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Activity of aqueous extract of *Panax notoginseng* flower buds against *Aedes albopictus* larvae and pupae

Dedicated to the memory of Prof. Ivano Morelli

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Abstract

The insecticidal activity of aqueous extract of *Panax notoginseng* (Burkill) F. H. Chen (Araliaceae) flower buds against *Aedes albopictus* Skuse (Diptera Culicidae) larvae and pupae was tested and quali-quantitative profile of principal saponins of the active tested aqueous extract were investigated by LC-ESI/MS/MS. *P. notoginseng* aqueous extract (35% of saponins/dried plant extract) showed the highest activity both against larvae and pupae at 1.2% concentration (100% of mortality) with statistically significant differences relative to controls at 0.4%, 0.2%, and 0.1% concentrations. The results obtained suggested that there is a relation between insecticidal activity and the presence of triterpene saponins. The application of crude plant extracts, instead of pure triterpene saponins as insecticidal formulation, could find better applicability considering that the purification of compounds from plant extracts would require too much time and cost.

Key words: *Panax notoginseng*, Araliaceae, Saponins, *Aedes albopictus*, Antimosquito activity.

Introduction

Aedes albopictus Skuse (Diptera Culicidae), known as Asian tiger mosquito, is an Asiatic insect, widespread in all continents and recorded in Italy for the first time in 1990 (Sabatini *et al*., 1990). The Asian tiger mosquito is a daytime outdoor ectoparasite, showing high anthropophly. It is reported as potential vector of dengue virus, encephalitis virus, and several others arbovirus (Boromisa *et al*., 1987; Mitchell, 1995; Shroyer, 1986). Recently it was an outbreak of chikungunya fever due to the bite of *Ae. albopictus* (Angelini *et al*., 2007) in the province of Ravenna, Italy. Moreover, it is an intermediate host of filarial species such as *Dirofilaria immitis* (Leidy), *Dirofilaria repens* Railliet et Henry, and *Setaria labiatopapillosa* (Alessandrini) (Cancrini *et al*., 1995; Cancrini *et al*., 2003). Water living larvae are active feeders on fine particulate organic matter. Nowadays the mosquito control is mainly directed against larvae and, only if necessary, against adults. This is because the adults control shows temporary results, and with negative impact on the environment, while larval treatment is more localized in time and space, resulting less dangerous. Common insecticids on market are synthetic molecules or natural preparations such as a crystal protoxin of *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) produced during the sporulation process. The latter is the most used worldwide, because it is considered to be harmless for humans, fishes, and other not dipteran insects. Unfortunately, the insecticidal activity of *Bti* is lost after 24 hours, and it is completely ineffective against eggs and pupae.

Considering the future possible development of resistance to currently used insecticides and with the aim to discover environmental pacific natural formulations that are active against the pupal and larval stages of mosquitos, we prepared aqueous extracts of *Panax notoginseng* (Burkill) F. H. Chen (Araliaceae) flower buds and assayed the extract against larvae and pupae of *Ae. albopictus*. In China, *P. notoginseng* is known as "San Qi" and it is largely used in the traditional medicine. The roots and the flowers of the plant are used as antibacterial, anti-inflammatory, antiseptic, cardiotonic, diuretic, haemostatic, and hypoglycaemic remedies (Yeung, 1985) and the flowers buds are used in Chinese traditional medicine as ipotensive, anticancer, to treat vertigo, dizziness, tinnitus, and laryngopharyngitis (Brown, 1995). Previous phytochemical investigations on *P. notoginseng* flower buds led to the isolation of several dammarane-type triterpene saponins, that are typical constituents of the genus *Panax* (Yoshikawa *et al*., 2003; Taniyasu *et al*., 1982).

The relationship between insecticidal activity and the presence of triterpene saponins was investigated in a recent study (Santiago *et al.*, 2005) wherein larvicidal activity of four monodesmoside saponins isolated from *Pentaclethra macroloba* (Willdenow) Kuntze and of one bidesmoside saponin from *Cordia piauhiensis* Fresen was tested against *Aedes aegypti* (L.). Moreover the larvicidal activity of commercial saponins extracted from bark of *Quillaja saponaria* Molina was studied on third-fourth instar larvae of *Ae. aegypti* and *Culex pipiens* L. (Pelah *et al*., 2002).

With the recent development of electrospray ionization mass spectrometry coupled with liquid chromatography (LC-MS) it has become technically and economically feasible to analyse crude plant extracts providing high resolution and rapid compound identification also without the need to isolate individual constituents (He, 2000).

In this work we describe the insecticidal activity of *P.*

notoginseng aqueous extracts flower buds against larvae and pupae of *Ae. albopictus*, giving also a qualitative and quantitative profile by LC-ESI/MS/MS of the principal saponins contained in the active aqueous extract.

Materials and methods

Chemicals

HPLC grade methanol (MeOH), and acid acetic (CH_3COOH) were purchased from J.T. Baker (Mallinckrodt Baker, Phillipssburg, NJ, USA). HPLC grade water (18 mΩ) was prepared by a Milli-Q^{50} purification system (Millipore Corp., Bedford, MA, USA). Standard ginsenosides Rb1, Rb2, Rc, and Rd were purchased from EXTRASYNTHESE (Genay Cedex, France).

Plant material

Dried flower buds of *P. notoginseng* of commercial use were purchased in Sapa (Vietnam) in May 2004. A voucher specimen (N°5883/1) is deposited at the Herbarium Horti Botanici Pisani, Nuove Acquisizioni, Pisa, Italy. The powdered dried flower buds (90.8 g) were heated at 100 °C in 1000 ml of water for 2 hours to afford the decoction of the plant that was lyophilized to yield 17.5 g of aqueous residue.

Collection of insects

Ae. albopictus eggs were collected from ovitraps placed in selected areas of Pisa (Italy) where the presence of mosquitoes was observed during previous inspections. Masonite strips (3x15 cm) were positioned vertically in black pots filled with 350 ml of water to provide a suitable surface for eggs deposition. Pots were checked for eggs presence once a week. To stimulate egg hatching obtain the strips dried at room temperature for 3 days, then were placed individually into plastic trays containing chlorine-free tap water from which 1st stage larvae were obtained, and the strips were left to dry again. This alternating wet and dry procedure was repeated twice (Toma *et al*., 2003).

Insecticidal assays

Batch of 20-25 fourth instar larvae or pupae were placed in a small beakers, containing 25 ml of distilled water. The lyophilized extract was poured into 500 ml beaker and diluted with 225 ml of distilled water and stirred vigorously until complete dissolution with a glass rod to form a solution. The average temperature of the water was approximately 25 °C. After 15-30 minutes of the dilutions, previously prepared in beaker with preparation of the test concentrations, the previously prepared in beaker with 25 ml of water, mosquito larvae were added. The lyophilized concentrations used of the decoction were 1.2%, 0.8%, 0.6%, 0.4%, 0.2%, 0.1%. Two controls were performed with deionised water, and two with 50 ml deionised water plus 1.92 mg finely ground *Bti* 600 UTI/mg, the quantity used in the test was calculated considering the amount of one pill in 50 l of water. After a period of 24 hours the alive and the dead larvae and pupae were counted. In recording the percentage mortalities for each concentration, the moribund and dead insect in both replicates were combined. Larvae were considered dead when they showed immobility even if probed with a needle in the siphon or in the cervical region. Moribund larvae were those incapable of rising to the surface (within a reasonable period of time); they might show discolouration, unnatural positions, tremors, uncoordination or rigour. Each bioassay was repeated 20 times.

Statistical analyses

Data were subjected to ANOVA in which different concentration levels (1.2%, 0.8%, 0.6%, 0.4%, 0.2%, 0.1%, control and *Bti* and stage (pupae or larvae of *Ae. albopictus*) with their interactions were considered as fixed effects and percentage mortality as the independent variable. The concentrations that caused 50% mortality (LC_{50}) were determined for pupae and larvae. Statistical analysis was undertaken using the statistical package JMP (SAS, 2002).

LC-ESI/MS analysis

LC-ESI/MS analyses were performed using a Surveyor LC pump, a Surveyor Autosampler, coupled with LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with Xcalibur 1.3 software. Analyses were carried out using a 2.1 mm x 50 mm i.d., X-BRIDGE ^{TM} C_{18} 3.5 µm column (Waters Corporation, Milford, MA, USA). The liquid phase was a mixture of MeOH (solvent A) and aqueous CH_3COOH 0.05% (solvent B). The solvent gradient was as follows: 0-20 min from 60% (A) to 80% (A); 20-25 min from 80% (A) to 100% (A). Elution was performed at flow rate of 0.3 µl/min. The volume of the injection was 3 µl for all the samples (standard of calibration and aqueous extract). The analyses were performed in Full Mass (FM) and Product Ion Scan (PIS) on positive ion mode. The ionization conditions were optimized on pure standards saponins and the following parameters were retained: capillary temperature, 280 °C; sheath gas flow rate, 80.00 au; auxiliary gas flow rate, 40.00 au; capillary voltage, 32 V; tube lens offset, 55.0 V; source voltage, 3.9 kV. N_2 was used as sheath and auxiliary gas.

A solution (0.5 mg/ml) of aqueous extract of *P. notoginseng* flower buds was prepared dissolving residue in HPLC grade water and 5 µl of this solution was injected for analysis each time. Triplicate injections for three times were repeated. In order to detect the saponins composition of the plant decoction, the sample preparation and HPLC analytical condition were adjusted to avoid any detectable degradation of these compounds during the performance.

All standards ginsenosides (Rb1, Rb2, Rc, Rd) injected in the same optimized condition gave the same response by electrospray ionization; considering that ginsenoside-Rb2 was the most abundant in our extract it was selected as external standard of calibration. Standard curve calibration was prepared over a concentration range of 1.9-250 µg/ml with eight different concentration levels each double of the previous. Triplicate injections were performed for each level and a weighed linear regression was generated. The curve of calibration with external standard was obtained using concen-

tration (μg/ml) with respect to area obtained from integration of the MS Base Peak extracting the value of area of ginsenoside-Rb2 for $[M+Na]^+$. The relation between variables was analysed using linear simple correlation. For the linear regression of the external standards, R^2 was 0.9996. For the quantification of the saponins the GraphPad Software, Prism 3.0 was used. The amount of the compounds was finally expressed both % weight saponins/weight dried flower buds aqueous extract and mg of saponin/g of dried flower buds.

Results

Table 1 shows of the activity of *P. notoginseng* aqueous extract against larvae and pupae of *Ae. albopictus* expressed as mortality percent. The highest activity both against larvae and pupae resulted at 1.2% concentration (100% mortality). This concentration was statistically significantly different from control and from 0.4%, 0.2%, and 0.1% concentrations. It is also evident that at 0.8 and 0.6% concentrations were not different from 1.2% both for larvae and pupae. Between 0.6% and 0.4% concentrations there were not significant differences against larvae while 0.2% and 0.1% concentrations were not different from control. Against pupae 0.4%, 0.2%, and 0.1% there were not statistically different but these concentrations were different from to the other tested concentrations. *Bti* activity caused 100% mortality on larvae but it was ineffective against pupae (100% mortality vs. 0% mortality of pupae; P<0.01). The LC_{50} of *P. notoginseng* flower buds aqueous extract of *Ae. albopictus* were calculated for larvae and pupae 0.355% and 0.486%, respectively.

To results obtained in the LC-ESI/MS/MS investigation of the *P. notoginseng* decoction (figure 1 and table 2) showed the presence principally of dammarane saponins **1**-**11** (see figure 2). Data showed the principal constituents of the extract were identified as notoginsenoside-Fa (peak A, **1**), notoginsenoside-Fc (peak B, **2**), ginsenoside-Rb1 (peak C, **3**), ginsenoside-Rc (peak D, **4**), ginsenoside-Rb3 (peak E, **5**), ginsenoside-Rb2 (peak F, **6**), ginsenoside-Rd (peak G, **7**), gypenoside XVII (peak H, **8**), notoginsenoside-Fe (peak I, **9**), notoginsenoside-Fd (peak L, **10**), ginsenoside-F2 (peak M, **11**). From a quantitative point of view (see table 3) the content of saponins in the aqueous extract of *P. notoginseng* flower buds is around 35% of dried extract weight equal to 67 mg of saponins/g of dried flower buds. The main saponin resulted ginsenoside-Rb2 (**6**) followed by notoginsenoside-Fd (**10**), and ginsenoside-Rc (**4**).

Table 1. Percentage mortality of *Ae. albopictus* larvae and pupae exposed to aqueous extract of *P. notoginseng* for 24 h.

	larvae % mortality ± SD	pupae % mortality ± SD
Control	0 C	0 B
Bti	100 A	0 B
1.2%	100 A	100 A
0.8%	90 A	80 A
0.6%	80 AB	75 A
0.4%	60 B	30 B
0.2%	25 C	20 B
0.1%	0 C	0 B

Different letters on the same column indicate significant differences (P<0.01).

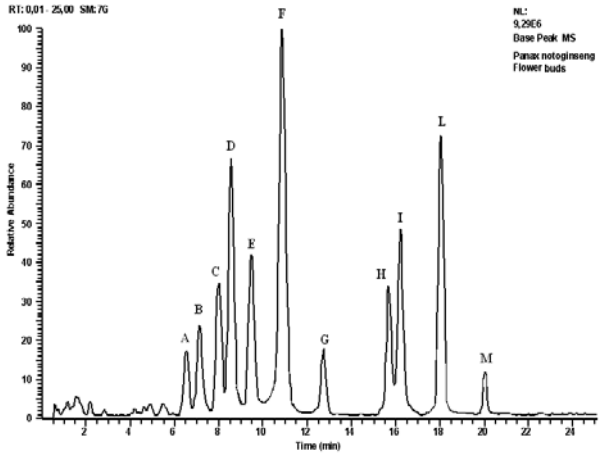
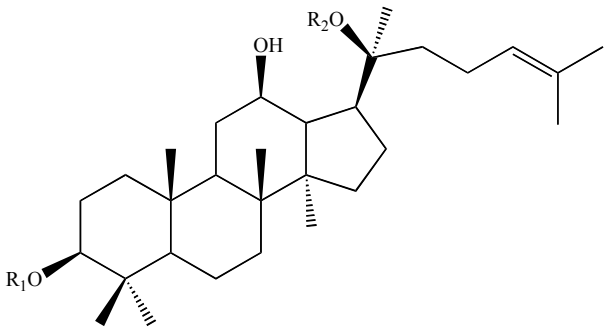


Figure 1. LC-ESI/MS Base Peak Chromatogram of aqueous extract of *P. notoginseng* flower buds (chromatographic conditions are reported in the text).

Table 2. MW, t_R, $[M+Na]^+$, MS^2 fragments of saponins from flower buds aqueous extract of *P. notoginseng*.

Peak	MW (u)	t_R (min)	Compound	$[M+Na]^+$ (*m/z*)	MS^2 fragments (*m/z*)
A	1240	6.5	(**1**) Notoginsenoside-Fa	1263	921, 719, 365
B	1210	7.2	(**2**) Notoginsenoside-Fc	1233	921, 497
C	1108	8.0	(**3**) Ginsenoside-Rb1	1131	789, 365
D	1078	8.6	(**4**) Ginsenoside-Rc	1101	789, 335
E	1108	9.5	(**5**) Ginsenoside-Rb3	1233	921, 789, 467
F	1078	10.9	(**6**) Ginsenoside-Rb2	1101	789, 335
G	946	12.7	(**7**) Ginsenoside-Rd	969	789
H	946	15.5	(**8**) Gypenoside XVII	969	365
I	916	16.2	(**9**) Notoginsenoside-Fe	939	789, 335
L	916	18.0	(**10**) Notoginsenoside-Fd	939	335
M	784	20.0	(**11**) Ginsenoside-F2	807	807, 627



	R_1	R_2
(**1**) Notoginsenoside-Fa	$Xyl^{1-2}Glc^{1-2}Glc$-	$Glc^{1-6}Glc$-
(**2**) Notoginsenoside-Fc	$Xyl^{1-2}Gcl^{1-2}Glc$-	$Xyl^{1-6}Glc$-
(**3**) Ginsenoside-Rb1	$Glc^{1-2}Glc$-	$Glc^{1-6}Glc$-
(**4**) Ginsenoside-Rc	$Glc^{1-2}Glc$-	$Ara(f)^{1-6}Glc$-
(**5**) Ginsenoside-Rb3	$Glc^{1-2}Glc$-	$Xyl^{1-6}Glc$-
(**6**) Ginsenoside-Rb2	$Glc^{1-2}Glc$-	$Ara(p)^{1-6}Glc$-
(**7**) Ginsenoside-Rd	$Glc^{1-2}Glc$-	Glc-
(**8**) Gypenoside XVII	Glc-	$Glc^{1-6}Glc$-
(**9**) Notoginsenoside-Fe	Glc-	$Ara(f)^{1-6}Glc$-
(**10**) Notoginsenoside-Fd	Glc-	$Xyl^{1-6}Glc$
(**11**) Ginsenoside-F2	Glc-	Glc-

Figure 2. Structures of saponins identified in aqueous extract of *P. notoginseng* flower buds.
[Glc: β-D-glucopyranosyl; Xyl: β-D-xylopyranosyl; Ara(f): α-L-arabinofuranosyl; Ara(p): α-L-arabinopyranosyl].

Table 3. Quantitative amount of *P. notoginseng* flower buds saponins by HPLC-ESI/MS analyses.

Compound	% saponin / dried extract	mg saponins / g dried flower buds
(**1**) Notoginsenoside-Fa	0.91	1.75
(**2**) Notoginsenoside-Fc	1.58	3.05
(**3**) Ginsenoside-Rb1	2.58	4.98
(**4**) Ginsenoside-Rc	4.24	8.18
(**5**) Ginsenoside-Rb3	3.51	6.77
(**6**) Ginsenoside-Rb2	10.22	19.72
(**7**) Ginsenoside-Rd	1.15	2.22
(**8**) Gypenoside XVII	2.09	4.03
(**9**) Notoginsenoside-Fe	2.56	4.94
(**10**) Notoginsenoside-Fd	5.32	10.26
(**11**) Ginsenoside-F2	0.68	1.31

Discussion

The LC-ESI/MS/MS analyses of *P. notoginseng* flower buds aqueous extract showed the presence of triterpene saponins in a good amount, circumstantially confirming the relationship between these secondary metabolites and the insecticidal activity seen in our bioassays. In fact, previous larvicidal investigations on natural products against larvae of *Ae. albopictus* showed a similar larvicidal efficacy of triterpene saponins (Santiago *et al*., 2005; Pelah *et al*., 2002).

The identification of each saponin into the decoction was accomplished by comparison with pure standards and literature information. In fact, the identity of ginsenoside-Rb1 (**3**), ginsenoside-Rc (**4**), ginsenoside-Rb2 (**6**), and ginsenoside-Rd (**7**) with peaks C, D, F, G in the LC-ESI/MS chromatogram, respectively, was confirmed injecting pure standards and comparing their retention time, molecular weight (obtained from the interpretation of FM experiments), and MS^2 fragments (obtained from PIS experiments) (figure 1 and table 2). The identity of the peaks A, B, E, H, I, L, and M was attributed on the basis of their fragmentation pattern and literature information. (van Breemen *et al*., 1995; Chan *et al*., 2000; Dou *et al*., 2001; Yoshikawa *et al*., 2003; Li *et al*., 2004).

The larvicidal and pupicidal activity of *P. notoginseng* flower buds makes it an attractive potential control agent. *B. thuringiensis* which has a good larvicidal activity but is totally ineffective against the pupal stage of *Ae. albopictus*.

The application of crude plant extracts as insecticidal product could be more efficient than attempting to apply pure compounds isolated from plant extracts which would be comparatively time consuming and costly.

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