Lethal and sublethal effects of triterpenes from *Junellia aspera* (Verbenaceae) on the grain storage insect *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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■ **ABSTRACT.** Duration of the pupal stage, toxic activity and nutritional indices induced by oleanolic acid (I), maslinic acid (II), and daucosterol (III) isolated from the native plant *Junellia aspera* and chemical derivatives, were determinated on larvae, and adults of *Tribolium castaneum* (Herbst). The current study shows that these triterpenes acts as acute toxic compounds when were applied topically and/or incorporated into the food of the red flour beetle. Nevertheless, no activity related with the nutritional status of this insect was produced.

KEY WORDS. Triterpenes. Toxic effect. *Tribolium castaneum*. *Junellia aspera*.

■ **RESUMEN. Efectos letales y subletales de triterpenos aislados de *Junellia aspera* (Verbenaceae) sobre el insecto de granos almacenados *Tribolium castaneum* (Coleoptera: Tenebrionidae)**. Se determinó la duración del estado pupal, actividad tóxica e índices nutricionales sobre larvas y adultos de *Tribolium castaneum* (Herbst) inducidos por ácido oleanólico (I), ácido maslínico (II) y daucosterol (III) aislados de *Junellia aspera* y derivados químicos. Se demostró que los triterpenos evaluados actúan como tóxicos agudos cuando se aplican por topicación y/o son incorporados en el alimento. Los compuestos ensayados no presentaron actividad relacionada con el estado nutricional de los insectos.

PALABRAS CLAVE. Triterpenos. Efecto tóxico. *Tribolium castaneum*. *Junellia aspera*.

INTRODUCTION

A number of species of plants have been reported to have several effects on stored-product insects (Ho *et al*., 1995). It is well accepted that natural products from plants may constitute new sources of insect pest control. Alternative strategies have included the search for new kind of insecticides, and the re-evaluation and use of traditional botanical pest control agents (Huang *et al*., 1999).

Junellia sp. (Verbenaceae) genus includes 45 endemic species to the Andes Mountain and Patagonian region of South America, it has been used as a medicinal plant by diverse andine cultures (Caldwell *et al*., 2000; Villagrán *et al*., 2003) and contain some triterpenoids (Pungitore *et al*., 2004). Oleanane triterpenoids are pentacyclic compounds with 30 carbon atoms, biosynthetically derived from the cyclization of squalene. This is a wide class of natural products whose structural diversity include several arrays of functional groups (Honda *et al*., 2000). Recent reviews include effects of triterpenoid as cancer chemopreventive, anti-ulcer and antidiabetic agents, angiogenesis inhibitors, and as inhibitors of the eukaryotic DNA polymerases. Particularly, oleanolic acid has been intensively investigated due to antifungal, anti-inflamatory, anti-HIV, diuretic, and anticancer properties (Kim *et al*., 2004). Nevertheless, there is little information about its action on insects. Insecticide properties have been report for other natural triterpenes, and theirs chemical derivatives (Reed *et al*., 1982; Lugemwa *et al*., 1990; Argandoña & Faini, 1993;

Gershenzon & Croteau, 1999; Natakani *et al*., 2002; Herlt *et al*., 2002; Rodríguez *et al*., 2003).

The present research was conducted to study the impact of different natural products from plants that grows in the Central-Western semi-arid area of Argentine on the development, survival and nutritional condition of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Red flour beetles attack stored grain products such as flour, cereals, meal, crackers, beans, spices, pasta, cake mix, dried pet food, dried flowers, nuts, seeds, (Weston & Rattlingourd, 2000) and is a major and common pest of indoor storage facilities with a world wide distribution.

We report nutritional, toxic and development alteration (enlargement of the pupation period) properties of three triterpenoids isolated from *Junellia aspera* (Gill. & Hook) (Verbenaceae) and four chemical derivatives, on larvae and adults of *T. castaneum*.

MATERIAL AND METHODS

Compounds. *J. aspera* dry aerial parts were chopped and macerated twice for seven days periods each time with MeOH at r.t. The solvent was evaporated under reduced pressure, and the residue taken up in $CHCl_3$ and partitioned against H_2O. The organic layer was dried (Na_2SO_4), concentrated, and the brown amorphous residue was purified by silica gel column chromatography. After several purifications oleanolic acid (I), maslinic acid (II) and daucosterol (III), were obtained. The compounds oleanolic acid acetate (IV), methyl oleanate (V), oleanonic acid (VI) and 3ß-acetoxyolean-12α-bromine-(28→13)-olide (VII) were obtained by chemical transformations (Pungitore *et al*., *in press*). All structures were confirmed by comparison of spectroscopic data with previously values (Agrawal & Jain, 1992; Mahato *et al*., 1992; Connolly & Hill, 2001).

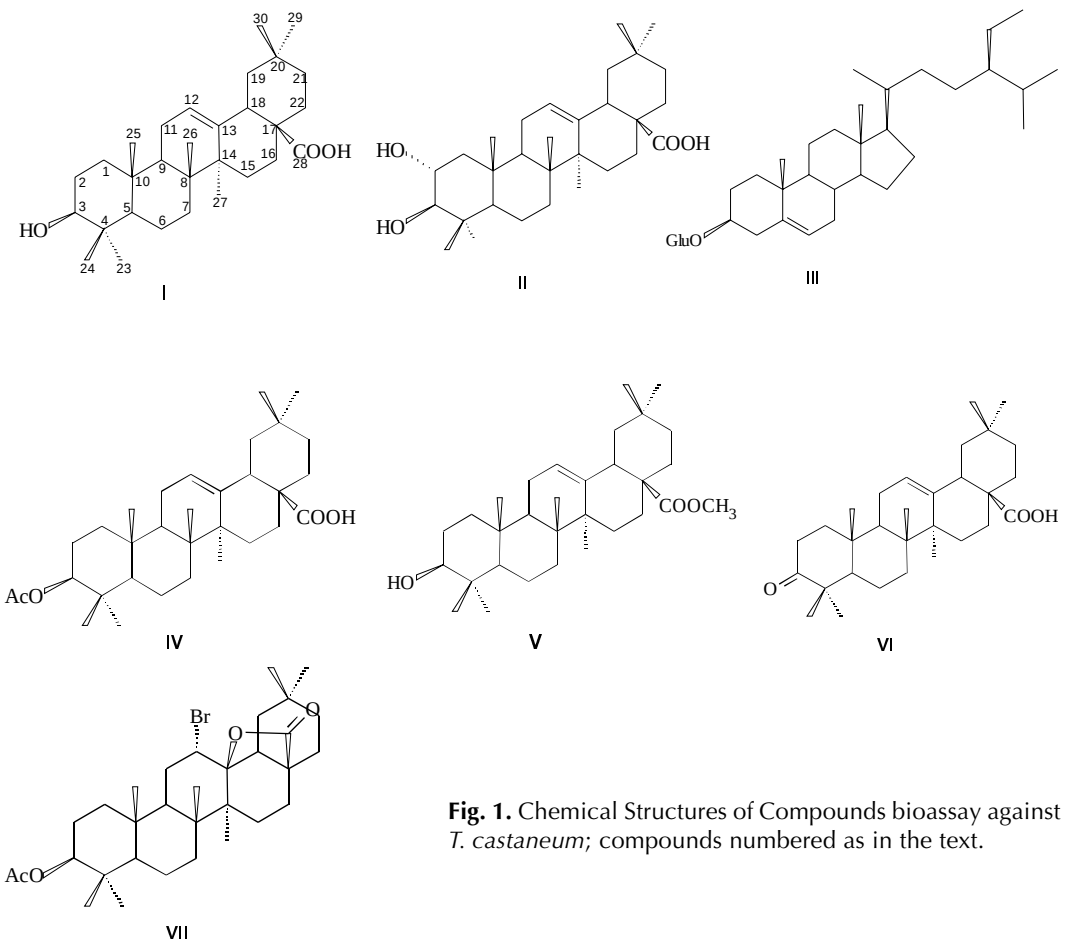

Fig. 1. Chemical Structures of Compounds bioassay against *T. castaneum*; compounds numbered as in the text.

Insects. *T. castaneum* used in tests were obtained from a colony established in the Laboratory of Zoology (San Luis National University, San Luis, Argentina). Cultures and experiments were carried out at 25 ± 1ºC, 65 % RH, and a 16:8 (L:D) photoperiod.

Laboratory assays

Topical Application. Fifth instars larvae of *T. castaneum* were randomly selected. Acetone test solutions of each compound were topically applied to the ventral surface of the thoracic segments with a Hamilton microsyringe (1µL/insect, equivalent to 60 mg/insect of the assayed compounds) (Carrizo *et al*., 1998). Controls were treated with the solvent alone. After treatment insects were placed into plastic vials (10 cm diameter x 7 cm height) containing food and held at 25 ± 1°C with a 16:8 (L:D) photoperiod. There were three replicates of ten larvae for each treatment. The duration of the pupal stage (days) was recorded, as well as the inhibition of the imaginal molt. Mortality was assessed every 24 hours for 60 days, and mortality was adjusted using the Abbott formula (Abbott, 1925). Insects were considered death when tactile stimuli elicited no visible normal reaction. Data were analyzed using the Kruskal-Wallis test, and Dunn's multiple comparisons test ($P < 0.05$). The program used for the analysis of the data was GraphPad Prism 4; GraphPad Software Inc.

Nutritional Indices. Flour discs (75 ± 8 mg/ disc) were prepared using 200 µl of a stirred suspension of wheat flour in water (20 g in 50 ml) (Huang *et al*., 2000). Using acetone as solvent, solutions of each compound (200 and 400 µg/ disc) were applied. Controls were treated using the solvent alone. The solvent was allowed to evaporate for 24 hours. Two flour discs of the same treatment were weighed and placed in a plastic vial (diameter 3 cm, height 2 cm). Ten weighed and unsexed adults of *T. castaneum* were added to each vial. Five replicates were set up for each compound and control. After 5 days, flour discs and live insects were weighed again. Nutritional indices were calculated as previously described by Huang *et al*., (2000): relative growth rate (RGR) = (A–B)/B x day^{-1}, where A is the weight of live insects on the fifth day (mg)/number of live insects on the fifth day. B represent the original weight of insects (mg)/number of insect at the beginning of bioassay. Relative consumption rate (RCR) = D/ B x day^{-1}, where D is biomass ingested (mg)/number of live insects on the fifth day. Efficiency of conversion of ingested food = (ECI) (%) = (RGR)/ (RCR) x 100. Feeding Deterrence Index (FDI) (%) = [(C-T)/C] x 100, where C is the consumption of control discs, and T the consumption of treated discs. Mortality by consumption was recorded at the end of the experimented (5 days), and adjusted using the Abbott formula (Abbott, 1925). Insects were considered death when tactile stimuli elicited no visible normal reaction. Data were analysed using the Kruskal-Wallis test, and Dunn's multiple comparisons test ($P < 0.05$). The program used for the analysis of the data was GraphPad Prism 4 program; GraphPad Software Inc.

RESULTS AND DISCUSSION

Toxic (antibiosis) and deterrent (antixenosis) modes of action have been suggested as responsible for the activity of several triterpenoids (Ortego *et al*., 1999). Compounds evaluated herein produced toxic effects when were applied directly on insects' surface (Table I) and when were incorporated into their food (Table II). Results revealed a general mode of action of these compounds against *T. castaneum* mainly related with a toxic action. No nutritional effects were observed for the triterpenes evaluated.

Oleanolic acid (I) produced an acute toxic effect when was applied topically to the larvae of *T. castaneum* (Table I), but did not cause effect when was incorporated into the food of adults (Table II). This indicates that I acts mainly by contact on *T. castaneum* larvae, but do not produce an important effect when is ingested by adults. It has been informed that I has an inhibitory effect on certain human cytochrome P_{450} enzymes (Kim *et al*., 2004). If we considered that in general terms the structure and mechanism of P_{450} enzymes have indeed been conserved from bacteria (Feyereisen, 1999), it is possible to think that I deactivates these mixed function oxidases when is applied directly on the cuticle of the larva, enhancing its toxic properties. On the other hand, after being ingested the activity of oleanolic acid disappears, possible by degradation due to some gut enzyme.

Adults fed with oleanolic acid (I) did not show nutritional alterations or antifeedant activity (Table II). This lack of action was already exhibited by *Spodoptera litura* F. (Lepidoptera: Noctuidae) in a leaf disk bioassay (Mallavadhani *et al*., 2003). Nevertheless, some action for this triterpene was recorded against *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) producing a moderate feeding attractant activity and a postingestive toxicity in a flour disk bioassay, but no effect when was applied topically (Pungitore *et al*., 2005). Compound II was the only one that produced a significant increment on the pupal stage duration (Table I). According to this, we can postulate that maslinic acid interfere with the normal development of *T. castaneum*, producing some alteration on the pupal stage duration. New investigations at the microscopic, enzymatic, and cytological level are necessary in order to determine whether the effect observed is related with hormonal disorders or with a growth regulator effect. This effect have already been observed with other natural products on this and other insects (Carrizo *et al*., 1998; García, *et al*. 2003). Maslinic acid (II) caused high mortality when was ingested by *T. castaneum* adults throughout 5 days. In contrast with our results, when *S. oryzae* adults were fed with flour disks containing compound II at a 200 and 400 µg/disk dose, it produced an important mortality after 10 and 20 days respectively (Pungitore *et al*., 2004). This results makes *T. castaneum* a more sensible target than *S. oryzae* to the triterpenes evaluated herein.

Table I. Duration of the pupal stage and insecticide activity of compounds 1-7 against *T. castaneum* larvae

Chemical (60 µg/insect)	Duration of pupal instar (day±SD) [a]	% Mortality (±SD) [b]
I	*	100 (0.0) [§§]
II	11 (2.0) [††]	70 (16.68)
III	9 (1.8)	49 (6.30)
IV	*	100 (0.0) [§§]
V	*	100 (0.0) [§§]
VI	*	96 (6.30) [§]
VII	9 (0.7)	89 (0.0)
Control	8 (1.0)	8 (13.29)

a, Kruskal-Wallis test (*K-W* = 13.62; df = 3;*P* = 0.003), data followed by [††] are significantly different according to Dunn's test ($P < 0.01$).
b, Kruskal-Wallis test (*K-W* = 24.82; df = 7; *P* = 0.0008), data followed by [§,§§] are significantly different according to Dunn's test ($P < 0.05$); ($P < 0.01$).
*, No data due to high mortality.

Notably it was observed that the highest bioactivity belongs together with compounds that possess the oleanane skeleton. Daucosterol (III) is the only triterpenes that do not belong to the oleanane group and its toxic action was below 50%.

Compound VI at the two doses evaluated and VII at its major dose, presented high values of feeding deterrent activity (Table II), but this antifeedant activities were not reflected in nutritional indices values. Due to this we prefer to consider this antifeedant activity as provisional, and continue researching the effect of oleananes on *T. castaneum* feeding behaviour.

It is important to mention that we have proved the antifeedant action of compounds I-VII with other model insects (data not shown) (e.g. *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), *Myzus persicae* (Sulzer) (Homoptera: Aphidae), *Rhopalosiphum padi* L. (Homoptera: Aphidae), *S. frugiperda* (Smith) and

Table II. Nutritional and feeding deterrence indices of *T. castaneum* adults in a flour disks bioassay.

Chemical (μg/disk)	RGR (mg/mg/d) (±SD)[a]	RCR (mg/mg/d) (±SD)[a]	ECI% (±SD)[a]	FDI%	% Mortality (±SD)[b]
I (200)	-0.005 (0.0196)	0.133 (0.0290)	-5.04 (14.66)	4	13 (30.1)
I (400)	-0.006 (0.0306)	0.126 (0.0317)	-9.39 (23.86)	12	18 (25.0)
II (200)	*	*	*	*	97 (4.8)†
II (400)	*	*	*	*	100 (0.0)†
III (200)	*	*	*	*	100 (0.0)†
III (400)	*	*	*	*	100 (0.0)†
IV (200)	*	*	*	*	100 (0.0)†
IV (400)	-0.014 (0.0069)	0.164 (0.0345)	-8.83 (4.08)	-21	0 (0.0)
V (200)	-0.0284 (0.0200)	0.076 (0.0849)	-57.82 (52.75)	39	32 (14.1)
V (400)	-0.028 (0.01993)	0.055 (0.0217)	-56.31 (46.83)	58	32 (14.1)
VI (200)	-0.029 (0.019)	0.042 (0.0080)	-68.89 (50.69)	68	39 (19.7)
VI (400)	0.000 (0.0329)	0.044 (0.0123)	-10.37 (73.67)	67	54 (14.1)
VII (200)	-0.012 (0.0215)	0.126 (0.0174)	-11.03 (18.53)	11	6 (15.5)
VII (400)	-0.011 (0.0142)	-0.012 (0.2793)	-9.92 (16.67)	107	9 (15.1)
Control	-0.009 (0.0361)	0.093 (0.0992)	-1.80 (44.16)	---	8 (8.3)

[i%] Treatments did not present a significant difference with the control.
*, No data due to high mortality.
[2%] Kruskal-Wallis test (*K-W* = 64.22; df = 14; $P < 0.0001$); data followed by † are significantly different according to Dunn's test ($P < 0.05$).

S. littoralis (Boisduval), but none of them presented antifeedant activity. This reveals that compounds I-VII are not active as feeding deterrents on pests generally used to evaluate the presence of biological activity of natural products on insects. Nevertheless, some effects on *T. castaneum* is presented herein and on *S. oryzae* was already reported (Pungitore *et al*., 2004).

CONCLUSIONS

The current study shows that triterpenes I-VII acts as toxic compounds when were applied topically and/or incorporated into the food of *T. castaneum*, and that did not present any important activity related with the nutritional status of it. Oleanolic acid (**I**), a major component of *J. aspera*, posses insecticidal effects on *T. castaneum* when was applied directly on the cuticle, and that compound II caused an increase in the duration of the pupal stage. Therefore, formulation and feasibility of application of these compounds in grain warehouses need to be investigated further. Likewise, more specific studies on the mode of action also will contribute to a better understanding of their bioactivity in insects.

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