Secondary Metabolites and Insecticidal Activity of *Anemone pavonina*

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The insecticidal properties of the crude extracts of the leaves and flowers of *Anemone pavonina* were evaluated on *Pheidole pallidula* ants and showed significant levels of activity. Bioassay-guided fractionations led to the isolation of the butenolide ranunculin (**1**) as the active principle. Chemical investigations of the extracts showed them to contain as major components the sitosterol glycopyranoside lipids **2**–**5** and the glycerides **6**–**8**. The structures of the metabolites were elucidated, following acetylation and hydrolysis of the natural products, by interpretation of their NMR and mass spectral data. The uncommon lipid metabolites **2**–**8** were isolated for the first time from the genus *Anemone* and this is the first report of insecticidal activity of the *Anemone* metabolite ranunculin against ants.

Key words: Insecticidal Activity, *Anemone pavonina*, *Pheidole pallidula*

Introduction

Many species of the plant family Ranunculaceae possess an array of insecticidal and antifeedant compounds. The genus *Anemone* (Ranales, Ranunculaceae) comprises perennial herbs and is represented with 17 species in Europe in 4 sections whereas 7 species in 2 sections are native in Greek mainland and Crete (Tutin, 1964). The species *A. pavonina* Lam. hybridizes in many Mediterranean regions with the central Mediterranean species *A. hortensis* L. giving the putative hybrid *A. fulgens* Gay, which is unable to breed (Maia and Venard, 1976). In the limited number of reports, species of the genus *Anemone* have been found to contain acetates of -amyrin and -sitosterol (Connolly and Hill, 2002) along with the saponins of oleanolic acid (Zhang *et al.*, 1997), hederagenin saponins (Wang *et al.*, 1997), as well as the butenolide ranunculin, which on fermentation yields glucose and protoanemonin.

A. *pavonina* is growing in many habitats of Attica and Crete (Strid, 2002) where it is the dominant flowering plant in the winter until mid spring. As a result of the armature of compounds that protect its precious tissues from insects, the fresh stem and leaves were used by ancient Greeks in the protection of the feeds from insects and in the preparation of pharmaceuticals (Raven and Stearn, 1990).

The omnivorous ant Nylander is abundant in the Mediterranean region and in many locations it is an effective competitor of the arboricolous myrmicine ant *Crematogaster scutellaris* (Olivier) (Cammaerts and Cammaerts, 1996, 1998).

In many Mediterranean ecosystems *A. pavonina* coexists with the ant *Pheidole pallidula* but field observations showed consistent repellency of the plant against ants. The ants never collected achenes of the plant and flowering plants were never visited by the *P. pallidula* workers or scouts, in spite of the fact that *A. pavonina* dominated the herbaceous plains of many Mediterranean-type open shrublands.

In continuation of our investigations towards the discovery of natural products with insecticidal activity or insect repellency from Greek endemic and Mediterranean plant species (Roussis *et al.*, 2000; Christodoulopoulou *et al.*, 2005), triggered by our field observations, we were able to collect and study the perennial herb *A. pavonina* growing wild in many regions of Greece.

† Deceased during his studies.

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Materials and Methods

Plant material

Anemone pavonina Lam. belongs to the family of Ranunculaceae and is a perennial herb, growing wildly in mountainous areas. The plant was collected from the slopes of Mt. Kithairon, Attica, Greece during the flowering period (February–March) and after separation leaves and flowers were kept, till chemical processing, in a well-aerated dark chamber. Voucher specimens are kept at the Herbarium of the Department of Pharmacognosy and Chemistry of Natural Products, University of Athens, Athens, Greece (MT-321).

Insect collection and bioassays

Ants were collected from a Mediterranean habitat on the west slope of Mt. Hymettus, Kalopoula district and Mt. Kithairon, Erenia district, Attica, Greece. Whole nests were excavated and transferred to the laboratory where they were kept for four months in plexiglass boxes at room temperature in the greenhouse. The ants were fed either twice a week with artificial food made with honey, grinded dry liver, egg yolk and grinded corn flakes and pellets of dog food or three times a week with fresh fragments of cockroaches. The ants used in the bioassay were introduced in the arena (Petrakis *et al*., 2005) and left in the middle for 24 h for acclimatization.

The extracts were submitted to bioassay-guided fractionation by normal phase chromatography. Each fraction was tested by three individual groups of ant minor workers. The fraction showing the greater killing efficiency was further fractionated until we were able to isolate and identify the active metabolite by means of spectroscopic methods (NMR, MS).

The bioassay of the extracts/compounds of *A. pavonina* was carried out by an adaptation of the fabric contact assay (Kwon and Ahn, 2002). For these experiments 22 mg and 25 mg from the extract of leaves and flowers, respectively, were dissolved in 100 μl of solvent (CH_2Cl_2/MeOH). The quantities that were applied on filter paper disks (3 cm) were calculated so that the surface density of the application would approximate the natural contents (*i.e.* 3.97% and 4.25% extract weight/dry leaf weight, respectively). The disks, after the evaporation of the solvents, were impregnated with a saturated sugar solution and were placed in Petri dishes (5 cm and 9 cm) along with 6 minor and 8 major workers. There were 20 iterations (10 with each type of extract) of the Petri dishes with minors and 16 (8 with each type of extract) for majors of *P. pallidula* ants. Control dishes were prepared with 100 μl of solvent. Mortality was recorded every 10 h for a period of 2 d.

The same procedure was followed in the bioassay-guided fractionation procedure until the fraction containing ranunculin (**1**) was proven to be the killing agent. Furthermore, 10 μg of pure ranunculin were dissolved in 1 ml of dichloromethane/methanol (approx. natural concentration) and evenly spread on a disk of filter paper (3 cm). In each Petri dish 5–9 ants were introduced in seven repetitions and the percentage mortality was estimated after 24 h.

Extraction and isolation

Quantities of the plant tissues (leaves dry weight: 178 g; flowers dry weight: 69 g) were separately exhaustively extracted at room temperature with mixtures of CH_2Cl_2/MeOH (3:1). The organic extracts after evaporation of the solvents afforded dark oily residues (leaves: 7.2 g; flowers: 3.1 g). The crude solvent extract of the leaves, that showed comparable insecticidal activity with the flower extract at the preliminary evaluation, was subjected to vacuum column chromatography (VCC) on silica gel using cyclohexane with increasing amounts (10% increments) of EtOAc and afterwards increasing amounts of MeOH (10% increments). Bioassays showed fraction IX (40% EtOAc in MeOH) (937 mg) as the most active fraction which was further purified by VCC on silica gel using 15% MeOH in CH_2Cl_2 as eluent. The most active fractions of this separation were subjected to medium pressure liquid chromatography (MPLC) with 10% MeOH in CH_2Cl_2 to afford pure metabolite **1** (93.1 mg).

The less polar fraction VI (540 mg) of the initial separation (10% MeOH in EtOAc) showed in the respective ^{1}H NMR spectrum interesting resonances and it was decided to analyze it further for the isolation of its constituents even though it did not exhibit significant ant toxicity. Since in the spectrum the presence of sugar moieties was evident the residue was acetylated with AcOAc in pyridine to afford the corresponding acetylated metabolites in the reaction mixture. Repetitive separations by normal phase HPLC with isocratic elution systems (10–20% EtOAc in hexane) resulted in the isola-

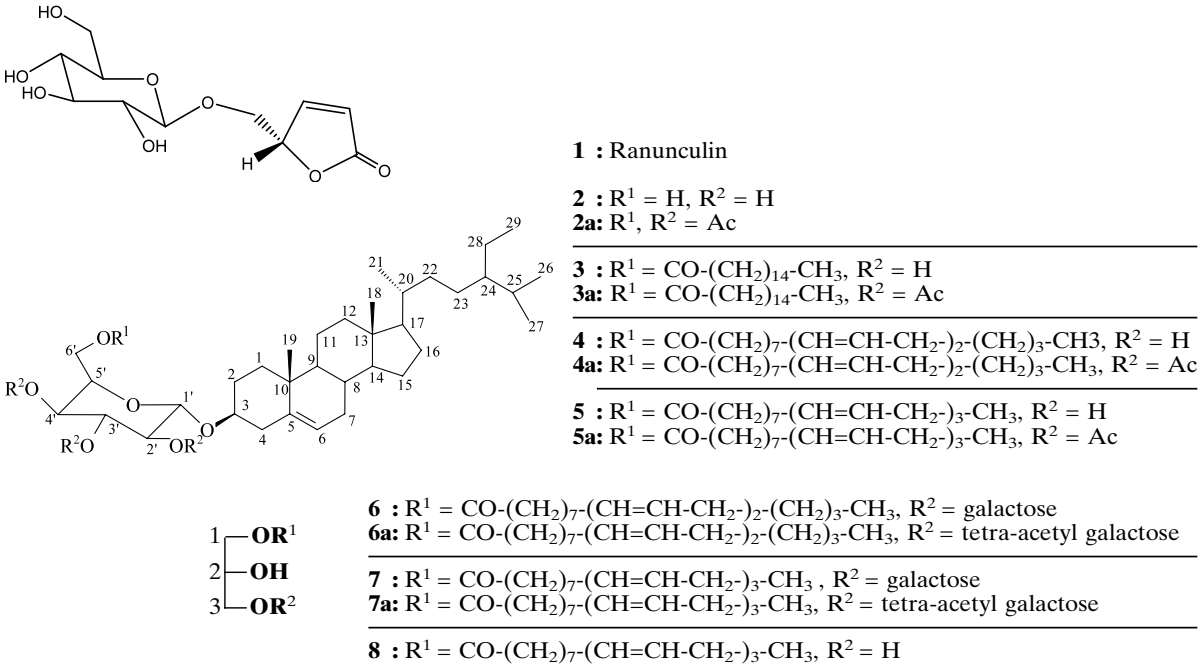

Fig. 1. Isolated metabolites.

tion of derivatives **2a** (7.0 mg), **3a** (4.1 mg), **5a** (6.2 mg), **6a** (8.3 mg), **7a** (7.8 mg).

Fraction V (167 mg), eluted with 40% hexane in EtOAc, was further purified by normal phase HPLC (40% EtOAc in hexane) to afford metabolite **8** (3.2 mg).

Quantities (1–3 mg) of acetates **2a**–**7a** and metabolite **8** were separately hydrolyzed by 1 M HCl in MeOH (10 ml) under overnight reflux. The concentrated reaction mixtures were partitioned between H_2O and CH_2Cl_2 and the resulting organic layers, after drying over $MgSO_4$, were analyzed by GC-MS for the identification of lipid moieties (Fig. 1).

Chemical analysis

Optical rotations were measured using a Perkin-Elmer model 341 polarimeter and a 10 cm cell. IR spectra were obtained using a Paragon 500 Perkin-Elmer spectrophotometer. NMR spectra were recorded using a Bruker AC 200 and a Bruker DRX 400 spectrometer. The 2D experiments (^1H-^1H COSY, HMQC, HMBC, NOESY) were performed using standard Bruker microprograms. High resolution mass spectra data were provided by the University of Notre Dame, Department of Chemistry and Biochemistry, Notre Dame, Indiana. EIMS data were recorded on a Hewlett Packard 5973 mass selective detector. Vacuum column chromatography (VCC) separations were performed on Kieselgel 60H (Merck), TLC analyses were performed with Kieselgel 60 F_{254} (Merck aluminum support plates) and spots were visualized upon spraying with 5% H_2SO_4 in MeOH and heating. HPLC separations were conducted using a Pharmacia LKB 2248 model and GBC LC-1240 refractive index detector with a Spherisorb S10W (Phase Sep; column size, 10×250 mm) column.

Statistical analysis

Corrections for control mortality were made by using Abbott's formula (Abbott, 1925). For the corrected values below zero a further correction of McCullogh and Nelder (Throne *et al.*, 1995) was applied. The mortality of insects was checked at regular intervals and the dead ants were counted. Since time observations are correlated, the probit and logit models of multiple observations over time developed by Throne and coworkers (Throne *et al.*, 1995) were applied. The mortality of the worker types (majors, minors) and the plant organs (flowers, leaves) were compared and tested

by the Scheffé contrast test (Johnson and Wichern, 1998).

Results and Discussion

According to the phylogeny of Ranunculaceae (Hoot, 1991; Hutchinson, 1969) based on characters of 24 genera, the family Ranunculaceae is divided into two tribes on the basis of the size of chromosomes (*i.e.* Ranunculoideae and Thalictroideae). The first tribe is further subdivided into four species groups, namely *Anemone*, *Delphinium*, *Helleborus* and *Cimifuga*. Previous studies have shown members of the group *Anemone* and members of the phylogenetically distant genus *Helleborus* to be ecdysteroid producers, which have been suspected to play a decisive role in the configuration of the invertebrate predator fauna (Dinan, 1998; Dinan *et al.*, 2002).

P. pallidula ants were chosen, in this study, as the model organism for the evaluation of insecticidal activity because they are one of the major predators in the ecosystems in that *A. pavonina* is growing and are consistently avoiding contact with the plants. Individually assayed against *P. pallidula* ants, extracts of the leaves and flowers of *A. pavonina* showed the former to have comparable levels of insecticidal activity (Table I). The bioassay-guided separation of the leaves extract by vacuum column chromatography and normal-phase high pressure liquid chromatography led to the isolation of metabolite **1** which was proven to be the active principle of the extract. Spectral studies based mainly on NMR analyses and mass spectra and comparison with reported values (Camps *et al.*, 1982) led to the identification of metabolite **1** as the butenolide ranunculin (Fig. 1). Ranunculin has been reported in the past as constituent of other *Anemone* species (Ruijgrok, 1963). The antifeedant and insecticidal properties of protoanemonin and ranunculin have been shown in the past for a number of other insects (Dinan *et al.*, 2002).

In the bioassays, ants exposed to flowers extracts had a LT_{50} mortality of (17.4 ± 2.2%) while those exposed to leaves extract had a LT_{50} value of (18.9 ± 1.9%). The probability of contrast comparisons according to Scheffé is given in Table I, in which the masking of the plant organs by the ant worker types cannot be avoided.

In both types of extracts – *i.e.* from leaves and flowers – the mortality of ants was roughly the same. In this way the plant tissue conforms to the statement that ontogenetically the perianth segments are modified leaves (Cronquist, 1988). Indeed, the influence of plant organs on the differential mortality of worker types (*i.e.* the so-called "statistical interaction") is not significant (*d.f.* 1 = 1; *d.f.* 2 = 38; $F = 0.023$; $P = 0.881$ ns). The comparison probabilities in Table I can show the insignificance of differences in mortality among plant organs despite the masking effect of the mortalities of majors and minors of *P. pallidula* (*e.g.* all the significant bold faced values while $P < 0.751$ ns, among majors exposed to flowers and leaves, and $P < 0.529$ ns, among minors).

In the general model below it was attempted to separate the effects of ants and plant organs:

LT_{50} = *constant* + (worker type) + (plant organ) + (worker type) · (plant organ).

It was found that $r = 0.81$; *d.f.* 1 = 1, *d.f.* 2 = 38; $F = 4.17$; $P = 0.052$ ns for all worker types and plant organs while for workers only, after the effect of the plant organs has been removed, it is *d.f.* 1 = 1; *d.f.* 2 = 38; $F = 69.09$; $P < 10^{-4}$.

From the fourteen measurements taken, for minor workers it was found that $t = 0.654$; *d.f.* = 13; $P = 0.525$ ns and for major workers $t = 0.252$; *d.f.* = 13; $P = 0.805$ ns. It is possible that the basic secondary chemistry is the same for leaves and flowers of *A. pavonina* and for this the mortality curves

Table I. Matrix of pairwise probabilities according to the Scheffé method[a].

	Flowers – major workers	Flowers – minor workers	Leaves – major workers	Leaves – minor workers
Flowers – major workers	1			
Flowers – minor workers	**0.000**[a]	1		
Leaves – major workers	0.751	**0.000**	1	
Leaves – minor workers	**0.006**	0.529	**0.000**	1

[a] Significance of differences is given in bold.

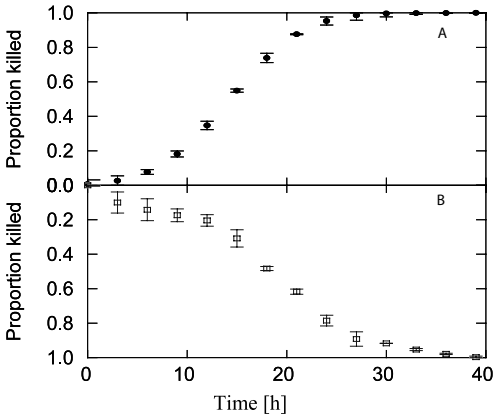

Fig. 2. The proportions of major (A) and minor (B) workers killed within given times of exposure. The error bars correspond to standard deviation.

are very similar for both types of extracts. In what follows the mortality data are pooled together.

The minor workers data was fitted to the probit model. The fit was highly significant (r^2 = 0.998; F = 0.0027; *d.f.* 1 = 2; *d.f.* 2 = 14; $P < 10^{-4}$). The actual mortality data together with the standard error bars are shown in Fig. 2B. It can be seen that above 90% mortality the error bars are very narrow indicating that almost all groups presented the same level of mortality. The LT_{50} for minor workers is 18.1 while for majors it is 15.2. For majors the data was fit to the logistic model in a significant way (r^2 = 0.990; $F = 8 \cdot 10^{-4}$; *d.f.* 1 = 2; *d.f.* 2 = 14; $P < 10^{-4}$). For majors the model curve values are shown in Fig. 2A. It can be seen that the error bars are much more narrow than the ones for minors indicating that majors have more homogeneous behavioral ways to feed and search unlike the fact that state that bigger ants have a richer behavioral repertory and accordingly they are more heterogeneous in their behavior (Holdobler and Wilson, 1990).

The natural content of ranunculin in the leaves of *A. pavonina* was found to be 0.34% (w/dry w). The bioassay showed 88.4% (mean standard deviation of seven values 11.70%) mortality.

Chromatographic separations aiming at the isolation/identification of the other secondary metabolites of *A. pavonina* leaves led to fractions that by their NMR characteristics were evident to contain polar lipid derivatives. In an attempt to handle them easier and purify these metabolites, quantities of the fractions were acetylated and the corresponding, less polar reaction mixtures were fractionated to allow the isolation of the acetylated metabolites **2a**–**7a** (Fig. 1). Analyses of the NMR data showed compounds **2a**–**5a** to be sitosterol glucopyranosides esterified at the 6′ hydroxy group by a series of different lipids. The mass spectra of the compounds and the GC-MS comparison of the lipid methyl esters, as afforded following methanolysis, with commercially available standards led to the identification of **2a** and **3a** as acetylated sitosterol-3-*O*-*β*-D-glucopyranoside and sitosterol-3-*O*-6-palmitoyl-*β*-D-glucopyranoside (Santyanarayama *et al.*, 1991), **4a** and **5a** as sitosterol-3-*O*-6-linoleoyl-*β*-D-glucopyranoside and sitosterol-3-*O*-6-linolenolyl-*β*-D-glucopyranoside (Hasimoto *et al.*, 1991). The NMR spectra of the acetates **6a**, **7a** and metabolite **8** indicated them to be galactosyl glycerols, esterified with different fatty acids. Methanolysis of the lipid chains and GC-MS analyses led to the identification of **6a** and **7a** as 3-galactosyl-1-linoleoyl-glycerol and 3-galactosyl-1-linolenolyl–glycerol (Kwon *et al.*, 1998) and **8** as linolenyl acid glyceride (Vlahov, 1998). Comparison of the NMR spectra before and after acetylation showed the complete absence of acetyl methyl resonances; so it was concluded that the natural products **2**–**7** are deprived of any acetyl groups. Comparison of the spectral data of the isolated metabolites with the literature values confirmed their identification.

Metabolites **2**–**8** have never been reported from the genus *Anemone*. Compounds **4** and **5** have been isolated only once before, from *Edgeworthia chrysantha* (Thymelaeaceae) (Hasimoto *et al.*, 1991), and they were shown to possess potent piscicidal activity against killie-fish. Galactosylacylglycerol derivatives **6** and **7** have also been reported only one time from *Hydrocotyle ramiflora* (Umbelliferae) (Kwon *et al.*, 1998) and *Sonchus arvensis* (Compositae) (Baruah *et al.*, 1983), respectively. Members of this chemical class have shown anti-inflammatory (Kikuchi *et al.*, 1982) and platelet aggregation inhibitory activity (Fusetani and Hasimoto, 1975; Oshima *et al.*, 1994).

Ants constitute one of the most serious problems in agriculture, storehouses and in household areas and the management of their populations with environmentally compatible means still remains a major scientific challenge. The promising levels of activity exhibited by ranunculin might lead, following future studies on several other ant species, to the development of a pest control system based partially or solely on this botanical formulation.

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