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Original Article

Ecotoxicity of Terpenoids Oleanolic and Ursolic Acids to *Pomacea canaliculata* and a Non-target Species

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Abstract

We assessed the ecotoxicity of oleanolic (OA) and ursolic acid (UA) on juveniles of *Pomacea canaliculata* and *Danio rerio* larvae using 96-hour acute assays. Concomitantly, we determined the mutagenic activity of both acids by *Salmonella*/microsome assays with TA98 and TA100 strains in the absence and presence of S9 mammalian metabolic activation. In P. canaliculata assays, the LC50 values were not significantly different throughout the experimental period, ranging from 12.53 to 11.81 mgL^{-1} for OA and 13.05 to 11.82 mgL^{-1} for UA. In *D. rerio*, LC50 values were significantly different at 24 and 48 hours of exposure, ranging from 0.42 to 1.21 mgL^{-1} for UA and 0.55 to 4.81 mgL^{-1} for OA. In the *Salmonella*/microsome assay, only UA exhibited a direct mutagenic base-pair substitution response at 6.25 mgL^{-1}, which decreased in the presence of *in vitro* rat liver S9 metabolic activation. In view of the results observed, the LC50 necessary for *Pomacea* eradication may lead to fish larvae mortality. The mutagenesis detected in UA extracts could impact populations lacking a mammal metabolic system. OA and UA pose risks to non-target species, with UA also showing mutagenic potential. Further assessment is needed before their use in pest control.

Keywords: Aglycones; Bioactivity; Ecotoxicology; Golden apple snail; Molluscicide; Mutagenicity

INTRODUCTION

Triterpenes are bioactive compounds widely found in plants. The isomers oleanolic acid (OA) and ursolic acid (UA) (Figure 1A and B) are examples of this class of molecules, commonly found in flowers, leaves, barks, and fruits, either in their free acid form or aglycones of saponins. These compounds often co-occur as free acids or as aglycone precursors of triterpenoid saponins in several plants (Jie 1995, 2005; Jäger *et al*., 2009).

Traditionally, OA and UA have been used in folk medicine for their vast array of beneficial biological activities. These include cytoprotective and hepatoprotective functions, antitumoral activity, modulation of gene expression in inflammatory processes, as well as anti-inflammatory, antioxidant, antimicrobial, antifungal, antiprotozoal, and trypanocidal properties (Jie, 2005; Ferreira *et al.,* 2010; Pironi *et al.*, 2018). These compounds serve as natural larvicides and biopesticides, highlighting their potential utility against various harmful organisms (Silva *et al.,* 2016). Additionally, these isomers are abundantly present in nature in different plant materials, serving as a physical barrier against water loss and pathogens. They exhibit bioactive properties against herbivory and may mediate significant ecological interactions through allelopathy (Szakiel, Ruszkowski & Janiszowska, 2005; Szakiel & Mroczek, 2007).

As demand rises for sustainable alternatives to manage agricultural pests, secondary metabolites from plants have gained significant attention for their potential applicability and ecological safety (Roy, 2024). These compounds exhibit a range of biological activities, including insecticidal properties. At the same time, this

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makes them suitable for biopesticide development (Diwan, Tiwari & Soni, 2024). Plant essential oils as biopesticides have also gained traction due to their target specificity and minimal ecological impact, as evidenced by a notable increase in related research publications in recent years (Gupta *et al.,* 2023). Applying OA and UA in agriculture offers promising benefits, particularly as natural pesticides. However, the effects of their widespread use on non-target species must be thoroughly understood (Glare & Nollet, 2023; Chowdhury *et al.*, 2023).

One significant pest, the Golden Apple Snail, *Pomacea canaliculata* Lamarck, 1822 (Figure 1C), is a freshwater gastropod native to southern South America and is listed among the world's 100 worst invasive species. *P. canaliculata* poses a substantial threat in various agricultural settings, especially rice paddies, across endemic regions and parts of Asia, including China, the Philippines, and Japan (Lowe *et al.*, 2000; Pastorino & Darrigan, 2012). Natural bioactive compounds present viable alternatives to conventional control methods in managing such invasive species in agricultural and urban environments (Chauhan, Jabran & Mahajan, 2017; Rimando & Duke, 2006). Given the increasing need for effective pest control in cultivated areas, plant-based bioactive agents such as OA and UA represent promising alternatives (Schneiker *et al.,* 2016). Nonetheless, comprehensive toxicity assessments remain essential due to their potential toxic effects on non-target organisms.

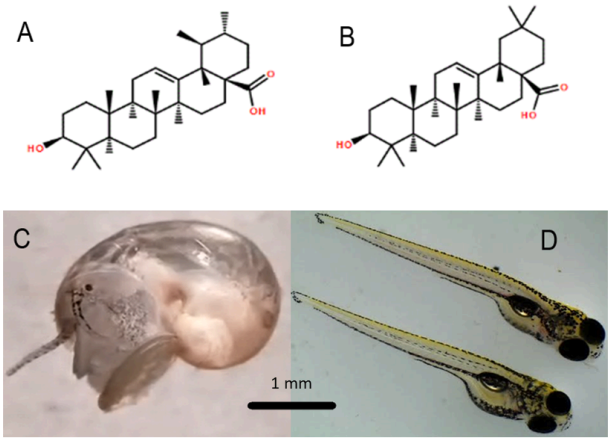

Figure 1. Molecular structure of (A) oleanolic acid and (B) ursolic acid, respectively. The molecular structures of the two acids were taken from the AK Scientific website. (C) *P. canaliculata* juveniles; (D) *D. rerio* larvae. Bar scale = 1mm

Zebrafish (*Danio rerio*, Figure 1D) is a globally recognized non-target model organism in ecotoxicological studies. Its small size, ease of maintenance, and high sensitivity to environmental contaminants make it an ideal candidate for assessing toxicological impacts (Stelzer *et al*., 2018). This species is commonly used for monitoring ecological impacts in both embryonic and larval stages (Freiry *et al.*, 2014; Braunbeck *et al.*, 2014; Pourshaban-Shahrestani, Rezazadeh & Hassan, 2025; Nipu *et al.*, 2025).

The *Salmonella*/microsome assay is widely employed in genetic toxicology for evaluating mutagenicity in natural products, environmental mixtures, foodstuffs, pharmaceuticals, and physical agents. Known for its predictive accuracy, it serves as an initial screening tool to determine the mutagenic potential of diverse chemicals and is commonly required for regulatory approval of new drugs and biocides, both synthetic and natural (Mortelmans & Zeiger, 2000; Claxton, Umbuzeiro & DeMarini, 2010).

Given the bioactivity of OA and UA, it is critical to investigate their potential impacts on non-target aquatic organisms to ensure safe applications as biocontrol agents and understand their environmental implications. This study is the first to evaluate the ecotoxicological and mutagenic effects of OA and UA on both target and non-target species, providing valuable insights into their environmental risks within the broader scope of environmental risk assessment.

We hypothesized that both acids would exhibit ecotoxic effects on *P. canaliculata* and *D. rerio*, with potential mutagenic activity, particularly in the absence of metabolic activation. This study aimed to examine the acute ecotoxicological effects and mutagenic activity of oleanolic and ursolic acids on the Golden Apple Snail and zebrafish, a non-target model organism. In addition, the mutagenic effects of these compounds were assessed using the *Salmonella/*microsome assay.

MATERIALS AND METHODS

Substances

Oleanolic acid (CAS number: 508-02-1; Purity > 98% - HPLC) and Ursolic acid (CAS number: 77-52-1; Purity > 98% - HPLC) were purchased from AK Scientific, Inc., Union City, CA 94587, USA. For the dilution, 20 mg of each compound were weighed separately and diluted in 1 mL of Dimethylsulfoxide (DMSO) 99% Sigma (CAS number 67-68-5) (stock solution 20.000 mgL^{-1}). Then, 500 µL of stock solution was pipetted into 4.5 mL of DMSO to prepare the stock solution (2.000 mgL^{-1}) for all concentrations used in the toxicity assays. Niclosamide (Sigma Aldrich; N350-50g; Lot: #047M4046V; CAS: 50-65-7) was used as a positive control.

Dosage Preparation

From the stock solution (2.000 mgL-1), all concentrations used in the assays were prepared

separately, under constant agitation, and each was adjusted to the aqueous solution in the 10 mL wells. Negative controls, H^2O and DMSO at a concentration of 1% were pipetted for both assays. For the *Salmonella* assay, initial concentrations were prepared in 10 mL of stock solution with a concentration of 25 mgL^{-1} of each compound, separately. After preparation, the samples were filtered using Sartorius Minisart® filters (0.45 µm). From the filtered content, 100 µL were pipetted for each corresponding concentration and applied in the mutagenicity assays.

Snails

P. canaliculata spawn was collected in the city of Viamão, State of Rio Grande do Sul, Brazil (-30.314747'S; -50.911911'W) (SISBIO - Biodiversity Authorization and Information System, Collection License: 61181; SISGEN - National System for the Management of Genetic Heritage and Associated Traditional Knowledge: A60F399). After collection, it was transported and kept in the laboratory for hatching. They were subsequently placed in a growing room (relative humidity mean 65% ± standard deviation 5), maintained under natural photoperiod (12h light/12h dark), in five-liter aquariums containing natural spring water (temperature 25 ºC ± 5; dissolved oxygen 6.98 mgL^{-1} ± 5; conductivity 293 mS ± 4; pH 7.5 ± 1.5; hardness 50 mgL^{-1} ± 0.25 of CaCO3). The juveniles used in the experiments had a shell size of 2.5 ± 0.5 mm and were up to 30 days old after hatching.

Fish

D. rerio larvae were bred from wild matrices kept in the Ecotoxicology Laboratory (Center of Ecology, Universidade Federal do Rio Grande do Sul). Test organisms were used until 14 days after hatching. During this period, the larvae were maintained in aquariums with demineralized and reconstituted water (conductivity 185 mS; pH 7.5; temperature 25 ºC; hardness 49 mgL^{-1} of CaCO3; photoperiod of 12h light/12h dark) and fed *Paramecium* sp. daily.

Toxicity Assays

***Mollusks*:** to determine the definitive concentrations, pilot assays were conducted to check the concentration-response curve of each aglycone separately. Toxicity assays were performed in six-well culture plates, with concentrations individually adjusted to the volume of the solutions in the 10 mL well, prepared with Demineralized and Reconstituted Water (DRW) (conductivity 185 mS; pH 7.5; average temperature 25 ºC) under constant agitation. All assays were static (without solution exchange, without feeding), covering a 96-hour experimental period; results were checked every 24 hours (Brackenbury, 2016). Nominal concentrations of the applied concentrations of OA and UA were [30; 25; 20; 15; 12.5; 7.5; 5; 3.75; 1.875; 0.93 mgL^{-1}] in addition to the negative controls (H^2O = DRW) and DMSO (1%) for all controls as a way to standardize the procedure across all assays. Positive control = Niclosamide: 0.5; 0.25; 0.125; 0.0625; 0.031; 0.015 mgL^{-1}, (LC50 24h: 0.15 mgL^{-1}; 48h: 0.09 mgL^{-1}).

The concentrations applied in the definitive assays expressed the best concentration-response curves for toxicity. Microplankton nets were placed in each well to prevent animals from escaping in all assays. Lethality was evaluated based on the animal shedding from the shell, mucous secretion, discoloration, absence of retraction, and the absence of a heartbeat confirmed under the microscope. Animals considered dead or immobile were counted and removed from the assay. All assays were performed with six replicates for each concentration, with five individuals per well (N = 30) (Brackenbury, 2016).

***Fish*:** pilot assays were conducted to check the concentration-response curve of each aglycone separately to determine the definitive concentrations. All toxicity assays with *D. rerio* larvae were performed in six-well plates, with the concentrations of each compound adjusted to 10 mL and prepared with demineralized and reconstituted culture water (hardness: 49 mgL^{-1} of CaCO3; conductivity: 189 mS; pH: 7.5; average temperature 25 ºC) under constant agitation. All assays covered 96 hours of exposure. The solution was changed at 48 hours (OECD, 2013), which requires the sample to be replaced at least every 48 hours to maintain dissolved oxygen levels within the minimum required for the species. Mortality was checked every 24 hours. The OA concentrations were [8; 4; 2; 1; 0.5; 0.25; 0.125 mgL^{-1}] and UA [2; 1; 0.5; 0.25; 0.125 mgL^{-1}], in addition to the negative controls (H^2O = DRW) and DMSO (1%) for all controls as a way to standardize the procedure across all assays. The concentrations applied in the definitive assays represented the best toxicity concentration-response curve from the previously performed pilot assays, with concentrations ranging from 100 to 0.0625 mgL^{-1}. Lethality was determined based on immobility and/or lack of response to mechanical stimulus. Animals considered dead or immobile were counted and removed from the assay. All assays were performed with six replicates for each dosage, with five individuals per well (N = 30), including the H^2O (N = 15) and DMSO (1%) (N =15) controls. Fish batch sensitivity is periodically assessed using KCl as a reference substance. Tests are only conducted when sensitivity remains within the acceptable range.

***Salmonella/microsome assay*:** mutagenicity and cytotoxicity were evaluated using the Kado microsuspension assay (Kado, Langley & Eisenstadt, 1983) modified from the *Salmonella*/microsome assay (Maron & Ames, 1983; Vargas, Motta & Henriques, 1993). *Salmonella enterica* serovar Typhimurium derived from the LT2 parental strains auxotrophic for histidine TA98 and TA100 were used. Mutagenesis was analyzed in TA98 (frameshift mutation) and TA100 (base-pair

substitution) in the absence and presence of liver metabolic fraction from male Sprague-Dawley rats (induced by Aroclor 1254, Moltox S.A., USA). All assays were performed in duplicate for each concentration, including negative controls, DMSO (1%), and liquid nutrient medium. The respective positive controls for each strain, in the presence and absence of metabolism: TA98-S9 (4-oxide-nitroquinoline: 4NQO, 0.5 µg/plate, CAS 56-57-5), TA100-S9 (sodium azide: SAZ, 0.5 µg/plate, CAS 26628-22-8, Sigma Chemical Company); TA98 and TA100 (+S9) (2-aminofluorene: 2AF, 0.5 µg/plate, CAS 153-78-6, Sigma Chemical Company). A confirmatory experiment was carried out whenever the response was positive or inconclusive. Mutagenesis was analyzed in the non-toxic linear portion of the concentration-response curve for both compounds [25; 12.5; 6.25; 3.125; 1.56; 0.78; 0.39 mgL^{-1}] defined within a range similar to that used in the *P. canaliculata* and *D. rerio* toxicity assays.

Statistical Analysis

Lethal Concentrations (LC50) (Lethal Concentration 50% ± CI: 95%) ($p < 0.05$) of the snails and zebrafish larvae assays were estimated using the Trimmed Spearman-Karber Program version 1.5 (TSK) (Hamilton, Russo & Thurston, 1975). Sample mutagenicity analyzed by *Salmonella* assay was considered positive when the curve of concentration-response presented a significant ANOVA among the dosages and a positive concentration-response curve ($p \leq 0.05$). These curves were analyzed in their linear portion by *Salanal Software* (*Salmonella* Assay Analysis, version 1.0, Research Triangle Institute, RTP, NC, USA), using the linear regression or Bernstein models (Bernstein *et al*., 1982). Mutagenic potential was estimated by the slope of the regression curve expressed in the number of revertants (rev)/mgL^{-1}. Cell survival (TA98) was analyzed comparing the different concentrations of the compounds after 72 hours of incubation. Samples were considered cytotoxic when the percentage of cell survival was less than 60% of colonies compared to the negative control (Vargas, Motta & Henriques, 1993).

To compare the toxicity ranges, the lower and upper limits of the confidence intervals of the LC50 values for the effects of the two compounds (OA/UA) on *P. canaliculata* juveniles and *D. rerio* larvae were arranged over the 96-hour experimental period. Asterisks (*) indicate statistically significant differences in sensitivity, as estimated by the LC50 values and their respective confidence intervals ($p < 0.05$).

RESULTS

Both acids demonstrated similar LC50 values in bioassays with *P. canaliculata* snails. For OA, these ranged from 12.53 to 11.81 mgL^{-1}, while for UA, they ranged from 13.05 to 11.82 mgL^{-1} (Table 1). This study is the first to report the molluscicidal effects of OA and UA on juvenile *P. canaliculata*. Notably, no mortality was recorded in the negative control groups (H^2O and 1% DMSO) throughout the experiment. Results at 72 and 96 hours were consistent (Table 1, Table 2, Figure 3). Although no statistically significant differences were detected between LC50 values across exposure times for either compound in snail bioassays, the estimated values confirm molluscicide potential within a toxicity range of 13.05 to 11.81 mgL^{-1} during the 96-hour testing period. Despite differences in isometry, with overlapping confidence intervals, OA and UA displayed comparable effects on *P. canaliculata* across all experimental time points.

Significant differences in LC50 values were observed between OA and UA in *D. rerio* larvae at 24 and 48 hours, with both compounds showing statistically significant toxicity at these time points. No statistical differences were observed at 72 and 96 hours, as indicated by overlapping confidence intervals. OA LC50 values ranged from 4.81 mgL^{-1} at 24 hours to 0.55 mgL^{-1} at 96 hours, while UA values ranged from 1.21 to 0.42 mgL^{-1} over the same periods. By 72 hours, both compounds showed nearly identical LC50 values, with UA exhibiting marginally lower toxicity (Figure 3, Table 1). No mortality was observed in the negative controls.

In the *Salmonella*/microsome assay, only UA demonstrated a direct base-pair mutagenic response (10.38 ± 3.362 rev/mgL^{-1}) at a concentration of 6.25 mgL^{-1}, showing a doubling of the mutagenicity index (MI) relative to the negative control. However, this effect decreased in the presence of *in vitro* rat hepatic metabolism (Table 3). Neither compound exhibited significant mutagenic activity for other assays, including the TA98 strain, as indicated by revertant counts per plate. Additionally, no cytotoxicity was observed for either compound at any tested concentrations (Table 3).

Table 1. Lethal Concentration (LC50) values in mgL^{-1} with estimates of the lower and upper confidence intervals (c.i. 95%) obtained in the toxicity assays with the target and the non-target species submitted to the aglycones.

	24 hours			**48 hours**			**72 hours**			**96 hours**		
P. canaliculata	*inf*	**LC50**	*sup*	*inf*	**LC50**	*sup*	*inf*	**LC50**	*sup*	*inf*	**LC50**	*sup*
OA	11.31	**12.53**	13.88	11.01	**12.23**	13.59	10.78	**11.97**	13.29	10.61	**11.81**	13.51
UA	12.98	**13.05**	14.22	11.52	**12.6**	13.79	11.12	**12.32**	13.65	10.57	**11.82**	13.2
D. rerio	*inf*	**LC50**	*sup*	*inf*	**LC50**	*sup*	*inf*	**LC50**	*sup*	*inf*	**LC50**	*sup*
OA	4.32	**4.81**	5.36	4	**4.51**	5.08	0.76	**0.88**	1.01	0.41	**0.55**	0.73
UA	1.07	**1.21**	1.34	0.81	**0.81**	1.09	0.68	**0.78**	0.89	0.3	**0.42**	0.6

Note: (OA = Oleanolic acid; UA = Ursolic acid). Concentrations of OA and UA applied in the *P. canaliculata*: [30; 25; 20; 15; 12.5; 7.5; 5; 3.75; 1.875; 0.93 mgL^{-1}]. Concentrations applied in the *D. rerio* larvae (OA): [8; 4; 2; 1; 0.5; 0.25; 0.125 mgL^{-1}], (UA): [2; 1; 0.5; 0.25; 0.125 mgL^{-1}]. inf = lower limit of the estimate; sup = upper limit of the estimate. LC50: Lethal Concentration values at 50% of the population of exposed organisms.

Table 2. Effects of oleanolic acid and ursolic acid on the cumulative mortality (%) of *Pomacea canaliculata* and *Danio rerio* at different concentrations and exposure times

	Oleanolic acid				**Ursolic acid**			
	P. canaliculata							
(mgL^{-1})	**24 h**	**48 h**	**72 h**	**96 h**	**24 h**	**48 h**	**72 h**	**96 h**
0,93	0	0	0	0	0	0	0	3,3
1,875	0	0	0	0	0	3,3	3,3	3,3
3,75	0	3,3	3,3	3,3	0	3,3	3,3	3,3
5	0	0	0	3,3	0	3,3	6,7	6,7
7,5	23,3	26,7	26,7	30	0	3,3	3,3	3,3
12,5	73,3	73,3	80	80	73,3	80	80	83,3
15	43,3	46,7	46,7	46,7	53,3	53,3	53,3	53,3
20	83,3	83,3	83,3	83,3	66,7	66,7	66,7	66,7
25	100	100	100	100	100	100	100	100
30	100	100	100	100	100	100	100	100
Water	0	0	0	0	0	0	0	0
DMSO (1%)	0	0	0	0	0	0	0	0
	D. Rerio							
(mgL^{-1})	**24 h**	**48 h**	**72 h**	**96 h**	**24 h**	**48 h**	**72 h**	**96 h**
0,125	0	3,3	3,3	23,3	0	0	3,3	23,3
0,25	0	0	3,3	26,7	0	3,3	6,7	46,7
0,5	0	0	10	36,7	3,3	10	13,3	43,3
1	0	0	63,3	90	30	46,7	66,7	100
2	0	0	100	100	100	100	100	100
4	23,3	33,3	100	100	100	100	100	100
8	100	100	100	100	100	100	100	100
Water	0	3,3	6,7	6,7	0	0	0	0
DMSO (1%)	0	0	0	0	0	3,3	3,3	33,3

Note: DMSO (1%) (100µl); Water = negative control; h= hours; mgL^{-1}= concentrations;

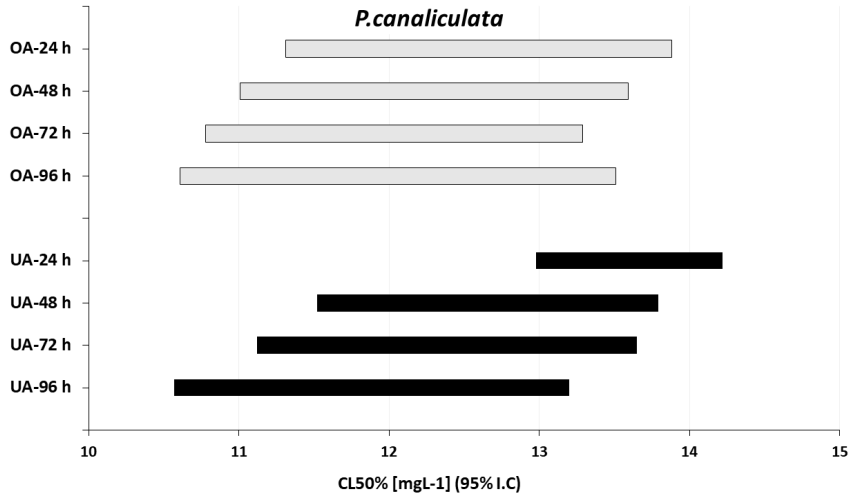

Figure 2. Ordination of the total lower and upper limits of LC50 estimates of the effects of the two compounds (OA = oleanolic acid; UA = ursolic acid) on *P. canaliculata* snails during the 96-hour experimental period. There was no statistical significance between the toxicity of the two aglycones to *P. canaliculata*.

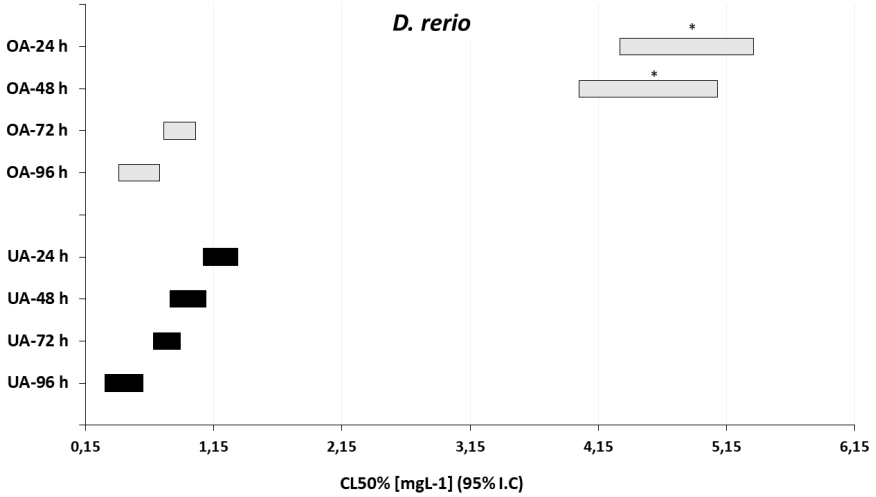

Figure 3. Ordination diagram of the total lower and upper limits of LC50 estimates of the effects of the two compounds (OA = oleanolic acid; UA = ursolic acid) on *D. rerio* larvae during the 96-hour experimental period. * = statistical significance ($p < 0.05$).

Table 3. Summary of the results obtained with the S. thyphimurium strains (TA98 and TA100) in the microsuspension assay (+/- S9) With the oleanolic and ursolic acids.

Oleanolic acid	TA98 - S9			TA98 + S9			TA100 - S9		TA100 + S9	
	Mean ± SD[c]	MI	SUR (%)	Mean ± SD	MI	SUR (%)	Mean ± SD	MI	Mean ± SD	MI
DMSO [a]	32.5 ± 13.44	1		34.5 ± 6.36	1		69 ± 4.24	1	77.7 ± 7.64	1
Positive control [b]	106.5 ± 3.54	3.28		220 ± 0	6.38		752 ± 53	10.91	655 ± 21.2	8.43
0.39 mgL^{-1}	27 ± 0	0.83	100	33.5 ± 3.54	0.97	100	75.5 ± 19.09	1.12	92.5 ± 9.19	1.19
0.78 mgL^{-1}	24 ± 5.66	0.74	100	32 ± 2.83	0.93	100	86.5 ± 0.71	1.25	89 ± 5.66	1.15
1.56 mgL^{-1}	34.5 ± 6.36	1.06	100	29.5 ± 10.61	0.86	100	93.5 ± 2.12	1.36	95.5 ± 2.12	1.23
3.12 mgL^{-1}	33 ± 7.07	1.02	100	32 ± 1.41	0.93	100	106 ± 2.83	1.54	87.5 ± 10.61	1.13
6.25 mgL^{-1}	27 ± 0	0.83	100	30 ± 7.07	0.87	100	94 ± 9.9	1.36	84 ± 12.73	1.08
12.5 mgL^{-1}	28 ± 0	0.86	100	35 ± 8.49	1.01	100	90 ± 16.97	1.30	87 ± 18.38	1.12
25 mgL^{-1}	26.5 ± 7.8	0.82	100	31.5 ± 9.19	0.91	100	110 ± 42.43	1.16	96 ± 1.41	1.24
Ursolic acid	**TA98 - S9**			**TA98 + S9**			**TA100 - S9**		**TA100 + S9**	
	Mean ± SD	**MI**	**SUR (%)**	**Mean ± SD**	**MI**	**SUR (%)**	**Mean ± SD**	**MI**	**Mean ± SD**	**MI**
DMSO [a]	32.5 ± 13.44	1		34.5 ± 6.36	1		72.5 ± 10.15 B [d]	1	77.7 ± 7.64	1
Positive control [b]	106.5 ± 3.5	3.28		220 ± 0	6.38		752.5 ± 53	10.91	655 ± 21.2	8.43
0.39 mgL^{-1}	29.5 ± 14.85	0.91	100	42 ± 1.41	1.22	100	120 ± 30.64 B	1.65	92 ± 0	1.18
0.78 mgL^{-1}	28 ± 7.07	0.86	100	40 ± 8.49	1.16	100	115 ± 22.82 B	1.58	82.5 ± 14.85	1.06
1.56 mgL^{-1}	24 ± 2.83	0.74	100	39.5 ± 2.12	1.14	100	131.75 ± 47.34 B	1.82	88 ± 11.31	1.13
3.12 mgL^{-1}	19.5 ± 6.36	0.6	100	40.5 ± 9.19	1.17	100	124.25 ± 46.24 B	1.71	88.5 ± 12.02	1.14
6.25 mgL^{-1}	34.5 ± 10.61	1.06	100	38.5 ± 0.71	1.12	100	147.75 ± 47.59 B	2.03	91.5 ± 16.26	1.18
12.5 mgL^{-1}	17 ± 0	0.52	100	32.5 ± 0.71	0.94	100	¤	1.31	81 ± 7.07	1.04
25 mgL^{-1}	26 ± 0	0.8	100	33.5 ± 4.95	0.97	100	¤	1.63	90.5 ± 19.19	1.17
Rev/mgL^{-1}							10.38 ± 3.36			

Note: [a] DMSO (1%) (100µl); [b] Positive control according to each assay: (-S9) 0.5 µl/plate of 4-NQO for TA98 and 0.25 µl/plate of sodium azide for TA100, (+S9) 0.50 µl/plate of 2AF for TA98 and TA100; [c] Data expressed in the mean of revertant colonies ± standard deviation (SD); [d] Bernstein model are indicated (B). **MI:** Mutagenic Index (mean of revertants per plate of sample/mean of revertants of negative control). The mutagenic potential was estimated by the slope of the regression curve expressed in the number of revertants(rev)/mgL^{-1}. **SUR:** Percentage of cell survival (number of revertants observed in the assay performed on the sample and the negative control).

DISCUSSION

The indiscriminate use of conventional pesticides in agricultural systems shows a lack of concern for the environment. Their known undesired direct and indirect effects on the fauna and flora have increased the demand for natural biocides (Cavoski, Caboni & Miano, 2011). In response, natural compounds like terpenoids have garnered attention as potential alternatives, as they are widely found in various plant materials and offer abundant and diverse bioavailability (Jie 1995, 2005; Pollier & Goossens, 2012; Woźniak, Skąpska & Marszałek, 2015).

The class of terpenes comprises several molecules like monoterpenoids, sesquiterpenoids, and triterpenoids, including the aglycones oleanolic and ursolic acids, which have multiple biological effects, depending on the conditions in which they are tested. The OA and UA have become the object of intense research due to the extent of their pleiotropic and multispectral effects on various biological models, as well as their low toxicity and many forms of bioactivity. The results of our study indicate efficacy and, consequently, underscore the relevance of investigating the aglycones as an alternative for the conventional chemical control of *P. canaliculata*.

The effectiveness of OA and UA may differ depending on the type of bioactivity and biological model being tested. Isomerism may or may not be a key factor, demonstrating a relationship between the structural-molecular form and the toxicological properties of compounds in different biological models (Sun *et al.*, 2006). The effects of purified saponins from *Quillaja saponaria*, as well as its precursors (prosapogenins and aglycones), elicited distinct responses in various toxicological models (Jiang *et al.,* 2018). The juvenile *P. canaliculata* individuals analysed in this study had already developed respiratory organs, including rudimentary yet fully functional lungs and gills. These organs serve as the primary sites for physiological maintenance and interaction with xenobiotics in the

organism (Koch, Winik & Castro-Vazquez, 2009; Seuffert & Martín, 2009). The structure of epithelial cells in the respiratory organs is affected by compounds that go into chemical complexation, causing the loss of the ciliary structures and leading to organ failure. Also, toxicity to the digestive gland causes mucus production, leading to immobility and death (Kruatrachue *et al.,* 2011). There is still no definitive explanation of how aglycones interact with cellular receptors in the respiratory tissues of submitted animal models. For example, the effect of UA on *Leishmania amazonensis* seems to be related to programed cell death like apoptotic mechanisms and autophagy presented major ultrastructural changes, cytoplasm vacuolization and associated disorganization mechanisms, and nitric oxide production (Yamamoto *et al.*, 2015).

The use of natural compounds, such as terpenoids, has garnered attention due to their bioactivity and potential application in pest control, particularly against *P. canaliculata.* This is largely attributed to their broad bioavailability and lower toxicity compared to synthetic products. However, results from the present study on oleanolic acid (OA) and ursolic acid (UA) reveal a complex relationship between efficacy and ecotoxicological risks. While OA and UA exhibited toxicity against *P. canaliculata* (LC50 11.81–13.05 mg L^{1}), their high sensitivity in *D. rerio* larvae (LC50 0.42–4.81 mg L^{-1}) and UA's direct mutagenicity in *Salmonella* highlight significant concerns regarding non-target organisms.

Comparatively, extracts from *Ilex paraguariensis* (Brito, Gosmann & Oliveira, 2018; Brito *et al*., 2021) and *Azadirachta indica* (Nik-Amalia *et al.,* 2023) showed higher LC50 values (24.75–53.65 mg L^{-1}), suggesting lower immediate potency but also reduced acute impacts on fish, such as *Rhamdia quelen* (LC50 17.98 mg L^{-1}). In contrast, extracts from *Chimonanthus* (He *et al*., 2024) and *Aegiceras corniculatum* (Yang *et al.*, 2025) demonstrated greater molluscicidal efficacy (LC50 0.19–14 mg L^{-1}), with mechanisms of action involving histopathological damage and enzymatic deregulation (e.g., AChE inhibition).

These findings indicate that, while OA and UA are promising for their pleiotropic effects and toxicity against *P. canaliculata*, their application in aquatic environments warrants caution due to potential risks to non-target species, such as fish, and the mutagenic potential of UA. Alternatives like *Chimonanthu*s and *A. corniculatum* may offer greater selectivity; however, the lack of data on mutagenicity and chronic effects underscores the need for further investigation. Therefore, the potential of biopesticides must balance efficacy, specificity, and ecological safety. Research should prioritize compounds with well-elucidated mechanisms and minimal impact on sensitive organisms, such as fish larvae.

For *D. rerio* embryos, the values from the assays with aglycones were similar to the assays with purified saponins, confirming that terpenoid precursors have high biological activity (Jiang *et al.,* 2018). In the present work, the LC50 values estimated for *D. rerio* larvae may result from biological factors strictly related to their higher sensitivity compared to embryos and adult individuals of the same species. Larvae are highly sensitive because of their large ionoregulatory surface area; therefore, physiological maintenance occurs strictly through the epidermis and gills by passive diffusion, and the epidermal extension is the main route of exposure (Rombough, 2002; Fol *et al.,* 2017). The cutaneous, gill, and intestinal cell epithelium consists of mitochondria-rich ionocytes responsible for gas exchange, ion regulation, and homeostatic maintenance (Ng *et al.*, 2019; Lin *et al.*, 2006). These sites contain highly sensitive cells and were the main physiological areas to undergo exposure to aglycones. Furthermore, isomeric differences may help explain the different results of the acute toxicity of each aglycone tested on non-target *D. rerio* larvae. The effects of UA on *Aedes aegypti* larvae, was observed that the compound had better biological activity in its original form compared to the molecule modified by semi synthesis. Their results emphasize that application of UA in its natural form is better in terms of extraction yield and bioactivity compared to synthesized compounds (Silva *et al*., 2016). Comparing the results, the more pronounced toxic effects of both acids on the larvae were certainly due to their higher sensitivity in comparison to the snails, which have remarkable physiological robustness (Hayes *et al*., 2015).

Phytochemicals have a broad and complex range of interactions, which may have protective or pro-mutagenic synergistic effects that will depend on the chemical affinity, structure, and applied concentrations. Overall, terpenoids exhibit antimutagenic properties mediated by reactions that require hepatic metabolism and indirect mutagenic substances. Thus, these listed effects may possibly be related to the protection and benefits to cells and genetic material through the interaction with these compounds found in medicinal plants. The UA, for example, may act on aflatoxin B, an indirect mutagenic agent, significantly reducing its mutagenic potential tested on the TA98 and TA100 strains (Young *et al.*, 1994). In the micronucleus assay with Balb-c rats, both OA and UA presented antimutagenic activity, significantly reducing the number of micronuclei compared to the positive control, which shows a protective effect on superior cells and again is characterized as an indirect mutagenic action (Resende *et al.,* 2006). Furthermore, antimutagenesis was observed in assays mediated by the P450 metabolic

activation on aflatoxin B1 and 2-aminofluorene. Their efficacy appears to depend on hepatic metabolism, with evidence suggesting that terpenoids reduce the mutagenic potential of these substances (Horn & Vargas, 2003, 2008).

New applications for naturally occurring compounds are continuously explored, looking into bioactivity in different organisms, aiming at possibly using raw materials where these substances are very abundant, and therefore considered by-products (Newman & Cragg, 2016). Particularly, terpenoids such as oleane and ursane-type aglycones have many pharmacological and biological activities, without prominent toxicity, being promising for the development of new technologies (Cargnin & Gnoatto, 2017; Jäger *et al*., 2009). The exact action mechanism of the acids remains only partially elucidated, requiring further studies that describe their forms of action, as well as the chemical-molecular interaction responsible for both their new and previously described biological activities (Wang *et al*., 2010).

Both compounds, OA and UA, demonstrated acute toxicity in bioassays with *P. canaliculata* and *D. rerio* larvae. Additionally, UA exhibited direct base-pair substitution (TA100) mutagenic activity in the *Salmonella*/microsome assay, which decreased in the presence of *in vitro* S9 to spontaneous mutation values. Our findings suggest that, compared to its isomer oleanic acid, ursolic acid possesses more pronounced bioactivity. Consequently, studying higher organisms, incorporating cell cultures, and especially *in vivo* tests, becomes imperative to analyze the risks or advantages of using natural compounds as drugs and agricultural products.

The biocidal effects of both compounds were more pronounced on non-target *D. rerio* larvae compared to *P. canaliculata* juveniles, displaying direct mutagenicity in the *Salmonella* assay. Mutagenesis could affect populations lacking a fully developed metabolic system. Given the greater impact on *D. rerio*, our results should be considered in the application of OA and UA as biocides in aquatic environments. These findings suggest that the concentrations necessary for effective control of *P. canaliculata* may pose risks to non-target species like fish larvae, and the mutagenicity observed in UA may have implications for organisms with undeveloped detoxification systems. We emphasize the importance of expanding toxicity research to include non-target organisms at different trophic levels and investigating response levels to induce chronic toxicity and genotoxicity in higher organisms.

In the present study, the toxic effects observed in *P. canaliculata* and *D. rerio* must be interpreted in light of the distinct exposure regimes applied to each species, which represent an important limitation. The snail assays were conducted under static conditions, simulating a single application, a relevant approach that reflects potential field-use scenarios. The chemical stability of oleanolic acid (OA) and ursolic acid (UA) in water, as demonstrated in previous studies (Castellano *et al*., 2022), supports the use of this exposure design. Conversely, the fish bioassays followed a semi-static protocol, with solution renewal every 48 hours, as required by OECD Guideline 203, to ensure adequate water quality for *D. rerio* larvae. This difference may have increased the effective exposure of fish to the test substances, potentially intensifying the observed toxic effects. Consequently, while our results indicate higher sensitivity in *D. rerio* larvae, this outcome may have been influenced by both the differential exposure conditions and the inherent sensitivity of the model organism. Future studies should consider the standardization of exposure designs across species to improve comparability and ensure more robust ecotoxicological risk assessments.

CONCLUSION

The effects of oleanolic acid and ursolic acid were more significant for *D. rerio* larvae compared to *P. canaliculata* juveniles, indicating direct mutagenicity in the *Salmonella/*microsome assay at the tested dosages for UA. However, the different modes of exposure, continuous immersion for snails versus semi-static exposure for fish larvae, may have contributed to the heightened sensitivity observed in larvae. It is therefore possible that this difference in exposure dynamics elevated the toxicity in fish. Consequently, the biocidal use of these molecules for pest control in aquatic environments requires careful evaluation, considering not only the intrinsic toxicity of the compounds but also the specific exposure conditions of non-target species.

CREDIT AUTHOR STATEMENT

FCB: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Writing – original draft, review and editing. **IKP:** Investigation, Data curation, Validation, Writing – review and editing. **AA:** Conceptualization, Formal analysis, Methodology, Project administration, Validation, Writing – original draft and review. **VMFV:** Formal analysis, Funding acquisition, Investigation, Project administration, Software, Resources, Supervision, Validation, Writing – original draft and review.

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Declaration of interest statement
The authors have no conflict of interest.

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AVAILABILITY OF DATA AND MATERIALS

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

COMPLIANCE WITH ETHICAL STANDARDS AND ETHICAL APPROVAL

This research used the spawning of mollusks collected in a natural environment with due legal permission, according to Brazilian legislation and Animal Use Ethics Committee of UFRGS. *Pomacea canaliculata*: Collection license SISBIO (61181) and SISGEN (A60F399); *Danio rerio*: CEUA UFRGS, 2014, Project number: 25232.

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