Trends in Biochemical Engineering (2020) 1: 20-29
Clausius Scientific Press, Canada

DOI: 10.23977/trebe.2020.11002
ISSN 2616-1710

Anti-TMVActivity of Atisine-type Diterpene Alkaloids in Spiraea Japonica Associates with Down-Regulating the Expression of TMV Coat Protein

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Keywords: Atisine-type Diterpene alkaloids, Tobacco mosaic virus (TMV), Coat protein, Curative activity

Abstract: Four atisine-type diterpene alkaloids, including hsp-X40, hsp-X35, hsp-X31 and hsp-X26, were derivatives of diterpenes isolated from the ethanol extract of *Spiraea japonica* and then their chemical structures were determined with spectroscopy techniques. The result indicated that four compounds at a concentration of 100μg/mL can inhibit the infection of TMV to tobacco through *in vivo* curative activity, with the curative rate of 55.4±1.5% of hsp-X26, 53.2±1.9% of hsp-X31, 58.1± 2.0% of hsp-X35, 60.3±1.2% of hsp-X40, which was lower ($p< 0.05$) than that of the positive control Ningnanmycin (55.2±1.9%). However, the four compounds have not shown a better protective effect for tobacco against the TMV infection. The compound hsp-x40 with the diterpene amino acid ester group exhibited its IC50 value was 76.7 ± 2.4 μg/mL, suggesting that diterpene amino acid ester group may be as the important functional group for the anti-TMV activity of atisine-type diterpene alkaloids. The molecular experimental results showed that four active compounds can down-regulate the expression of TMV coat protein, indicating that the anti-TMV activity of atisine-type diterpene alkaloids in *spiraea japonica* associates with down-regulating the expression of TMV coat protein.

1. Introduction

Tobacco mosaic virus (TMV) was a widely studied plant virus. It was estimated that the economic losses caused by TMV each year exceed $100 million [1]. Currently, the main antiviral agents used were morpholine hydrochloride and Ningnanmycin [2]. These medicaments generally sprayed for prevention and treatment at the early stage of onset and have a protective effect, however, the curative effect was not significant. Previous studies have illustrated that the secondary

metabolites of some plants have an excellent inhibitory effect on plant viruses [3-7] and were safe, non-toxic, disease-resisting and economical. Therefore, finding natural compounds with good inhibitory effect on plant virus diseases from plant resources have become a hot research topic around the world [8-11].

In the previous study, 16 compounds from the ethanol extract of the entire plant of Spiraea japonica var acuminata Franch were isolated and identified including 8 atisine-type diterpene alkaloids, 3 diterpenes, 2 new alkaloids, 2 new diterpenes, and 1 new natural product. Some of them were chemically modified, and a total of 39 compounds were obtained. In this study, the half-leaf method was used to screen the activity of 39 compounds against the tobacco mosaic virus through *in vivo* protective effect and *in vivo* curative effect.

When the concentration of compounds was 100 μg/mL, 39 compounds did not exhibit better protective effect for tobacco against the TMV infection *in vivo* .When the concentration of compounds were 100 μg/mL, four compounds, including hsp-X40, hsp-X35,hsp-X31 and hsp-X26, showed better curative effect for tobacco against the TMV infection *in vivo.* The concentration gradient was further set 25 μg/mL, 50μg/mL, 100 μg/mL and 200 μg/mL, meanwhile, the inhibition rates of four diterpenoids *in vivo* curative effect of TMV was calculated. Among them, the diterpene compound hsp-X40 had the highest inhibition rate (78.1 ± 2.4%) when the concentration of compound was 200 μg/mL, which was higher than that of the positive control group (Ningnanmycin). The molecular experimental results showed that four active compounds can down-regulate the expression of TMV coat protein, indicating that the anti-TMV activity of atisine-type diterpene alkaloids in *spiraea japonica* associates with down-regulating the expression of TMV coat protein.

2. Materials and methods

2.1 Tobacco

Nicotiana glutinosa was the host of TMV. Its seeds were preserved and cultivated in float tray in the Key Laboratory of Natural Product Chemistry, Chinese Academy of Sciences, Guizhou Province. Health plants with similar age, size and weight with 4 ~ 6 leaves were selected as experimental materials. Nicotiana tabacum K326 (K326) was the host of systematic infection of TMV .Its seeds were preserved and cultivated in float tray in the Key Laboratory of Natural Product Chemistry, Chinese Academy of Sciences, Guizhou Province. Health plants with similar age, size and weight with 4 ~ 6 leaves and were selected as experimental materials

2.2 Tested virus

The common strain of tobacco mosaic virus (TMV) U1 was kept in the refrigerator with a temperature of -20°C in the Key Laboratory of Natural Product Chemistry, Chinese Academy of Sciences, Guizhou Province. TMV was diluted to 32 μg/mL with 0.01 mol/L phosphate buffer (PBS) before use.

Instruments

All solvents were obtained from industrial-grade distillation, including 200–400 mesh silica gel (Qingdao Ocean Chemical Co., Ltd.), Lichroprep RP-C18 gel (40-63 μm; Merck, Darmstadt, Germany, and Sephadex LH-20 (40-70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden), silica gel GF254 (China Qingdao Haiyang Chemical Industry Co., Ltd.), semi-preparative high-performance liquid chromatography (HPLC), Waters column (diameter 10–100 mm), Ultraviolet spray (Dragondorff), spectrometer (Bio-Rad FTS-135), digital polarimeter (JASCO DIP-370), mass spectrometer (Brook HCT / E and Waters Autospec Premier P776), in spectrometer

(INOVA-400 MHz NMR), refrigerated centrifuge, multi-functional microplate reader, and dual-color infrared laser scanning film imaging system.

2.3 Reagents

The reagents include on-denatured tissue/cell lysate (Solarbio), BCA protein concentration determination kit (Solarbio), Reagent TMV (agdia) ultra-sensitive ECL chemiluminescence kit (Biyuntian), alkaline phosphatase labeled goat anti-rabbit IgG (H + L), Goat Anti-Rabbit IgG (Doctor Biotech), F (ab ') 2 fragment, FITC Goat Anti Mouse IgG (H + L) (Immunoway), Plant actin Monoclonal Antibody (Mix) (Immunoway), BCIP/NBT alkaline phosphatase color reagent (Biyuntian), and Ningnanmycin 8% liquid agent (Liangye brand).

2.4 Preparation of Chemical Compound [12]

Spiraea japonica was collected in Songming County, Yunnan Province, People's Republic of China, and was identified as Rosaceae and Spiraea by Mr. Chen Yu in the Kunming Institute of Botany, Chinese Academy of Sciences. Besides, the specimen (GZH201001) was deposited in the Key Laboratory of Natural Product Chemistry, Chinese Academy of Sciences, Guizhou Province.

The powder of dried whole plant of S. japonica var. acuminate (20.0 kg) was extracted with 95% EtOH (20 L × 3) three times (80°C for 3 h, respectively). The combined EtOH extracts were concentrated under vacuum to give a crude residue (1.4 kg), which was suspended in water, adjusted to pH = 3 with solid tartaric acid, and extracted with petroleum ether to remove the chlorophyll (5 L × 3). The water layer was adjusted to pH = 10 with a saturated solution of Na_2CO_3 and then extracted with $CHCl_3$. The crude residue (94.3 g) was subjected to a silica gel column, eluted with $CHCl_3$–MeOH (from 1:0 to 0:1) to yield six fractions (A–E). Fr. A (11.0 g) was applied to a silica gel column (petroleum ether–EtOAc from 9:1 to 5:5) to obtain 5 (102.4 mg), 9 (2.3 g), and 11 (86.3 mg). Fr. B (4.2 g) was purified by a silica gel column chromatography (petroleum ether–acetone from 9:1 to 8:2) to obtain 10 (1.9 g). Fr. C (2.5 g) was applied to a RP-C18 column chromatograph (MeOH–H_2O from 40:60 to 100:0) to obtain three sub-fractions (C1–C3). Compound 3 (134.7 mg) was obtained from Fr. C1 (356 mg) by a silica gel column chromatography (petroleum ether–EtOAc 8:2). Fr.C2 was separated by a Sephadex LH-20 (MeOH), and then chromatographed on a silica gel column (petroleum ether–acetone 8:2) to yield 2 (76.4 mg) and 7 (206.0 mg). Fr. C3 was purified by a silica gel column (petroleum ether–acetone7:3) to get 4 (54.6 mg) and 6 (35.3 mg), respectively. Fr. E (1.1 g) was purified by a Sephadex LH-20 (MeOH) and chromatographed on a silica gel column (petroleum ether–acetone from 8:2 to 6:2) to obtain two fractions, which were then purified by semi-preparative HPLC using a X-Bridge Prep C18 (10 × 250 mm, 5 μm) column, to afford 1 (22.1 mg), 8 (95.1 mg), 12 (78.8 mg), and 14 (42.3 mg), respectively. The structure of the isolated compounds was modified.

2.5 Screening of 39 compounds TMV-inhibiting activity

The tested compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with 0.1% tween aqueous solution to the required concentrations. The solution of an equal concentration of DMSO was used as a negative control group (CK). The positive control group was diluted with 8% commercial antiviral agent, Ningnanmycin.

Screening of *in vivo* protective effect: Nicotiana glutinosa with similar age, size and weight with 4 ~ 6 leaves were selected as experimental materials. The compound solution of 100 μl and 100 μg/mL was evenly wiped on the left side of the leaves of Nicotiana glutinosa while the solvent of 100 μl (DMSO + 0.1% Tween) was evenly wiped on the right side of leaves of Nicotiana

glutinosa. After 24 hours, the tobacco mosaic virus (TMV) of 100 μl and 32 μg/mL was evenly rubbed and inoculated on both sides of the leaves of Nicotiana glutinosa. Simultaneously, a negative control group (DMSO + 0.1% Tween) and a positive control group were set. According to the above method, each compound was screened three times repeatedly and placed in a light incubator (cultivation condition was 28 °C). After 72 hours, the number of spots on leaves of Nicotiana glutinosa was recorded, and the inhibition ratio of compound to TMV was calculated.

Screening of *in vivo* curative effect: Nicotiana glutinosa with similar age, size and weight with 4~ 6 leaves were selected as experimental materials. The tobacco mosaic virus (TMV) of 100 μl and 32 μg/mL was rubbed and inoculated on both sides of the heart leaf tobacco. After 2 hours, the compound solution of 100 μl and 100 μg/mL was evenly wiped on the right side of leaves of Nicotiana glutinosa, while the solvent (DMSO + 0.1% Tween) was evenly wiped on the left side of leaves of Nicotiana glutinosa. Simultaneously, a negative control group (DMSO + 0.1% Tween) and a positive control group were set. According to the above method, each compound was screened three times repeatedly and placed in a light incubator (cultivation condition was 28°C), After 72 hours, the number of spots on leaves of Nicotiana glutinosa was recorded, and the inhibitory ratio of compound to TMV was calculated. Four compounds with better inhibition of TMV activity in the *in vivo* curative effect were selected from 39 compounds: hsp-X26, hsp-X31, hsp-X35, and hsp-X40. The concentration gradient was further set to 25 μg/mL, 50 μg/mL, 100 μg/mL, and 200 μg/mL; the inhibition rate of each compound at different concentrations was calculated, and then 50% inhibiting concentration was calculated [14].

2.6 Molecular Experimental [13]

The common smoke K326 with similar age, size and weight with 4 ~ 6 leaves were selected as experimental materials and placed in a dark room for 1 night. Each common tobacco K326 was selected with 3 leaves of similar size; each leaf was rubbed and inoculated with 100 μL, 32 μg/mL TMV. After 2 hours, 100 μL, 200 μg/mL compounds hsp-X26, hsp-X31, hsp-X35, hsp-X40 were sprayed evenly on the leaf surface of each ordinary tobacco K326 inoculated; this process was repeated 3 times for each compound. Simultaneously, a negative control group and a positive control group were set. They were sampled after 72 hours.

Preparation of non-deformable tissue/cell lysate: Add 10μL of PMSF 1mL of lysate, making the final concentration of PMSF as 1mM.Weigh 0.1g sample, grind it to a liquid state with liquid nitrogen, add 1mL of non-denatured tissue/cell lysate, then put it into a refrigerated centrifuge, centrifuge at 12000 r/min for 5min, finally take the supernatant. Total protein concentration determination. BCA protein concentration determination kit is used to determine protein content.

Prepare 15% lower layer glue and 5% upper layer glue, and insert an 11-hole tooth comb. After the gel is completely solidified, put the gel into the electrophoresis tank and add the electrophoresis solution. Then, add a certain volume of sample to each well. After the electrophoresis at 80 V for 30 min, perform electrophoresis at 100 V for 1.5 h. After the electrophoresis, activate the PVDF membrane with methanol, put the gel and membrane into the electrophoresis tank in a certain order, and perform electrophoresis at 220 MA for 2 h. After the film transfer was completed, the PVDF film was placed in the solution of 3% Alb for 1.5 h. After blocking, wash the membrane three times with TBS solution, cut the PVDF membrane from an appropriate position, incubate the PVDF membrane containing 40 KDa with the primary antibody of plant actin, incubate the PVDF membrane containing 17 KDa with the primary antibody reagent TMV of the tobacco mosaic virus coat protein (dilution ratio: 1: 200) at 4°C for 12 h overnight After 12 h, wash the primary antibody 3 times with TBS and use plant actin as the internal reference gene at a dilution ratio of 1: 3000; then, add the second antibody FITC Goat Anti Mouse IgG (H + L) of plant actin (dilution ratio: 1:

2000) and the secondary antibody of tobacco mosaic virus protein (dilution ratio: 1: 1000) for 1.5 h of incubation after 1.5 h, wash it with TBS buffer to terminate the reaction scan the PVDF membrane containing 40 kD by a dual-color infrared laser scanning film imaging system, and add BCIP/NBT alkaline phosphatase color reagent to the PVDF membrane containing 17 kDa , finally,observe the results after 15 min.

2.7 Statistical analysis

SPSS 21.0 software was used to analyze the difference through a single-sample T-test. In the experiment, the general linear model was used to analyze the data. The significant difference was expressed as P <0.05 (T-test), and the very significant difference was expressed as P <0.01 (T-test).

3. Results and analysis

3.1 Structural identification of the four compounds, as can be seen from Figure 1

Four atisine-type diterpene alkaloids, including hsp-X40, hsp-X35, hsp-X31 and hsp-X26, were derivatives of diterpenes isolated from the ethanol extract of *Spiraea japonica.*

Hsp-x26, Colorless powder ^{1}H NMR (400MHz, $CDCl_3$) δH: 6.00 (1H, d, J = 1.6 Hz), 5.30 (1H, d, J = 1.6 Hz), 4.14 (1H, dd, J = 4.4, 12.4 Hz), 3.65 (2H, t, J = 4.8 Hz), 2.80 (1H, s), 2.64 (1H, d, J = 11.2 Hz), 2.37-2.56 (4H, m), 2.22 (1H, d, J = 11.2 Hz), 2.13 (2H, m), 1.90-1.95 (2H, m), 1.54-1.80 (9H, m), 1.39-1.44 (2H, m), 1.25 (1H, s), 1.14-1.17 (1H, s), 1.03 (1H, m), 0.84 (3H, s); 13C NMR (100 MHz, CDCl3) δC: 203.8, 146.4, 118.5, 70.6, 60.7, 59.9, 57.8, 53.5, 50.2, 47.8, 43.0, 40.8, 39.5, 39.2, 35.8, 33.5, 27.5, 26.3, 25.3, 24.6, 23.0, 18.8. HREIMS m/z 359.2452 [M] + ($C_{22}H_{33}NO_3$, calcd.359.2460). It was identified as (3S, 8R, 10R, 10aR)-10-hydroxy-14-(2-hydroxyethyl)-8-methyl-12-methylenedodecahydro-3, 10a-ethano-4b, 8-(methanoiminomethano)phenanthren-11-one

Hsp-x31, Colorless powder ^{1}H NMR (400MHz, $CDCl_3$) δH: 5.93 (1H, d, J = 1.6 Hz), 5.91 (1H, d, J = 1.6 Hz), 5.23 (1H, d, J = 1.6 Hz), 5.22 (1H, d, J = 1.6 Hz), 4.90 (1H, d, J = 1.6 Hz), 5.67 (1H, d, J = 1.6 Hz), 4.20 (1H, s), 3.83-3.93 (5H , m), 3.68-3.74 (2H, m), 3.46-3.51 (2H, m), 3.19-3.27 (2H, m), 3.048-3.07 (2H, m), 3.78-3.82 (2H, m), 1.09 (3H, s); 13C NMR (100 MHz, $CDCl_3$) δC: 200.8, 148.2, 117.3, 95.1, 91.1, 85.7, 83.4, 67.8, 67.3, 64.9, 63.1, 51.0, 49.2, 47.4, 45.5, 44.3, 43.1, 43.0, 40.6, 36.8, 36.7, 35.7, 35.3, 35.1, 33.7, 29.9, 29.8, 29.6, 27.4, 24.5, 24.0, 23.5, 23.3, 22.8, 22.7, 20.6, 20.3; HREIMS m/z 355.2137 [M] + ($C_{22}H_{29}NO_3$, calcd.355.2147). It was identified as (4R,8aR,10R,10aR,13S)-4-methyl-12-methylenedodecahydro-4,14b,10-(epiethane[1,1,2]triyl)-10a, 13-ethanoisochromeno[4,3-g]oxazolo[3,2-a]azocin-11(1H)-one.

Hsp-x35, Colorless powder ^{1}H NMR (pyridine-d6, δH inppm, J in Hz, 400 MHz): 7.25 (s), 7.60 (s), 7.31 (s), 4.98 (1H, s), 4.96 (1H, s), 4.21 (1H, dd, J = 2.0, 11.6 Hz), 4.04 (1H, d, J = 11.6 Hz), 3.92 (1H, brs), 3.80 (s), 3.68 (1H, dd, J = 4.0, 10.8 Hz), 0.91 (3H, s). 13C NMR (pyridine-d6, δC in ppm, 500 MHz): 165.3, 162.3, 156.4, 133.3, 124.2, 123.9, 123.1, 113.0, 108.8, 81.6, 78.3, 62.4, 61.2, 59.9, 55.6, 54.3, 48.2, 47.5, 42.0, 41.8, 40.4, 38.7, 36.7, 33.6, 27.4, 27.1, 26.9, 26.6, 23.0, 16.4. HRESIMS m/z 596.1980 [M + Na] + ($C_{22}H_{33}NO_3$, calcd. 596.1988). It was identified as 2-((1R,6S,8aR,9R,11S)-9,11-dihydroxy-1-methyl-12-methylenedodecahydro-8a,6-ethano-1,4a-(methanoiminomethano)phenanthren-14-yl)ethyl 3-bromo-5-methoxybenzoate.

Hsp-X40,Yellow powder ^{1}H NMR (500MHz, $CDCl_3$) δ: 5.03 (1H, brs), 5.00 (1H, brs), 4.22 (2H, m), 3.90 (1H, brs), 3.68 (1H, dd, J = 6.0, 13.5 Hz), 2.76 (1H, d, J = 13.5 Hz), 2.44 (1H, d, J = 13.5 Hz), 2.33 (1H, brs), 0.93 (6H, t, J = 8.5 Hz) 0.79 (3H, s). 13C NMR (500MHz, CDCl3) δ: 176.5, 155.8, 108.9, 81.2, 77.7, 61.9, 60.3, 57.3, 53.5, 52.7, 47.7, 46.6, 43.9, 41.4, 40.0, 38.2, 35.8, 33.4, 29.6, 26.8, 26.4 , 26.3, 26.0, 24.7, 23.0, 22.2, 21.7, 15.2. HRESIMS m / z 497.3350 [M+Na] +

($C_{22}H_{33}NO_3$, calcd.497.3355). It was identified as (2S)-2-((1R, 6S, 8aR, 9R, 11S)-9, 11-dihydroxy-1-methyl-12-methylenedodecahydro-8a, 6-ethano-1, 4a-(methanoiminomethano) phenanthren-14-yl) ethyl 2-amino-4-methylpentanoate.

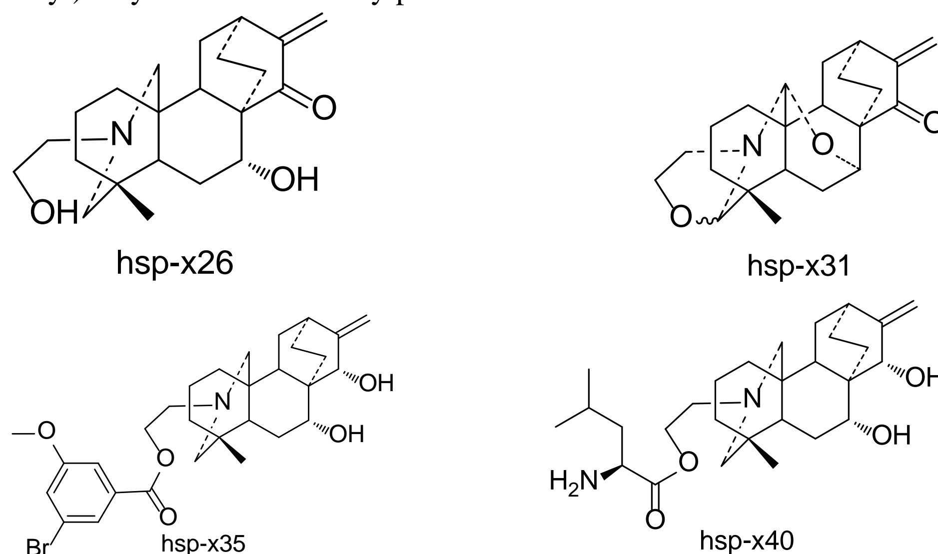

Figure. 1 Structure diagram of four compounds

3.2 The inhibition rates of 39 compounds (100 µg/mL) on TMV in vivo protection effect and in vivo curative effect (Table 1)

It can be seen from Table 1 that the inhibition rates of 39 compounds *in vivo* protection were lower than that of the positive control Ningnanmycin (70.2±2.5%),when the mass concentration ratio of the test sample was 100 µg/mL. The inhibition rates *in vivo* curative effects of the 4 diterpene compounds hsp-X40, hsp-X35, hsp-X31, and hsp-X26) were all higher than those of the positive control agent Ningnanmycin (55.2 ± 1.9%), when the mass concentration of the test sample was 100µg / mL. Among them, diterpene compound hsp-X40 had the highest inhibition rate *in vivo* curative effect (60.3 ± 2.1%), followed by hsp-X35 (58.1 ± 2.0%). The difference between the chemical structures of the two compounds was that the diterpene of hsp-X40 linked to an amino acid ester, while the diterpene of hsp-x35 linked to 3-methoxy-4-bromobenzoate, besides, the curative effect inhibition rates of another two compounds, hsp-X26 (55.4 ± 1.5%), hsp-X31 (53.2 ± 1.9%) were higher than those the positive control group.

Table 1 Inhibition rate of TMV (100µg / mL) of 39 compounds for in vivo protection effect and in vivo curative effect

compounds	**inhibition ratio** %	
	In vivo protection effect	*In vivo* curative effect
hsp-x1	5.2±0.2**	12.3±0.5**
hsp-x2	6.3±0.2**	11.4±1.3**
hsp-x4	5.5±0.3**	12.8±0.7**
hsp-x5	5.8±0.2**	14.1±1.0**
hsp-x6	6.7±0.2**	13.8±1.0**
hsp-x7	7.8±0.3**	13.5±1.1**

hsp-x9	8.5±0.2**	13.1±1.0**
hsp-x11	7.7±0.4**	14.5±0.9**
hsp-x12	8.9±0.2**	13.8±0.9**
hsp-x13	6.9±0.4**	12.9±1.4**
hsp-x14	7.2±0.4**	13.7±1.2**
hsp-x15	8.3±0.4**	14.2±1.2**
hsp-x17	6.5±0.4**	13.5±0.8**
hsp-x21	7.6±0.3**	15.6±1.3**
hsp-x22	8.6±0.3**	16.5±0.9**
hsp-x23	7.6±2.7**	13.6±1.2**
hsp-x25	6.5±0.4**	14.3±1.3**
hsp-x26	**20.3±1.4****	**55.4±1.5**
hsp-x27	10.2±0.3**	22.3±1.8**
hsp-x28	9.8±0.4**	24.5±1.3**
hsp-x29	8.9±0.3**	28.4±2.2**
hsp-x30	9.5±0.4**	27.6±1.8**
hsp-x31	**18.5±0.5****	**53.2±1.9**
hsp-x32	9.5±0.3**	29.4±2.1**
hsp-x33	8.8±0.4**	26.7±1.6**
hsp-x34	9.4±0.4**	23.7±2.1**
hsp-x35	**17.8±1.0****	**58.1±2.0**
hsp-x36	8.4±0.4**	16.8±1.7**
hsp-x37	9.6±0.2**	17.2±1.5**
hsp-x38	10.4±0.4**	18.4±1.8**
hsp-x40	**19.4±0.8****	**60.3±2.1**
hsp-x41	9.7±0.2**	23.5±1.7**
hsp-U2	10.3±0.3**	11.4±1.2**
hsp-U4	11.5±1.0**	17.6±1.4**
hsp-U7	5.6±0.3**	14.7±1.3**
hsp-U8	6.4±0.4**	15.6±1.6**
hsp-U9	7.3±0.5**	16.8±1.0**
hsp-U10	7.8±0.6**	19.5±1.7**
NM	70.2±2.5	55.2±1.9
negative control		

Note: "**" denotes that there was a significant difference between the anti-TMV rate of the compounds and the anti-TMV rate of the positive control (P <0.05 level).

3.3 Inhibition rates of TMV of 4 compounds in vivo curative effect under different concentration gradients

It can be seen from Table 2 and Fig. 2 that the gradient concentrations of the compounds hsp-X26, hsp-X31, hsp-X35, and hsp-X40 *in vivo* curative effect were 25 μg/mL, 50 μg/mL, 100 μg/mL, and 200 μg/mL, respectively. The diterpene compound hsp-X40 had the best inhibitory activity (78.1 ± 2.4%).When the concentration was 200 μg/mL, it was higher than that of the positive control group *in vivo* curative effect (62.1 ± 1.2%). The inhibition rate of hsp-X35 was 60.2 ± 1.5%, the inhibition rates of hsp-x31 and hsp-x26 *in vivo* curative effect were 55.3 ± 2.2% and 58.2 ± 1.9%, respectively. IC50 value of hsp-x26, hsp-x31, hsp-x35, and hsp-x40 were 105.5 μg/ml,

116.8 μg/ml, 99.1 μg/ml, 76.7 μg/ml. The IC50 value of Ningnanmycin was 85.4 μg/ml.

Table 2 Inhibition rates of TMV of 4 compounds in vivo curative effect under different concentration gradients

Compounds	inhibition rates %			
	25μg/mL	50μg/mL	100μg/mL	200μg/mL
hsp-x26	20.1±2.2	32.1±1.7	55.4±0.8	58.2±1.7
hsp-x31	21.3±1.2	31.2±1.5	53.2±1.7	55.3±1.3
hsp-x35	20.2±2.1	29.4±1.6	58.1±1.4	60.2±1.1
hsp-x40	19.2±1.2	30.3±1.9	60.3±1.2	78.1±1.0
NM	21.3±2.5	33.1±1.6	55.2±1.9	62.1±1.2
Negative Control	0	0	0	0

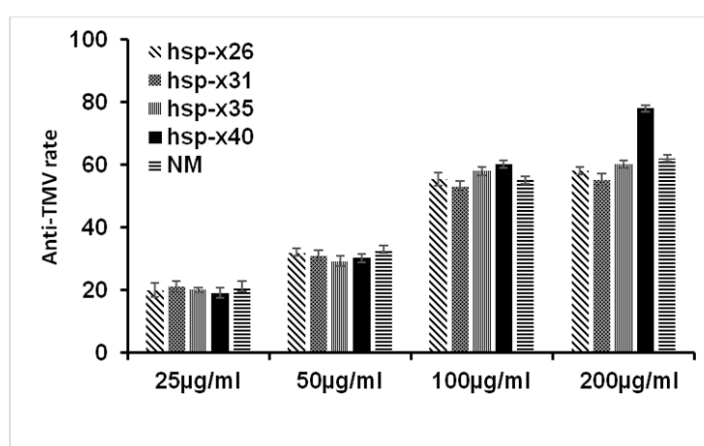

Figure. 2 Inhibition rates of TMV of 4 compounds in vivo curative effect under different concentration gradients.

3.4 Western blot results

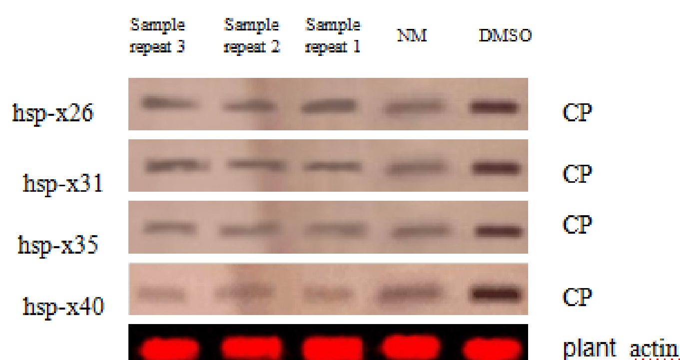

Figure. 3 The four active diterpene compounds inhibit TMV coat protein at a concentration of 200 μg/mL in vivo

As can be seen from Figure 3, the expression coat protein (TMV-CP) of TMV can be reduced by

Ningnanmycin and the four compounds. Among them, hsp-x40 had the least expression of coat protein (TMV-CP) of TMV.

4. Discussion

The tobacco mosaic virus was a plant virus. Plant viruses were usually composed of 5% nucleic acid and 95% protein. The protein surrounds the nucleic acid to form a protective shell, namely, coat protein (TMV-CP). The coat protein has the function of protecting the nucleic acid (RNA or DNA) from degradation in the virus [14]. The coat protein of the virus was the target of drugs to inhibit the replication of tobacco mosaic virus *in vivo* curative effect. It can inhibit or interfere with virus assembly by inhibiting the synthesis of coat protein, resulting in effectively controlling the virus reproduction. Therefore, the coat protein of the virus was an essential molecular target for antiviral agents [15].

A preliminary mechanism of compounds to inhibit TMV growth can be described as follows. First, it can be replicated in tobacco when tobacco mosaic virus invades tobacco; then, the compound acts on the coat protein of tobacco mosaic virus, preventing the virus from further assembling; As a result, virus replication could be inhibited .The specific process might be that the virus coat protein must form a certain degree of the polymer as the first step of virus assembly, and then be further assembled with virus nucleic acids to form complete virus particles. It may have an impact on the subsequent assembly steps if the antiviral agent interferes with the polymerization process of the coat protein of the virus, resulting in inhibiting the infection activity of the virus, making it difficult to synthesize the coat protein of tobacco mosaic virus [16].

In this study, the half-leaf spot method was used to screen the TMV-inhibiting effect of atisine-type diterpene alkaloids in Spiraea japonica *in vivo* protective effect and *in vivo* curative effect. *In vivo* protective effect test, the activity of 39 compounds in TMV-inhibiting was lower than that of the positive control group when the compound concentration was 100 μg/mL. In the *in vivo* curative effect test, there were 4 compounds (hsp-X26, hsp-X31, hsp-X35, and hsp-X40) whose TMV-inhibiting rates were higher than that of the positive control group when the compound concentration was 100 μg/mL .In the concentration gradient experiment, the inhibition rate of the TMV Spiraea Japonica diterpene alkaloid hsp-X40 against TMV was higher than that at other gradient concentrations and higher than that of the positive control group when the compound concentration was 200 μg/mL. It can be revealed that the TMV-inhibiting rate of hsp-X40 was the highest among gradient concentrations and was higher than that of the positive control group when the concentration was 200μg/mL. This result was further verified by western-blot showing that the expression ofTMV coat protein in the system-infected host common tobacco K326 was significantly down-regulated, when treated with hsp-X40 at the concentration of 200 μg/mL. It indicated that the amino acid ester of the compound hsp-x40 may have a strong interference on the polymerization process of TMV coat protein. Consequently, it is not conducive to the assembly of virions, resulting in affecting the expression of the viral coat protein of the virus [16]. However, how the diterpene alkaloid hsp-x40 affects the assembly process of virions remains to be further investigated.

Acknowledgements

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The article is supported by Guizhou Joint Fund LH [2016] 7410.

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