investigated. The safety issues of this compound for human health, its acaricidal mode of action, and formulations to improve its acaricidal potency and stability warrant further research. Field experiments are also necessary to confirm

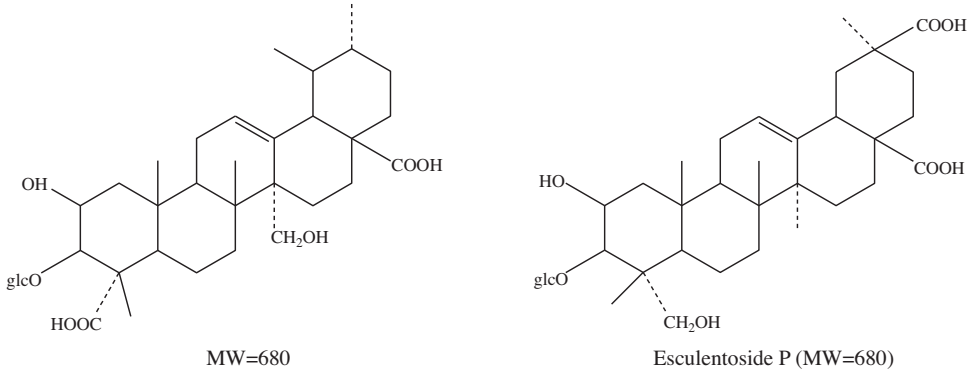

Fig. 4. Structures of esculentoside P and another esculentoside (both are isomerides).

its efficacy in reducing the carmine spider mite population under natural conditions.

In conclusion, this study presents a new promising acaricidal ingredient of *P. americana* identified as a type of oleanane triterpenoid saponin – esculentoside P, which is the most active component among phytolaccasaponins. As the carmine spider mite is susceptible to the composition of *P. americana* herein evaluated, use of natural products as alternative acaricides to control *T. cinnabarinus* should be considered. The results reported here also establish a strong foundation for further investigation of the efficacy of the acaricidal properties of natural product extracts. Esculentoside P from *P. americana* represents a new strategy for controlling *T. cinnabarinus*, thereby making it possible to develop new phytogenic acaricides.

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