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Cantharidin Component of Iranian Blister Beetles (Col: Meloidae) and their differences between Iranian and Exotic Species

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Abstract

Cantharidin is one of the most well- known compounds which has ever been fascinating in medicine due to its effects on human and domestic animals. It is produced naturally by beetles of family Meloidae and Oedemeridae, however a considerable spectra of other insects sequestered it too. Cantharidin along with the other analogue, Palasonin, which has a methyl group less than it, found in the hemolymph and all tissues of both cantharidin producing and cantharipilous taxa. Although, palasonon mostly found in low volume, some species bear it even in a higher amount than cantharidin; thus it may be regarded as a precursor for cantharidin synthesis in producing taxa. Measuring titre of both chemicals in Iranian blister beetles from Nahavand county, Hamedan Province and some other species from France, Italy and South Africa, we have tried to have an index to differentiate species or at least different poulations of the same species as it has already done for family Staphylinidae. In this way, cantharidin may not be a good inicator, but it seems more effective along with other analogues, chemicals and some simple physiological information. Cantharidin/Palasonin ratio (C:P) is a better index which seems efficient in dividing European species from similar South African ones. Palasonin itself can be used to diffrentiate Iranian species from all other studied groups. More chemicals used in this new developed method, more precise is the classification.

Key- words : Blister beetles, Meloidae, Cantharidin, Chemical Ecology, Iran

Introduction

Many arthropods are known to take up and sequester toxic secondary compounds without being damaged. On the contrary, they use these defensive chemicals of plant origin for their own purpose and may increase their individual fitness. Interesting association can be seen in nature between secondary chemicals and so- called pharmacophagous insects (18). Uptaken compounds are highly attractive to these insects; that is why they even take the desired chemical orally, detoxified and finally sequestered it. These compounds may help the pharmacophagous organism for better survivorship (5). The insect derived compound, Cantharidin, may be placed in such a category too (7).

Cantharidin, a terpenoid substance (Fig. 1) found in blister beetles (Coleoptera: Meloidae), is among the widely known insect natural products in the world (1, 7, 12, 16). Its reputation derives principally from description of its physiological activities, most notably as an aphrodisiac for human and livestock (14, 16, 17, 19). Cantharidin is also the blistering agent that earned these bettles their common name (7, 12). For more than 2000 years, blister beetles in powered or tincture form have been used medicinally in Europe, China, Korea and elsewhere as a diuretic and abortificient as well as an aphrodisiac (11, 12, 14, 17). LD_{50} in human is 10-50 mg/kg, but 0.5 mg lodged in the throat could cause blistering sufficient to produce death by suffocation (12). Cantharidin was purified and crystallized in 1810 by Robiquet from Spanish fly, *Lytta vesicatoria* (8, 12). Surprisingly, for a structure remarkable for its simplicity, it took 150 years of study to be fully synthesized (3, 8, 12).

It is a monoterpene anhydride which inhibits Protein Phosphatase 2A (PP2A). In order to activate the phosphorylated and thus inactivated odorant receptor proteins, the phosphate groups must be rapidly removed by PP2A (7, 9).

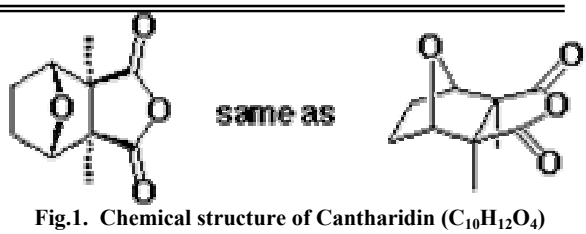

Fig.1. Chemical structure of Cantharidin ($C_{10}H_{12}O_4$)

In nature cantharidin is only known to be produced by blister beetles (Col: Meloidae) and false blister beetles beetles (Col: Oedemeridae) where it is found in haemolymph and various tissues (2, 4, 7, 18). In plants, cantharidin has not been discovered ; however, insecticidal seeds of the Indian tree , *Butea frondosa* (Leguminosea) contain demethyl cantharidin, where the 3-methyl group of cantharidin is missing (7, 13). The possibility that cantharidin is transferred from males into females during mating was suggested long ago by Beauregard in 1890 (7, 11). Transfer of cantharidin from males to females of meloid beetles has been demonstrated now (1, 3, 6, 15).

In the Present article, collecting of meloid beetles in the western province of Hamedan (Iran) along with the methodology of cantharidin detection and quantification is first described and then it is discussed if there is any difference in volume of cantharidin and its analogue between Iranian and exotic species. Finally, the possible application of these information in chemosystematism of blister beetles is briefly noted. Little work has been done so far in the world which is partly due to complex laboratory rearing of blister beetles.

Materials and Methods

Beetle Collection

Meloid beetles, collected in four different locations. Iranian species were collected in Nahavand county, Hamedan

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province at the latitude of 34,13 N and longitude of 48,21 E and the European ones (*Hycleus polymorphos* and *Mylabris quadripunctata*) in Cèvennès National Park, 80 Km Southwest of Ales, Southern France whereas Italian samples (*H. polymorphos*) were collected from Northern Italy, 40 km North of Torino. Apart from mentioned species, *Hycleus lunata* was also collected in Western Cape Province, about 75 km North west of Cape Town, South Africa. Elevation of field inspection in Iran, France, Italy and South Africa were about 1300-1550, 650-1105, 1150-1650 and 500-900 m respectively. All specimens were collected on different shrubs of family Astraceae, Compositeae and Leguminoseae during May- July 2001 in Iran, June 2002 in France, July 2002 in Italy and January & February 2002 in South Africa. Beetles were on the plants and those fallen on the ground were simply picked up, placed in small glass bottles individually and transported to the laboratory in electric isoterm containers , not to let the temperature rises above –10 °C. In the laboratory, materials were kept in –30 °C freezer for further studies.

All the species were identified by the available keys and later they were confirmed by comparing them to the types in Hungarian National History Museum (HNHM), Budapest (10).

Chemical Analysis

Studies of ecological, physiological and behavioural role of cantharidin in meloid beetles and other insect taxa within food web requires an effective means for cantharidin quantitation. Since the quantitation methodologies are important in the study and will be as well important in future experiments, we have described them in details.

Extraction

Prior to extraction, dry weight (DW) was determined for all samples. Whole body, reproductive organs, eggs, guts and body fragments were hydrolized in small fused test tubes with 100- 200 µl 6 N hydrochloric acid (HCl) at 120 °C for 4 hours in order to resolve biomatrix and to set the bound cantharidin free. Afterwards, an equivalent amount of chloroform was added and each sample was vigorously shaken on a Vortex mixer for 60 seconds. Then, samples were centrifuged at 2000 × g for 5 minutes. The organic phase was filtered and transferred into a vial. To reduce evaporation of chloroform during storage, a few droplets of deionised water added.

Quantitative Gas Chromatography

In order to detect and quantify total cantharidin, GC-MS with the following specification was used :

0.1-0.5 µl of each sample was injected splitlessly via a 1 µl Hamilton syringe into a Carlo Erba Vega Series 2, GC 6000 gas chromatograph equipped with a HT8 (non-polar) fused silica capillary column (Chrompack, FT 0.25 µ, ID 0.32 µm, OD 0.43 mm) which in turn connected to a Finnigan MAT Ion Trap Detector (ITD). Electron Impact Ionization (EI 70 ev) provides mass spectra with a characteristic fragmentation of cantharidin: the base peak with *m/z* 96 and two other fragments of *m/z* 128 and 96 (M+ : 196). Chromatographic conditions were as follows: initial temperature 60 °C, temperature increase of 10 °C/min up to 275 °C, an isoterm period of 10 minutes in 275 °C and then cooling down to 60 °c. Total mass Spectra analysed by X-Calibor 2000 and base peaks were compared by NBS registry of mass spectral data bank. Palasonin detection was done by a GC-Q equipped by an EI ionisation system with the following characters: HT5 fused cilica capillary column (Bounded phase, SGE; ID 25m × 0.32 mm, FT 0.1 µ). In order to prove detected chemical, technical form of the chemical supplied by SIGMA-ALDRICH Chemie GmbH, Taufkirchen, Germany was injected into the above mentioned gas chromatography system and the base peaks were compared to the sample.

Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopic characterization of cantharidin and palasonin is noteworthy for its simplicity in both spectral data and interpretation. Cantharidin possesses only four magnetically different hydrogen atoms, producing signals at 1.24 ppm (methyl), 1.78 and 1.80 ppm (methylene) and at 4.72 ppm (methine). Each NMR signal in both proton and carbon spectra is derived from both halves of the cantharidin molecule, providing unusual sensitivity.

Statistical analysis

Repeatability of experiments for one species was tested over five samples and an error of 4% was determined at the 95% confidence level. Cantharidin data were analysed by analysis of variance (two- way ANOVA; sex and species) for percentage of Cantharidin per beetle. Percent data were transformed (√% +0.59). Significant means were separated with Duncan's multiple range test at the P = 0.05 level.

Results

The results of our findings are presented in the following four tables:

Table 1. Titre of cantharidin in Iranian meloid species (Col: Meloidae) according to sex (Nahavand county, Hamedan province, 2001).

Species	Sex	Mean ± SD (%)	Range (%)	Mean ± SD (mg)	Range (mg)
Mylabris impressa	F	1.9 ± 1.5	0.5- 4.0	0.47 ± 0.28	0.12- 0.73
	M	5.7 ± 4.9	0.6- 12.6	1.24 ± 1.31	0.05- 3.23
Mylabris guerini	F	1.6 ± 1.0	0.4- 2.7	0.49 ± 0.30	0.15- 0.88
	M	2.8 ± 0.6	1.6- 3.5	0.70 ± 0.31	0.36- 1.07
Mylabris variabilis	F	0.4 ± 1.0	1.9	0.07 ± 0.11	0.29
	M	1.5 ± 1.64	0.12- 2.4	0.37 ± 0.30	0.03- 0.68
Mylabris schreibersi	F	1.34 ± 1.1	2.7	0.32 ± 0.26	0.07- 0.50
	M	5.73 ± 2.0	2.5- 8.8	1.14 ± 0.51	0.44- 1.89
Alosimus smyrnensis	F	1.35 ± 1.24	0.5- 3.0	0.52 ± 0.29	0.14- 0.69
	M	6.0 ± 5.2	0.6- 10.7	2.03 ± 2.07	0.10- 3.82
Muzimes iranicus	F	3.7 ± 2.9	1.8- 7.8	4.52 ± 2.52	2.18- 8.49
	M	5.4 ± 1.9	3.4- 8.1	5.21± 3.75	1.43- 10.12
Callydos alloushei	F	0.3 ± 0.2	0.35	0.06 ±0.05	0.13
	M	0.35 ± 0.17	0.3- 0.5	0.05 ± 0.02	0.03- 0.07
Croscherichia sp.	F	0.2 ± 0.1	0.55	0.11 ± 0.03	0.07- 0.18
	M	1.1 ± 0.6	0.46- 1.65	0.18 ± 0.11	0.10- 0.39
Lydoceras bilineatus	F	± 0.3	0.35	0.77 ± 0.54	0.08- 1.50
	M	0.4 ± 0.28	0.1- 0.8	0.82 ± 0.56	0.09- 1.51

Table 2. Cantharidin quantitation in different organs of a female *Mylabris impressa* (Col : Meloidae), 2001

Dissected Organ	Cantharidin (ng/mg) DW
foregut + hindgut	618
One piece of flight muscle	499
Complete gut (fmh)	3945
Bursa Copulatrix	7920
Ovary	11481
Fat Body	4454

Table 3. Titre of cantharidin in some meloid species according to sex, 2002.

Species	Sex	Mean ± SD (%)	Range (%)	Mean ± SD (mg)	Range (mg)
Hycleus polymorphos	F	1.6 ± 1.1	0.5- 2.9	0.52 ± 0.29	0.14- 0.79
(Southern France)	M	6.6 ± 4.8	0.7- 11.3	2.03 ± 2.07	0.13- 4.0
Mylabris quadripuctata	F	± 0.2	0.4	0.87 ± 0.95	0.09- 1.76
(Southern France)	M	0.5 ± 0.3	0.1- 0.9	0.84 ± 0.56	0.08- 1.57
Hycleus lunata	F	2.0 ± 1.5	0.5- 4.0	0.49 ± 0.28	0.14- 0.75
South Africa	M	6.6 ± 5.3	0.6- 12.7	1.25 ± 1.32	0.06- 3.38

Table 4. Volume of cantharidin, palasonin (ng/mg DW) and *C:P* ratio in genus *Hycleus* (Col: Meloidae), 2002.

Species	Location	Sex	No. of individuals	Volume of Cantharidin	Volume of Palasonin	*C:P* ratio
H. polymorphos	Northern Italy	F	14	484.2948	1224.5704	0.39
H. polymorphos	Southern France	F	23	343.77	1220.8541	0.28
H. nata	South Africa	F	31	58292.511	2067.0972	28.2

Discussion

Cantharidin which is the most important chemical in blister beetles, normally transferred by male to the females in almost all meloid species during mating. Therefore, it was necessary to measure separately the titre of cantharidin in both sexes.

Considering titre of cantharidin in Iranian and non- Iranian meloids, no significant difference is found (tables1 & 3). It seems that cantharidin may not be used as a chemical indicator to categorize family meloidae by a chemosystematic approach which has been already applied in rove beetles (Col: Staphylinidae).

One reason lies in the natural load of cantharidin in both sexes which is higher in males and the rest returns back to its transferase during copulation. In this way, a male before copulation has a high titre of the chemical, while its load will be decreased significantly just after copulation when most of the toxin transfers to the females' sexual organ as a nuptial gift. So, the virgin female which had low amount of toxin, would get it in a huge amount, which made the receptor of the highest titre of cantharidin. That is why, categorizing of meloids by only cantharidin quantitation seems impractical and other factores needed to interpret the data properly. Other chemical components of blister beetles can be used as indicator agents too. For example Palasonin (3-demethylcantharidin) which has a methyl group less than cantharidin (Fig. 2), is present in all species of genus *Hycleus*, from South Africa, Italy and France, but not in any of the mentioned Iranian species (table4). Palasonin has two optical isomers of them, (-) one isolated in 1967 from seeds of *Butea frondosa*, Leguminaceae (7).

Using a GC-Q system, palasonin was traced with base peaks of *m/z* 82 and 114 and molecular ion of M+ 182. Applying NMR system, the small difference in the chemical shifts of the exo and endo hydrogen signals does not permit assignment of these signals to specific hydrogen atoms. The geminal and vicinal coupling constants were too small to be accurately observed at 90 MHz.

The fact that palasonin has been found in meloids is of great importance. It may indicate the origin of cantharidin in insects; thus one may imagine palasonin as a precursor to be later shifted to famous toxin of meloid and oedemerid beetles, cantharidin. Of course, volume of palasonin in different meloid species is variable and more interesting, the point that cantharidin/palasonin (*C:P*) ratio is not fixed as well. However, many sources have already reported that the palasonin titre is always lower than cantharidin and only a traceable amount, we have hereby shown that it can not be always true. As indicated in table 4 , *C:P* Ratio between the two European species of genus *Hycleus* is different and less than the African one. Repeating of such experiments with other species or different populations may even lead to a reliable index to divide poulations or species. Another interesting point lies in the lackage of palasonin in many other genera of blister beetles. It is not known why only a small fraction of this family possesses palasonin and what the role of this moderately toxic substance is.

Concerning concentration of cantharidin in internal organs, current study (table 2) supports prior ones that highest concentration of cantharidin observed in sexual organs, particularly in ovary. It can also be concluded that total volume of cantharidin is a function of beetles' size, the fact reflected well in table 1. As table 1. shows, the highest level of cantharidin is seen in *Muzimes iranicus*, the large greenish- blue species found in Nahavand county, Hamedan province, Iran.

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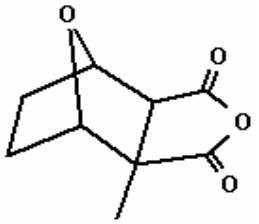

Fig. 2 Chemical structure of (-) Palasonin ($C_9H_{10}O_4$)

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