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# Cytotoxic and genotoxic effects of defence secretion of *Ulomoides dermestoides* on A549 cells

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ABSTRACT

*Ethnopharmacological relevance: Ulomoides dermestoides* (Fairmaire, 1893) is a cosmopolitan tenebrionid beetle reared by Argentine people who consume them alive as an alternative medicine in the treatment of different illnesses such as asthma, Parkinson's, diabetes, arthritis, HIV and specially cancer.
*Aim of the study:* To evaluate the cytotoxicity and DNA damage of the major volatile components released by *Ulomoides dermestoides* on human lung carcinoma epithelial cell line A549.
*Materials and methods:* The defence compounds of *Ulomoides dermestoides* were extracted with dichloromethane and analyzed and quantified by capillary gas chromatography. The toxicity effects of the beetle's extract against A549 cell line were evaluated. Cytotoxicity was evaluated by MTT test and Trypan blue assay and genotoxicity was evaluated by the comet assay. The synthetic compounds, individually or combined, were also tested in A549 cells and normal mononuclear human cells.
*Results:* The defence compounds of *Ulomoides dermestoides* extracted with dichloromethane (methyl-1,4-benzoquinones, ethyl-1,4-benzoquinones and 1-pentadecene as major components) showed cytotoxic activity on A549 cells demonstrated by MTT test and Trypan blue assay, with $IC_{50}$ values of 0.26 equivalent/ml and 0.34 equivalent/ml, respectively (1 equivalent = amount of components extracted per beetle). The inhibition of A549 cell proliferation with the synthetic blend (1,4-benzoquinone and 1-pentadecene) or 1,4-benzoquinone alone was similar to that obtained with the insect extract. 1-Pentadecene showed no inhibitory effect. Low doses of insect extract or synthetic blend (0.15 equivalent/ml) inhibited mononuclear cell proliferation by $72.2 \pm 2.7\%$ and induced significant DNA damage both in tumor and mononuclear cells.
*Conclusion:* Results of this study demonstrated that defence compounds of *Ulomoides dermestoides* reduced cell viability and induced DNA damage. We also concluded that the insect benzoquinones are primarily responsible for inducing cytotoxicity and genotoxicity in culture cells.


## 1. Introduction

The Oriental tenebrionid *Ulomoides dermestoides* (Fairmaire, 1983) (Coleoptera: Tenebrionidae) (synonyms: *Alphitobius*? *Dermestoides*; *Martianus dermestoides*; *Palembus dermestoides*) was first reported in June 2000, in Misiones, Argentina (Flores et al., 2002) where weevils were reared at home to be used as an alternative treatment for various diseases. Insects are distributed through the so-called "the chain of weevil", which is a solidarity network that promotes a treatment that involves the consumption of 4900 live insects for 140 days with the purpose of alleviating or cure diseases such as asthma, Parkinson's, diabetes, arthritis, HIV and cancer. It has been suggested that the weevil reaches the stomach where it dies and releases healing substances. However, there is no scientific work that supports these popular beliefs. Although popular use of beetles for treatment of various illnesses has been reported (Buzzi and Miyazaki, 1999; Chu et al., 1977; Costa-Neto, 2002), it is unknown which the insect compound/s carrying the potential healing effects are. Only a recent publication has described anti-inflammatory properties of hydroquinones and crude polar extract of *Palembus ocularis* (Wahrendorf and Wink, 2006). Anti-inflammatory effects of unknown components

*Abbreviations:* BQ, 1,4-benzoquinone; MBQ, methyl-1,4-benzoquinone; EBQ, ethyl-1,4-benzoquinone; C15:1, 1-pentadecene; VOC, volatile organic compound; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CGC, capillary gas chromatography; SB, synthetic blend; DI, damage index.

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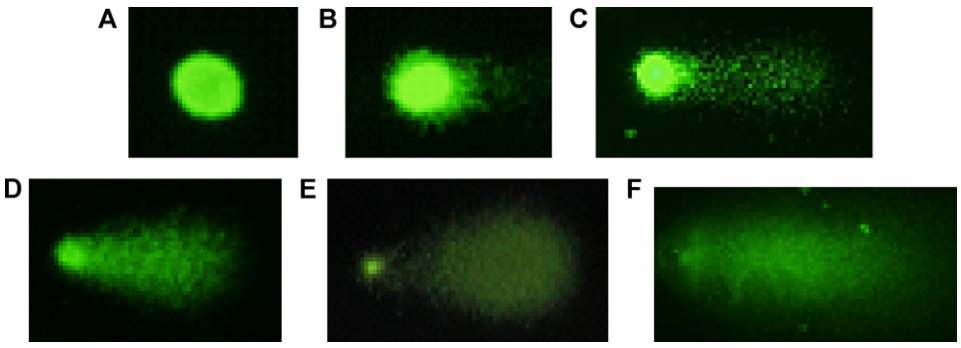


**Fig. 1.** DNA damage levels visualized by SYBR green staining of A549 cellular DNA after cell electrophoresis. Damaged DNA fragments exhibited comet-like tails. Cell at damage level degree 0 (A), degree 1 (B), degree 2 (C), degree 3 (D), degree 4 (E) and dead cells (F). Pictures A–F, 400×.

of the polar extract of *Ulomoides dermestoides* in rat and peripheral blood mononuclear cells were recently reported (Santos et al., 2009). The volatile organic compounds (VOC) released by stressed *Ulomoides dermestoides* contain methyl-1,4-benzoquinone (MBQ), ethyl-1,4-benzoquinone (EBQ) and 1-pentadecene (C15:1) representing more than 90% of the volatile blend (Villaverde et al., 2009). It was widely reported that many species of tenebrionids produce and secrete a defensive volatile blend with repellent and irritant properties against predators, containing mainly benzoquinones and alkenes (Attygalle et al., 1991; Attygalle et al., 1993; Blum, 1981; Eisner et al., 1998; Wirtz et al., 1978). Benzoquinones induce a broad spectrum of effects that range from being vitally important for homeostasis (vitamin K or ubiquinone) to extremely toxic or carcinogenic. However, most quinones have been widely reported as highly cytotoxic and/or genotoxic compounds due to the formation of reactive oxygen species (ROS) and covalent binding to macromolecules (Bolton et al., 2000; Fabiani et al., 2001; Hutt and Kalf, 1996; Monks and Jones, 2002; O'Brien, 1991; Pongracz et al., 1990; Ruiz-Ramos et al., 2005; Siraki et al., 2004).

The aim of this study was to evaluate the toxic effect of the volatile secretion of *Ulomoides dermestoides* on human lung carcinoma epithelial cell line A549. The toxic effect on normal human mononuclear cells was also evaluated. The cytotoxic effect was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay and Trypan blue method while genotoxicity was evaluated by using the comet assay.

## 2. Materials and methods

### 2.1. Reagents

Solvents (hexane and dichloromethane) were obtained from Carlo Erba (Milan, Italy). Triton X-100, dimethylsulphoxide (DMSO), 1,4-benzoquinone (BQ), 1-pentadecene (C15:1), Sybr green I, Trypan blue solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) and hydroxymethy aminomethane (Tris) were purchased from Sigma–Aldrich (St. Louis, MO).

### 2.2. Insects rearing

*Ulomoides dermestoides* was maintained at 27 ± 2 °C and 70 ± 5% relative humidity and fed on bran bread supplemented with peanut seeds. Pupae were sorted by sex and transferred to separate containers until eclosion, 30 days old adults were used.

Voucher specimens are deposited in División Entomología, Museo de Ciencias Naturales de La Plata, Buenos Aires, Argentina.

### 2.3. Extraction and quantification of the insect secretion

Five insects with an average weight of 10 mg were submerged in 300 μl dichloromethane for 20 min to obtain whole body extracts. The solvent extracts were transferred to another vial, reduced in volume under nitrogen, and the resulting extracts were analyzed and quantified by capillary gas chromatography (CGC) according to Villaverde et al. (2009) or resuspended in DMSO for cell treatment. A synthetic blend (SB) was prepared with BQ and C15:1, combined at a ratio similar to that present in the insect secretion.

### 2.4. Cell culture

The human lung carcinoma epithelial cell line A-549 was obtained from Dr. Amada Segal-Eiras (CINIBA, UNLP. Argentina). The cells were maintained in 75 $cm^2$ tissue culture flasks in Eagle's minimal essential medium (MEM) (Gibco, Invitrogen Corporation) supplemented with 10% fetal bovine serum (SFB) sterile-filtered (Natocor, Córdoba, Argentina) and 100 μg/ml streptomycin. Cells were routinely grown at 37 °C, cultured in a humidified atmosphere with 5% $CO_2$. For the assays and the continuous cell propagation, adherent monolayers at exponential growth phase (about 80% confluence) were harvested with trypsin (0.25%) in phosphate buffered saline (PBS).

Mononuclear cells were obtained from blood samples from healthy staff volunteers ($n = 3$). The research followed guidelines of the Declaration of Helsinki and Tokyo for humans, and was approved by the Comité de Bioética, Facultad de Ciencias Médicas, UNLP. Heparinized peripheral blood (10 ml) was collected under aseptic conditions. Mononuclear cells were isolated by density-gradient centrifugation on Histopaque-1077 (Sigma–Aldrich). Cells were then cultured in RPMI 1640 medium (Gibco, Invitrogen Corporation) supplemented with 10% fetal bovine serum (Natocor,

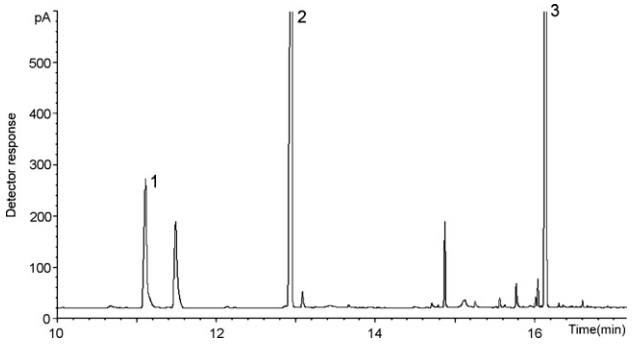


**Fig. 2.** Capillary gas chromatography profiles of *Ulomoides dermestoides* extract: 1, methyl-1,4-benzoquinone; 2, ethyl-1,4-benzoquinone; 3, 1-pentadecene.

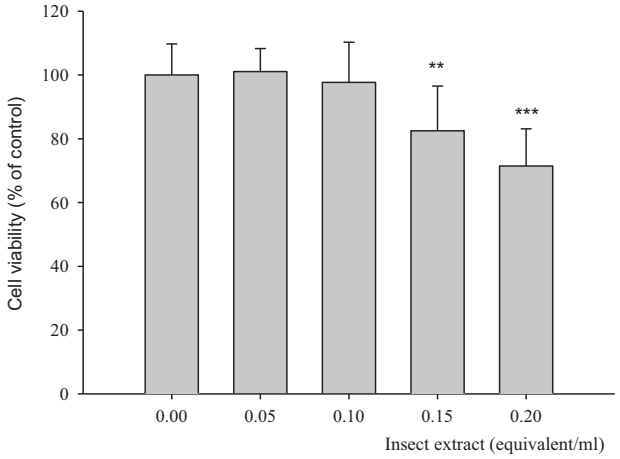


**Fig. 3.** Determination of insect extract cytotoxicity in A549 cells using the MTT assay. The cells were incubated with the indicated doses in MEM + SFB for 24 h. **Very significantly different ($p < 0.01$) from control. ***Extremely significantly different ($p < 0.001$) from control (unpair $t$-test). Data are reported as the mean ± standard deviation of 8–16 replicate wells/dose performed in 2–4 separate experiments, respectively.

Córdoba, Argentina), 100 units/ml of penicillin, 100 μg/ml of streptomycin (PAA Laboratories GmbH). Each culture was performed in quadruplicate in 48-well plates. Cells were incubated at 37 °C for 24 h under atmosphere containing 5% $CO_2$.

### 2.5. MTT assay

Cytotoxicity was measured using the MTT assay (Mosmann, 1983). For the assay, $8 \times 10^3$ A549 cells/well were seeded on 24-well plates, and the suspension was incubated at 37 °C for 24 h for cell attachment before the treatment. A549 cells and mononuclear cells were incubated with the insect extract, BQ, C15:1 or SB for 24 h. Both, the insect extract or chemicals, were dissolved in DMSO to a final concentration of 1% (v/v). DMSO was added to the cell culture used as control at final concentration of 1%. After the exposure time, the medium was removed and then MTT assays were performed. Cells from each well were incubated with 0.5 ml of MTT (0.5 mg/ml) in PBS at 37 °C for 3 h. After this, the MTT was removed from wells and 0.5 ml of acidified isopropanol (0.04 M HCl) was added. Absorbance of the reaction product (formazan) was measured at 560 nm with background subtraction at 640 nm using a microplate reader (Bio-Rad Laboratories, model 3550). Cell viability was expressed as percentage of the controls. The cytotoxicity of *Ulomoides dermestoides* extract, SB and BQ were considered according to the $IC_{50}$ values; $IC_{50}$ is the concentration of the tested compound required to produce 50% inhibition of cell growth after an exposure time of 24 h.

**Table 1**
Growth inhibition of A549 cells by *Ulomoides dermestoides* defence components.

| Compound tested | $IC_{50}$[a] (equivalent/ml) |
|---|---|
| Insect extract | 0.26 ± 0.04 |
| Benzoquinone | 0.31 ± 0.01 |
| Synthetic blend | 0.30 ± 0.07 |

Data represent the mean value ± standard deviation, $n = 4$. Statistical analyses were performed by one-way ANOVA and Tukey test. $IC_{50}$ values did not differ significantly from each other, $p > 0.05$.

[a] $IC_{50}$ is the concentration compound (insect extract or synthetic analogues) required to reduce the optical density to 50% of the control in the MTT assay. The values were estimated from non-linear regression analysis of dose–response curves as represented in Fig. 1. The $IC_{50}$ value obtained for the insect extract corresponds to 31.7 μM EBQ, 11.9 μM MBQ and 15.3 μM C15:1, and the $IC_{50}$ value for SB corresponds to 61.3 μM BQ and 17.7 μM C15:1.

### 2.6. Trypan blue assay

A549 cells were seeded in 25 $cm^2$ tissue flasks in 4 ml growth medium at densities of $3.5 \times 10^5$ cells and allowed to attach at 37 °C for 24 h cultured under humidified atmosphere with 5% $CO_2$. After that time, culture medium was replaced by fresh medium (control) or culture medium containing test compound. The analyses were carried out in triplicate. The flasks were then incubated for further 24 h. Cell viability was assessed by their ability to exclude Trypan blue (0.2%, w/v, final concentration) (Phillips, 1973), using a Neubauer chamber.

### 2.7. Comet assay

The comet assay was performed according to the method of Singh et al. (1988) with minor modifications (Tice et al., 2000). Briefly, conventional slides were covered with a layer of 180 μl 0.5% normal agarose (GIBCO-BRL). An amount of 75 μl of low melting point agarose (0.5%, w/v) (GIBCO-BRL) was mixed with approximately $1.5 \times 10^4$ cells suspended in 15 μl; the mixture was then layered onto the slides, and immediately overlaid with coverslips.

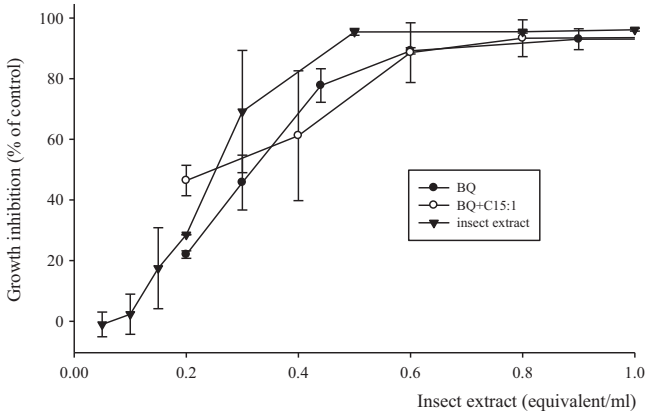


**Fig. 4.** Dose–response curve of A549 cells incubated with 1,4-benzoquinone (BQ), synthetic blend (SB) and insect extract, expressed as inhibition of cell growth according to the MTT test. Each point on the curve represents the mean value ± standard deviation of 8–16 replicate wells/dose performed in 2–4 separate experiments, respectively.

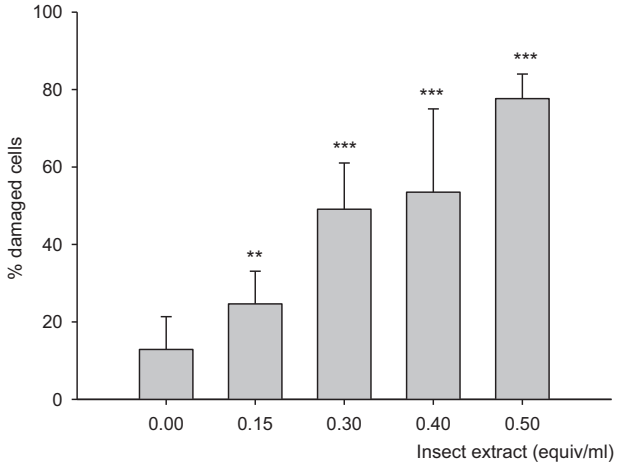


**Fig. 5.** Percentage of DNA damage of A549 cells evaluated by comet assay. Cells were incubated with the indicated insect extract concentration in MEM + SFB for 24 h. **Very significantly different ($p < 0.01$) from control. ***Extremely significantly different ($p < 0.001$) from control (unpair $t$-test). Data represent the mean ± standard deviation, $n = 15$ replicates of 100 cells each.

**Table 2**
DNA damage in A549 cells exposed to insect extract.

| Insect extract (equivalent/ml) | DNA damage level | | | | |
|---|---|---|---|---|---|
| | Degree 0 | Degree 1 | Degree 2 | Degree 3 | Degree 4 |
| Control | 87.1 ± 8.5 | 8.4 ± 6.5 | 2.8 ± 2.2 | 0.5 ± 0.7 | 0.3 ± 0.5 |
| 0.15 | 75.3 ± 8.4$^{ns}$ | 7.4 ± 6.5$^{ns}$ | 9.7 ± 5.1$^{***}$ | 4.4 ± 4.3$^{*}$ | 1.2 ± 1.3$^{ns}$ |
| 0.30 | 50.9 ± 11.9$^{***}$ | 25.3 ± 14.6$^{**}$ | 11.4 ± 3.5$^{***}$ | 8.1 ± 3.9$^{***}$ | 2.1 ± 1.1$^{**}$ |
| 0.40 | 46.5 ± 21.5$^{***}$ | 20.1 ± 16.3$^{ns}$ | 7.4 ± 2.7$^{*}$ | 13.7 ± 4.4$^{***}$ | 6.3 ± 2.3$^{***}$ |
| 0.50 | 22.3 ± 6.3$^{***}$ | 30.2 ± 12.0$^{*}$ | 27.2 ± 11.2$^{***}$ | 9.4 ± 5.3$^{**}$ | 1.2 ± 1.0$^{ns}$ |

Data represent the mean value ± standard deviation, $n = 15$ replicates of 100 cells each. Statistical analyses were performed by one-way ANOVA and Tukey Test.
$^{*}$ Statistically significant compared to the respective controls of each degree column, $p < 0.05$.
$^{**}$ Statistically significant compared to the respective controls of each degree column, $p < 0.01$.
$^{***}$ Statistically significant compared to the respective controls of each degree column, $p < 0.001$.
$^{ns}$ Statistically significant compared to the respective controls of each degree column, $p > 0.05$.

After agarose solidification at 4 °C for 10 min, the coverslips were removed and the slides were immersed overnight at 4 °C in fresh lysing solution (2.5 M NaCl; 100 mM $Na_2$ EDTA, 10 mM Tris, pH 10 containing 1% Triton X-100 and 10% dimethylsulphoxide, added just before use). The slides were equilibrated in alkaline solution (1 mM $Na_2$EDTA, 300 mM NaOH, pH > 13) for 20 min. Electrophoresis was carried out at 25 V and 250 mA for 30 min. After this, slides were neutralized by washing three times with Tris buffer (pH 7.5), 5 min each, and finally with distilled water; slides were then stained with 1/1000 Sybr Green I.

### 2.8. Image analysis

Fluorescence microscopy observations were performed with an Olympus BX51 microscope (Tokyo, Japan) equipped with a U-WIBA filter cube (excitation filter 460–490 nm, barrier filter 515–550 nm). Individual cells were analyzed and photographed using an Olympus DP70 digital camera using the ImagePro Plus (IPP$^{TM}$) v5.1 image analysis software (Media Cynernetics, Silver Spring, MA).

Based on the extent of strand breakage, cells were classified according to their tail length into five categories (Fig. 1), ranging from 0 (no visible tail, degree 0) to 4 (still a detectable head of the comet but most of the DNA in the tail, degree 4). Apoptotic and necrotic cells (without a detectable head) were included in a fifth group "dead cells".

Quantification of DNA damage was calculated using the damage index (DI) parameter defined by Güerci et al. (2008) expressing the degree of cell damage cumulatively for each insect extract dose evaluated.

Dd is damage degree and $P_{(Dd)}$ corresponds to the percentage of cells with damage degree (Dd).

### 2.9. Statistical analysis

Statistical analysis was performed using the one-way analysis of variance (ANOVA) and Tukey–Kramer multiple comparisons test with significance level set at $p < 0.05$ or unpair $t$-test (GraphPad inStat program).

## 3. Results

### 3.1. Extraction and quantification of the insect secretion

After dichloromethane extraction for 20 min, the amount of major VOC components detected by CGC (Fig. 2) was 16.6 ± 6.0 μg/insect (EBQ) ($1.66 \times 10^{-3}$, w/w), 5.6 ± 2.3 μg/insect (MBQ) ($5.6 \times 10^{-3}$, w/w), and 12.5 ± 1.6 μg/insect (C15:1) ($12.5 \times 10^{-3}$, w/w). Thus, 1 insect equivalent (the amount of components extracted per beetle) contained 0.122 μmoles EBQ, 0.046 μmoles MBQ and 0.059 μmoles C15:1.

### 3.2. Cytotoxic assays

As shown in Fig. 3, doses below or equal to 0.10 equivalent/ml of insect extract did not significantly reduce A549 cell viability, whereas higher doses significantly reduced viability.

From dose–response curves obtained by incubating the cells with insect extract, BQ and SB (Fig. 4), the corresponding $IC_{50}$ values were determined (Table 1).

The effect of insect extract, pure BQ or SB on growth inhibition of A549 cells did not differ significantly from each other, showing similar $IC_{50}$ values (Table 1). On the contrary, 1-pentadecene did not show any cytotoxic activity, even at a high concentration (3.00 equivalent/ml) (data not shown).

The effect of the synthetic blend on growth inhibition of mononuclear cells exposed to 0.15 equivalent/ml was 72.2 ± 2.7%. At higher concentrations 100% of cells were dead. Data are the mean ± standard deviation, $n = 4$.

By Trypan blue method, the $IC_{50}$ value of A549 cells treated with insect extract was 0.34 ± 0.06 equivalent/ml, not significantly different from $IC_{50}$ values obtained with MTT assay ($p > 0{,}05$).

### 3.3. Comet assay

Fig. 5 shows the percentage of A549 cells with DNA damage after being incubated with increasing doses of the insect extract. When cells were exposed to 0.15 equivalent/ml of insect extract, the percentage of damaged cells (24.6 ± 8.4 equivalent/ml) was very significantly increased when compared with controls ($p < 0.01$).

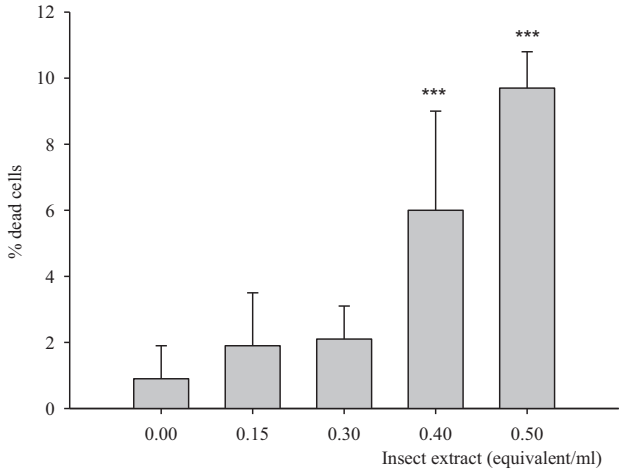


**Fig. 6.** Percentage of dead cells of A549 cells evaluated by comet assay. Cells were incubated with indicated doses of insect extract in MEM + SFB for 24 h. ***Extremely significantly different ($p < 0.001$) from control (unpair $t$-test). Data represent the mean ± standard deviation, $n = 15$ replicates of 100 cells each.

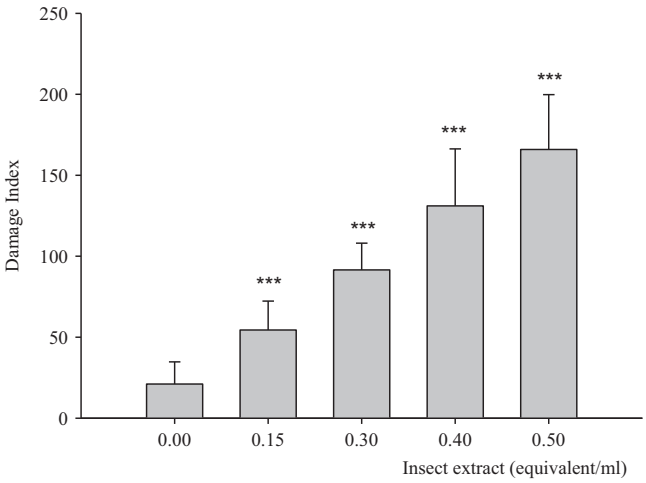


**Fig. 7.** Damage index of A549 cells obtained by comet assay. Cells were incubated with indicated doses of insect extract in MEM + SFB for 24 h. ***Extremely significantly different ($p < 0.001$) from control (unpair *t*-test). Data represent the mean ± standard deviation, $n = 15$ replicates of 100 cells each.

At higher concentration differences were extremely significant ($p < 0.001$).

Table 2 summarizes the DNA damage of cells incubated with different concentrations of *Ulomoides dermestoides* extract. Levels of DNA damage were scored from degree 0 to 4, their images are shown in Fig. 1. Cells treated with 0.15 equivalent/ml only showed significantly increased DNA damage, compared to control cells, at lesion degree 2 ($p < 0.001$) and degree 3 ($p < 0.05$). Cells exposed to 0.30 equivalent/ml showed a significant increase in all DNA damage levels, and cells exposed to 0.40–0.50 equivalent/ml showed a significant increase in cells at the highest DNA damage levels (degree 3 and degree 4, $p < 0.001$) (Table 2). As shown in Fig. 6 cells exposed to concentrations equal or above 0.40 equivalent/ml showed a significant increment in the percentage of dead cells (see Fig. 1F).Damage index was directly proportional to the concentration of *Ulomoides dermestoides* extract, showing extremely significant differences in DNA damage on A549 cells incubated with insect extract, compared with control cells ($p < 0.001$) even at the lowest doses of insect extract (Fig. 7).

Damage index of control mononuclear cells was 18.4 ± 4.2 while cells incubated with 0.15 equivalent/ml showed a damage index of 88.1 ± 19.8. Data are the mean ± standard error, $n = 9$ replicates of 100 cells each.

## 4. Discussion and conclusions

For the last 10 years in Argentina, *Ulomoides dermestoides* has been widely consumed alive as an alternative medicine to cure various diseases such as asthma, Parkinson's, diabetes, arthritis, cancer and HIV. However, there is no scientific work that guarantees the curative effect of insect ingestion.

Stressed *Ulomoides dermestoides* release benzoquinones (MBQ and EBQ) and the unsaturated hydrocarbon pentadecene as major VOC compounds (Villaverde et al., 2009). In order to evaluate the toxic effects of *Ulomoides dermestoides* VOC on human cells, we quantified the amount of major VOC components released per insect, and studied their effect on cell viability and DNA damage of A549 cells. Moreover to evaluate the effect of major compounds of insect extract on normal cells, cytotoxicity and genotoxicity were evaluated in mononuclear human cells.

In the present study it has been shown that doses of insect extract of 0.15 equivalent/ml and higher, significantly reduced tumor cell viability below 85% (Fig. 3) and above 70% in mononuclear cells. The similar $IC_{50}$ values obtained with insect extract, pure BQ and synthetic blend (Table 1), together with the lack of pentadecene cytotoxic activity, suggested that benzoquinones in the insect extract are primarily responsible for the inhibition of cell proliferation. Similarly, Wahrendorf and Wink (2006) concluded that in the polar extract of *Palembus ocularis*, containing hydroquinones and pentadecene as main components, the hydroquinones represented the active principle.

We also demonstrated the genotoxicity effect of *Ulomoides dermestoides* extract in culture mammalian cells A549 and mononuclear cells evidenced by the breakdown of DNA as assessed by comet assay (Figs. 5–7). According to Tice et al. (2000), it has been suggested that DNA breakdown occurs in double strands as well as in single strands. Evaluated parameters of DNA damage (percentage of damage cells, damage index, and DNA damage degree) showed that even when cells were treated with the lowest concentration tested that affected cell viability (0.15 equivalent/ml) exhibited significant DNA damage. In accordance with many other reports on genotoxicity of benzoquinones (Hiraku and Kawanishi, 1996; Pandey et al., 2009), methyl and ethyl benzoquinones of the insect extract might induce DNA damage by oxidative stress. Damage might occur through the formation of ROS such as hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (OH) (Ruiz-Ramos et al., 2005; Shen et al., 1996; Wiemels and Smith, 1999; Yardley-Jones et al., 1991). Also, increased DNA recombination was reported in Chinese hamster ovary cells exposed to BQ (Winn, 2003). Moreover, O'Brien (1991) reviewed numerous quinones that are widely used as anticancer compounds, and suggested that in rapidly dividing cells, such as tumor cells, quinones cytotoxicity can be attributed to DNA modification. Although benzoquinones produce deleterious cellular effects, if they are targeted to malignant tumors they might become useful therapeutically as anticancer agents (Siraki et al., 2004). To our knowledge, this is the first report on the effect of insect MBQ and EBQ on viability and genotoxicity in tumor cells. Present data may help to clarify the effects of *Ulomoides dermestoides* secretions on human health.

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