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Effect of structurally related flavonoids from *Zuccagnia punctata* Cav. on *Caenorhabditis elegans*

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Abstract

Zuccagnia punctata Cav. (Fabaceae), commonly called *jarilla macho* or *pus-pus*, is being used in traditional medicine as an antiseptic, anti-inflammatory and to relieve muscle and bone pain. The aim of this work was to study the anthelmintic effects of three structurally related flavonoids present in aerial parts of *Z. punctata* Cav. The biological activity of the flavonoids 7-hydroxyflavanone (HF), 3,7-dihydroxyflavone (DHF) and 2′,4′-dihydroxychalcone (DHC) was examined in the free-living nematode *Caenorhabditis elegans.* Our results showed that among the assayed flavonoids, only DHC showed an anthelmintic effect and alteration of egg hatching and larval development processes in *C. elegans.* DHC was able to kill 50% of adult nematodes at a concentration of 17 μg/mL. The effect on larval development was observed after 48 h in the presence of 25 and 50 μg/mL DHC, where 33.4 and 73.4% of nematodes remained in the L3 stage or younger. New therapeutic drugs with good efficacy against drug-resistant nematodes are urgently needed. Therefore, DHC, a natural compound present in *Z. punctata*, is proposed as a potential anthelmintic drug.

Keywords

7-hydroxyflavanone, 3,7-dihydroxyflavone, 2′,4′-dihydroxychalcone, anthelmintic effect, *Caenorhabditis elegans*

Introduction

With more than 9,000 described chemical structures, flavonoids represent a major sub-group of secondary plant compounds and possess a magnificent diversity with different structural and sterical properties (Williams and Grayer 2004). A broad spectrum of biological activities, such as anthelmintic, antioxidant, antimicrobial and anti-inflammatory, is attributed to these polyphenols that might benefit human and/or animal health and aging (Nijveldt *et al*. 2001; Ross and Kasum 2002; Katiki *et al*. 2011; Daglia 2012; Ndjonkaa *et al.* 2013; Menaa *et al*. 2014). The activities of flavonoids are dependent on their chemical structures. The position and the degree of hydroxylation have been demonstrated to be the most important for their biochemical and pharmacological actions (Rice-Evans *et al*. 1996; Kelly *et al*. 2002; Lamoral-Theys *et al*. 2010; Iglesias *et al*. 2014). Much of the knowledge regarding their beneficial effects was obtained from model organisms such as the nematode *Caenorhabditis elegans* (Strayer *et al.* 2003; Wilson *et al.* 2006; Kampkötter *et al.* 2007; Wink and Abbas, 2013).

Zuccagnia punctata Cav. (Fabaceae), commonly called *jarilla macho* or *pus-pus*, is a native shrub widely distributed in the semiarid and arid regions of western Argentina. The infusion and maceration in ethanol of the leaves are used in traditional medicine as foot antiseptic and against bacterial and fungal infections, asthma, arthritis and rheumatism (Ratera and Ratera, 1980; Toursarkissian, 1980). The leaves of the shrub are a rich source of phenolic compounds, being mainly flavonoid derivatives such as flavanones, flavones, chalcones and caffeoyl ester derivates (Pederiva and Giordano, 1984; Svetaz *et al*. 2004; Zampini *et al*. 2005; Agüero *et al*. 2010). 2′,4′-dihydroxychalcone was reported as one of the major constituents isolated from the leaf resin of *Z. punctata* (Zampini *et al*. 2005; Agüero *et al*. 2010).

The ethanolic extract of *Z. punctata* and 2′ ,4′ -dihydroxychalcone has shown efficient antibacterial activity against antibiotic multi-resistant bacteria isolated from cutaneous infection (Zampini *et al*. 2005) and phytopathogenic fungi *in vitro* (Svetaz *et al*. 2004); as well as antibacterial activity against *Streptococcus pneumoniae in vivo* (Zampini *et al*.

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2012). Other important activities of *Z. punctata* extract and its flavonoids have been reported, such as modulation of expression and activity of the best known membrane efflux pump, ABCB1 P- glycoprotein multidrug transporter, involved in drug resistance (Chieli *et al*. 2012) and cytoprotective effect on ethanol-induced gastroduodenal tract injuries in rats (de la Rocha *et al*. 2003). In addition, no acute toxicity of the hydroalcoholic extract and 2′,4′-dihydroxychalcone isolated from it has been determined in the *in vitro* comet assay test on human hepatoma HepG2 cells (Zampini *et al*. 2008). Moreover, 2′,4′-dihydroxychalcone showed inhibitory capacity on proliferation of human liver cancer cells (Loa *et al*. 2009). Morán Vieyra *et al*. (2009) demonstrated the scavenging capacity of $O_2^{\cdot-}$ by *Z. punctata* flavonoids (2′,4′-dihydroxychalcone > 7-hydroxyflavonol > 7-hydroxyflavanone) in aqueous systems at pH 7.4. Subsequently, the functionality of 2′,4′-dihydroxychalcone can be associated with their antioxidant capacity against different oxidant or aggressive species (Morán Vieyra *et al*. 2009).

The aim of this paper was to study the anthelmintic effect of structurally related flavonoids present in *Zuccagnia punctata* Cav. in *Caenorhabditis elegans*. We examined egg hatching, larval development and adult mortality, as well as lifespan of animals exposed to different concentrations of the compounds. *C. elegans* is a useful model organism for drug discovery and target identification because of its short life cycle, easy maintenance and good biological characterization (Artal-Sanz *et al*. 2006). Predominantly the worm is used for genetic studies. However, *C. elegans* may be also particularly useful in the development of anthelmintic drugs since it is evolutionarily closely related to parasitic worms (Geary and Thompson, 2001; Holden-Dye and Walker, 2007).

Material and Methods

General

Flavonoids: 7-hydroxyflavanone (HF), 3,7-dihydroxyflavone (DHF) and 2′,4′-dihydroxychalcone (DHC) were obtained from Indofine Chemical Company (New Jersey, USA). Levamisole was from Sigma-Aldrich (Germany) and 5-fluoro-2-deoxyuridine (FUdR) from Fisher Scientific. All solvents and reagents used were analytical grade.

Nematode strains and culture

The wild-type strain N2 (Bristol) was obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN) and maintained at 20°C on Nematode Growth Medium (NGM) supplemented with *Escherichia coli* strain OP50 (uracil requiring bacterial strain). Stock solutions of all natural products were made in dimethyl sulfoxide (DMSO, Sigma-Aldrich ≥99.5%). Each stock solution was added to M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 0.25 g $MgSO_4 \cdot 7H_2O$ in 1 l water) (Brenner 1974) in the anthelmintic and egg hatching assays or to autoclaved NGM before solidification (55–60°C) in lifespan and larval development assays, using *E. coli* OP50 as nematode food.

Anthelmintic assay

Age-synchronized N2 adult populations were prepared by harvesting eggs and worms from 3-day-old cultures, treating with alkaline hypochlorite (1 mL bleach, 0.5 mL 5N NaOH, 3.5 mL worm suspension) for 10 min, followed by centrifugation (1300×*g*) for 1 min. The eggs were washed with M9 buffer several times and transferred to fresh NGM plates seeded with *Escherichia coli* OP50. The plates were incubated at 20°C for 3 days, after which worms were harvested for testing. Worms were suspended in M9 buffer to obtain a final density of about 50 worms in 10 µl per replicate.

Tests were performed in 24-well plates containing different concentrations of the flavonoids (10, 25 and 50 µg/mL), in DMSO (1%), levamisole (1.2, 2.5, 5, 10 µg/mL), or DMSO (final concentration 1%) in a total volume of 500 µl/well of M9. Six replicates per treatment were made. The 24-well plates were covered with transparent plastic, and incubated at 20°C for 24 h. After incubation, nematodes were counted and determined as motile or non-motile using an inverted microscope. Nematodes were considered motile when they exhibited any movement, and as non-motile when there were no tail, head, or pharyngeal movements during 5 s of observation (Skantar *et al*. 2005). It is important to differentiate motility in adult nematodes from movement caused by larvae hatched from eggs inside the body of dead *C. elegans*.

The negative control group consistently showed 95–100% motile nematodes 24 h after incubation.

Egg hatching assay

Eggs were separated from the NGM/OP50 plates by washing with 5 mL of M9 buffer. Eggs were centrifuged at 2,000 rpm for 10 min, the pellet was dissolved in M9 buffer and the number of eggs was adjusted to 2,000 eggs/mL. About 200–300 eggs in 50 µL of M9 buffer were incubated for 1 h at 20°C in a 24-well tissue culture plate, after which the M9 buffer was discarded. Different concentrations of the flavonoids (10 to 200 µg/mL) in DMSO (final concentration 1%), levamisole (10 to 200 µg/mL) or DMSO were added to M9 buffer to obtain 500 µL of final volume into each of the 24 wells. Eggs were incubated for 12 h at 20°C and at the end of the incubation period, unhatched eggs and L1 larvae were counted and the percent hatched at different concentrations of pure compounds was calculated.

Larval development assay

Age-synchronized N2 adult populations were obtained by synchronization as described above. The eggs collected were

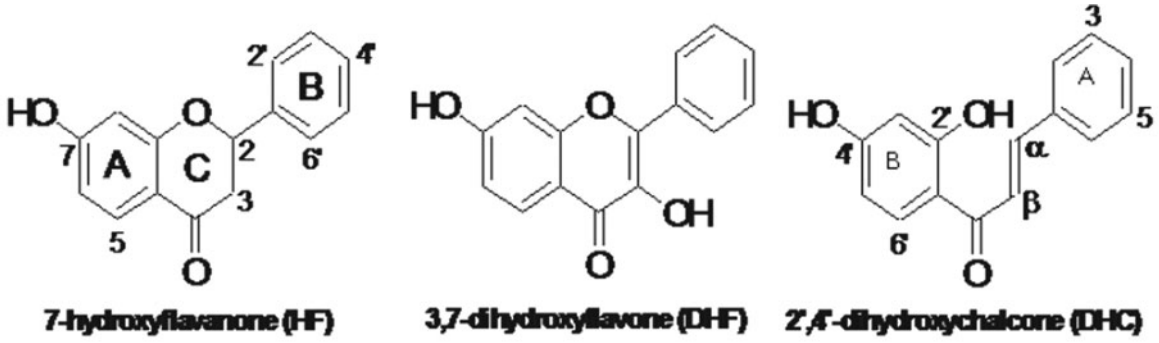

Fig. 1. Chemical structures of the flavonoid compounds studied

transferred to standard plastic Petri dishes containing fresh NGM, 10 to 200 µg/mL of the pure compounds in DMSO 1%, or DMSO 1% (negative control) and seeded with dead *E. coli* to avoid the possibility that the drug directly affects the *E. coli* and indirectly affects the worms (Collins *et al*. 2006). The development of these worm populations was visually assessed at 48 h post-transferral. Worms were categorized as L1–3, L4 or adult (Bull *et al*. 2007).

Lifespan assay

Median lifespan was defined as the time that 50% of the nematode had died. The lifespan of nematodes exposed to natural products was monitored and compared to controls.

For all lifespan studies, FUdR was also added to the NGM to a final concentration of 250 µM to prevent progeny production. FUdR has previously been shown to have no effect on *C. elegans* lifespan (Gandhi *et al*. 1980).

For all lifespan experiments, age-synchronized adult populations were prepared allowing 5–10 hermaphrodites to lay eggs for 4 h (Wilson *et al*. 2006). The eggs collected were transferred to standard plastic Petri dishes containing fresh NGM, 10 to 200 µg/mL of DHF and HF in DMSO (1%) and seeded with *E. coli*. The plates were incubated at 20°C for 2 days. L4 larvae (80–100 worms) were transferred with a platinum wire to NGM/FUdR containing/pure compound plates and seeded with OP50. DMSO-containing medium was included in each experiment as a control.

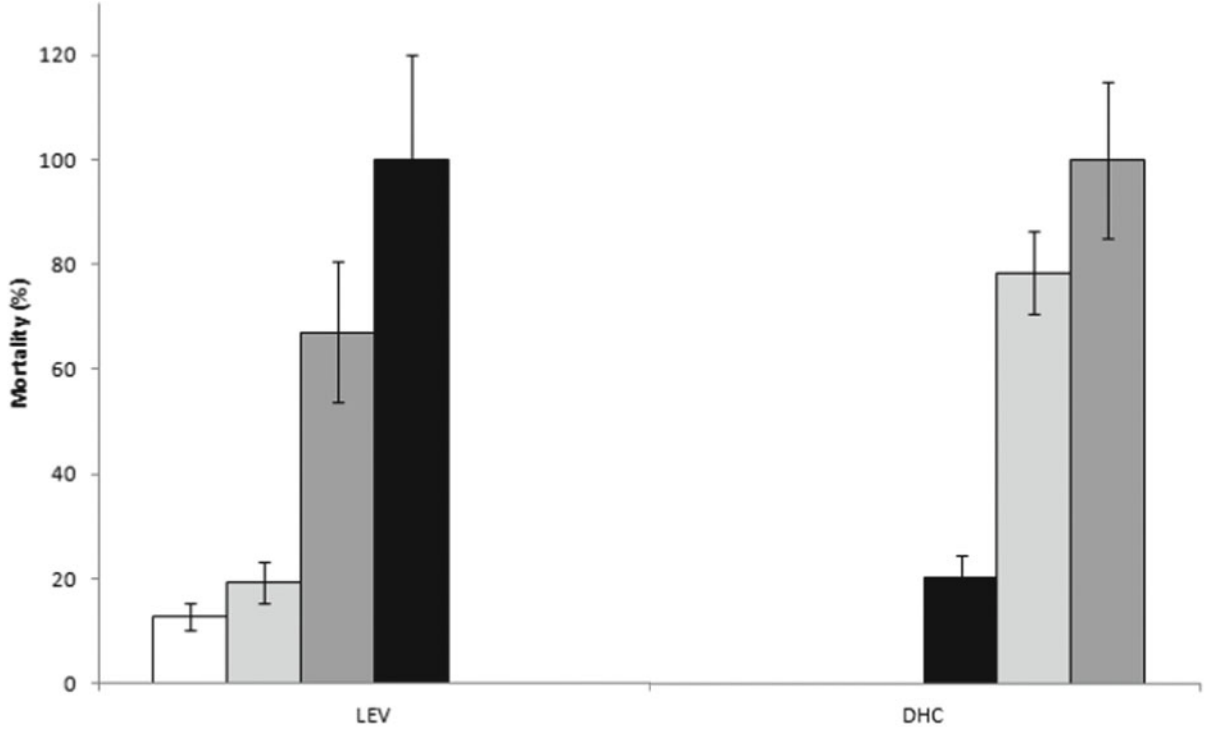

Fig. 2. DHC shows anthelmintic activity in a dose-dependent manner. Anthelmintic activity of 1.2 (□); 2.5 (▫); 5 (▪) and 10 (■) µg/mL levamisole (LEV) and 10 (■); 25 (▫); 50 (▪) µg/mL of DHC against adult *C. elegans*. Values (mean ± SD) are statistically different (Student's t-test), $p < 0.05$

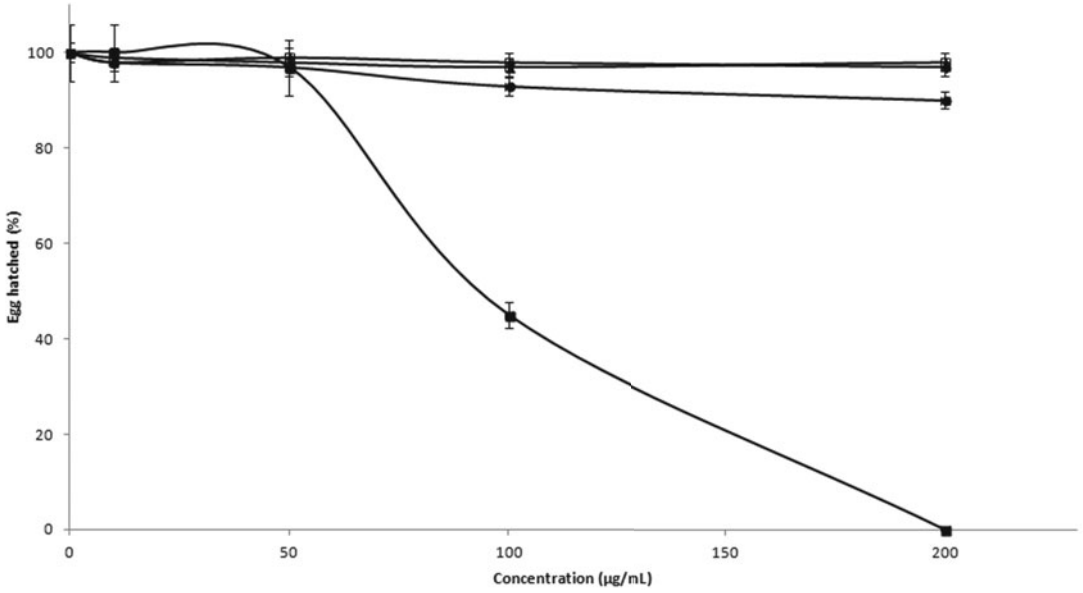

Fig. 3. DHC decreases normal egg hatching process Effect of different concentrations of flavonoids (DHC ■, DHF ▲, HF ♦) and levamisole (LEV, ●) on egg hatching process of *Caenorhabditis elegans*. Values (mean ± SD) are statistically different (Student's t-test), $p < 0.05$

Surviving and dead animals were counted daily until all nematodes had died. Nematodes that failed to respond to a gentle touch (with a platinum wire) were scored as dead. Worms that crawled off the plate, died because of bagging or protrusion of the gonad through the vulva were discarded from the initial data set. Each pure compound concentration was tested in duplicate and the assay was repeated twice.

Statistical analysis

Data analysis for lifespan experiment was performed with GraphPad Prism version 5.0 for Windows. Lifespan data were analyzed by plotting Kaplan-Meier survival curves and conducting Log-Rank tests. Values of $p<0.05$ were considered to be statistically significant. For anthelmintic and larval devel-

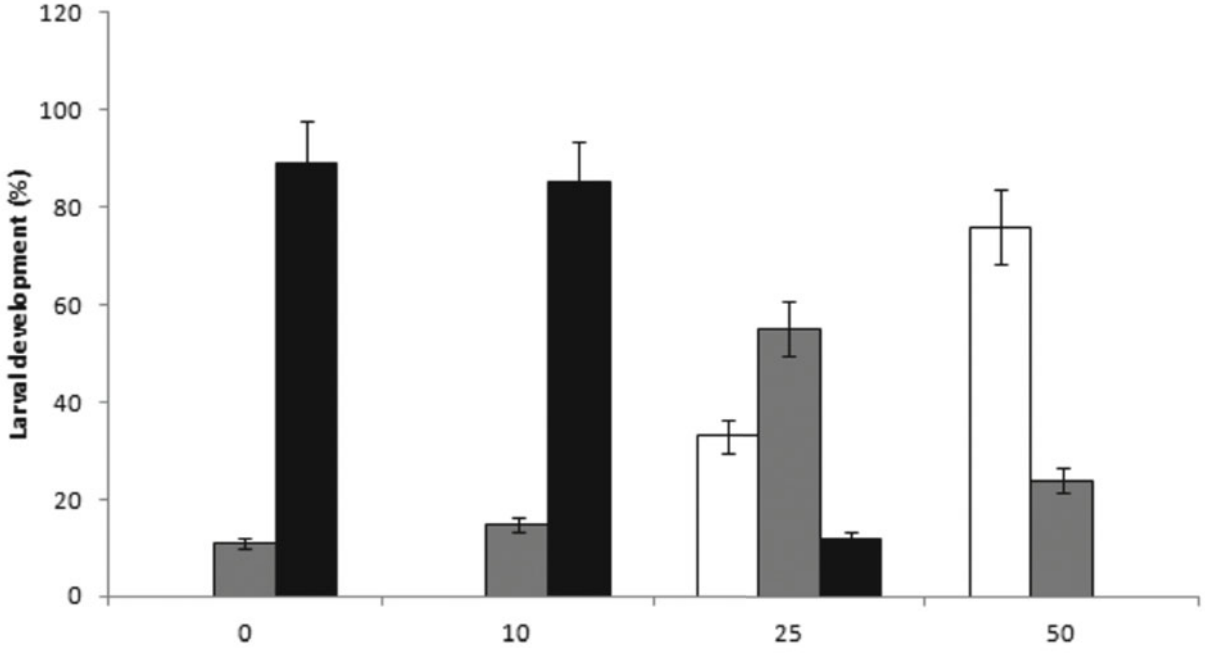

Fig. 4. DHC disturbs larval development process, Effect of different concentrations of 2′,4′-dihydroxychalcone in comparison to control (DMSO) on *Caenorhabditis elegans* development. L1-L3 (□); L4 (■) and adults (■).Values (mean ± SD) are statistically different (Student's t-test), $p < 0.05$

opment assays the statistical significance was determined using the Student's t-test (unpaired test used, significance level set at $p<0.05$).

Results

In the present study we evaluated the effects of three flavonoids (Fig. 1) present in *Z. punctata* on the free living nematode *C. elegans.*

In the evaluation of the effect of pure compounds in the anthelmintic assay, only DHC was active against adult *C. elegans*. The percentage of worm's mortality increased with increasing concentrations (Fig. 2). The LC_{50} of DHC, defined as the concentration required to kill half the members of the tested nematode population after 24 hours, was 17 µg/mL, and for levamisole, a known anthelmintic drug, was 4.7 µg/mL. Levamisole is a nicotinic receptor antagonist and elicits spastic muscle paralysis due to prolonged activation of the excitatory nicotinic acetylcholine receptors on body wall muscle (Aceves *et al*. 1970). DHC and levamisole 24-h LC_{50} results for *C. elegans* were calculated using LOESS regression and linear interpolation from the percent viability studies.

The effect of different concentrations of the three flavonoids and levamisole on egg hatching is summarized in Fig. 3. The dihydroxylated chalcone was the only active compound and the proportion of eggs hatching decreased with increasing concentrations. The lowest concentration of DHC required to inhibit egg hatching by 50% was 93 µg/mL. Levamisole did not affect the process up to 200 µg/mL. The highest concentration tested of DHC (200 µg/mL) caused 100% inhibition of the egg hatching process.

In the larval development assay, it was observed that *C. elegans* populations exposed from egg to adulthood on agar plates containing 25 and 50 µg/mL of DHC demonstrated an alteration in the percentage of worms that had reached L4 or

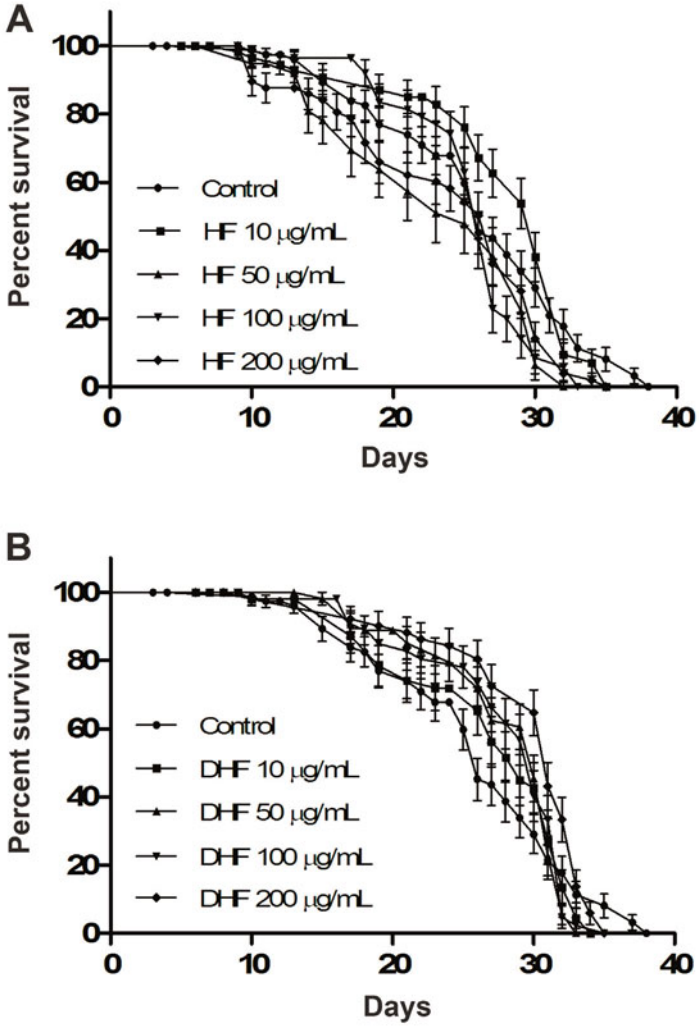

Fig. 5. Survival curves of *Caenorhabditis elegans* exposed to 10, 50, 100 and 200 µg/mL of 7-hydroxyflavanone (HF) (**A**) and 3, 7-dihydroxyflavone (DHF) (**B**) in comparison to control (DMSO). The figures represent the mean of two independent trials

adulthood after 48 h (Fig. 4). *C. elegans* populations exposed to these concentrations consisted of 33.4 and 73.4% L3 stage or younger, respectively, in comparison to control (Fig. 4). At 100 µg/mL of DHC, not all eggs hatched and larvae L1 present in the wells died after 24 hours. HF and DHF were ineffective to inhibit the larval development (data not shown).

Because DHF and HF did not have any effect in the assays above mentioned we tested their effect in the normal lifespan of *C. elegans*, on the basis of the knowledge that flavonoids can also extend lifespan of *C. elegans* (Grünz *et al*. 2012). In the lifespan assay, neither HF nor DHF modify the normal period of life of the nematode at the tested concentrations in comparison to control (Table 1 and Fig. 5). To determine whether the solvent DMSO itself had any effect on *C. elegans* lifespan, worms were also exposed to a final concentration of 1% DMSO. This concentration of DMSO did not have any adverse biological effects on the nematodes in our study as reported by other authors (Attar *et al*. 2011).

Table I. Effect of chemical structurally related flavonoids on *Caenorhabditis elegans* lifespan (days)

Concentration (µg/mL)	HF	DHF
	Lifespan (days)	
0	26	26
10	30	29
50	25	30
100	26	30
200	27	31

3,7-dihydroxyflavone (DHF) and 7-hydroxyflavanone (HF). All values were no statistically significant p 0.05 (Log-rank Test)

Discussion

The free-living soil nematode *C. elegans* is used as a system to screen products for their potential anthelmintic effect against small ruminant gastrointestinal nematodes. The Order Rhabditida, to which *C. elegans* belongs, is closely associated with the Order Strongylida, which contains the important trichostrongyle parasites of ruminants, including *Haemonchus contortus* and *Trichostrongylus* spp. If tested drugs are effective in *C. elegans* cultures at low concentrations, it is reasonable to assume that they may have anthelmintic activity against related nematodes, including *H. contortus* (Thompson *et al*. 1996; Katiki *et al*. 2011).

DHC can disrupt the life cycle of nematodes by preventing the hatching of eggs and the developing into larvae. DHC also showed an anthelmintic effect on adult *C. elegans* in the 24 h assay. Although the concentrations of 2´,4´-dihydroxychalcone needed to affect the nematode are greater than those needed to inhibit the growth of pathogenic bacteria (Zampini *et al*. 2005, 2008, 2011), difference can be explained by the impermeability of the nematodes´cuticules (Cox *et al*. 1981).

C. elegans has extensive physical and enzymatic xenobiotic defenses that may render many pharmacological tools ineffective. The physical barriers include a four-layered cuticle that lines its exterior and oral and rectal cavities (Cox *et al.* 1981) as well as an intestine through which solutes are rapidly pumped (Avery and Shtonda, 2003). The worm's genome is replete with predicted xenobiotic detoxification enzymes, including 86 cytochrome P450s, and 60 ATP-binding cassette transporters, many of which likely function as xenobiotic efflux pumps (Lindblom and Dodd, 2006). Compounds that are ineffective when applied to whole animals can readily antagonize their targets if they are provided with direct access (Jospin *et al.* 2002; Franks *et al*. 2002; Kwok *et al.* 2006). Hence, it is likely that *C. elegans* is generally resistant to exogenously applied pharmacologicals because they fail to accumulate to effective concentrations within its tissues. Only small molecules with structural features (Klekota and Roth, 2008; Horton *et al.* 2003), like 2´,4´-dihydroxychalcone, would accumulate to effective concentrations in the nematodes.

Young-Ah *et al*. (2006) reported that the presence of hydroxyl group in 4 position and the symmetry of hydroxylated substitutions in the B ring for flavonoids such as apigenin and its derivates could be responsible of larval growth inhibition of wild-type N2 worms. On the other hand, chalcones have been reported to have a broad range of biological activities such as antimalarial, antibacterial, antitumor, antioxidant, antihyperglycemic, anti-inflammatory, antileishmanial and anti-HIV (Attar *et al*. 2011; Shenvi *et al*. 2013; de Mello *et al*. 2014). Liu *et al*. (2003) reported that antileishmanial activity is favoured by chalcones with more hydrophilic character, between them 4´-hydroxychalcones. There are published reports on the nematocidal activity of chalcones. Laliberté *et al*. (1967) reported that chalcone and other related compounds showed high activity against zooparasitic nematodes: *Syphacia obvelata* and *Notodiaptomus dubius*. González and Estévez-Braun (1998) reported that chalcones and other similar aromatic compounds related to the Shikimate pathway, showed activity against phytoparasitic species like *Globodera pallida* and *Globodera rostochiensis*. Chalcones with different degrees of hydroxylation and methoxylation showed a significant activity *in vitro* against promastigotes and intracellular amastigotes of *Leishmania amazonensis* and the human malaria parasite, *Plasmodium falciparum* (Nowakowska, 2007). Recently, Attar *et al*. (2011), showed the nematocidal activity against *C. elegans* of ferrocenyl derivatives of chalcones and their organic analogs.

Lee *et al*. (2008) reported that some flavones have effects on *C. elegans* development. However, under our experimental conditions, DHF and HF (up to 200 µg/mL) did not show an anthelmintic effect on adults. Furthermore, exposure to these compounds did not alter normal physiological processes such as egg hatching, larval development and lifespan of *C. elegans*. One possible explanation is the lack of hydroxylated

substituents in the B ring and a hydroxyl group in 4 position of the molecule.

Polyphenols represent one of the most prevalent classes of compounds found in our daily diet. Over the last ten years, increasing attention has been dedicated to flavonoids because of their interesting biological activities. Flavonoids in general are believed to have no or little toxicity and have a long history of human consumption (Zampini *et al*. 2012). Very large doses of these compounds (up to 500 mg/kg) have been administered to animals, with little or no toxicity reported (Morris and Zhang, 2006). Zampini *et al*. (2008) assayed the DHC genotoxicity/antigenotoxicity in the *in vitro* comet assay test on human hepatoma HepG2 cells. The natural product neither affected cell viability nor induced DNA damage, indicating that it was not toxic. Additionally toxicity studies *in vivo* are being conducted in our laboratory.

Conclusion

In our study we tested the effects of DHC, HF and DHF from *Z. punctata* on the free-living nematode *C. elegans*. We observed that DHC can inhibit key biological processes like egg hatching and larval development in *C. elegans*. Anthelmintic effect on adults was also shown. Due to the appearance of drug-resistant worms, new therapeutic drugs with good efficacy and low toxicity are urgently needed. Therefore, DHC, a natural compound present in *Zuccagnia punctata*, could be proposed as a potential anthelmintic drug. Further studies of the molecular basis of chalcones anti-nematodal activity should provide useful information for developing more specific and more effective anthelmintic.

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