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ORIGINAL ARTICLE

Effect of *Zuccagnia punctata* Cav. (Fabaceae) extract on pro-inflammatory enzymes and on planktonic cells and biofilm from *Staphylococcus aureus*. Toxicity studies

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KEYWORDS

Zuccagnia punctata;
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Abstract *Zuccagnia punctata* Cav. (Fabaceae), a native plant from Argentina has been used traditionally as medicinal species. The aim of the study was to validate the antibiotic and anti-inflammatory potential of *Z. punctata* organic extract (ZpE) and the major compounds; 2′,4′-dihydroxy-3′-methoxychalcone (DHMC), 2′,4′-dihydroxychalcone (DHC), 7-hydroxyflavanone (7-HF) and 3,7-dihydroxyflavone (DHF); using an *in vitro* model. The antibiotic activity was determined using a broth microdilution method and the minimum inhibitory concentration (MIC) was determined. The extract and the isolation compounds affect the normal growth of all assayed *Staphylococcus aureus* strains. The MIC values for ZpE and isolated compounds were between 125 and 500 μg/mL and between 25 and 400 μg/mL, respectively, against all assayed strains. The inhibitory effect of extract and isolated compounds on biofilm formation and on pro-inflammatory enzymes ($sPLA_2$, COX-2, LOX) was analyzed. The compound DHC was the most active on $sPLA_2$ while DHF and DHMC showed the highest activity on LOX. Both the extract and pure compounds except DHMC were active against COX-2. It can be concluded that the phytocomplex and the pure compounds possessed antibiotic and anti-inflammatory activities under the conditions tested, and could be a good alternative therapy for infective and inflammatory processes.

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1. Introduction

Due to the difficulty of eradicating infections caused by *Staphylococcus aureus* by its high capacity to develop antibiotic resistance and virulence factors such as biofilm, the optional treatment with new antimicrobial agents is necessary. The phytomedicine is a very interesting therapeutic alternative because the plant extracts are multi-components that could act on multiple targets by different action mechanisms. Some countries have begun to look at their locally available traditional medicines as a means to drug new discovery.

Zuccagnia punctata Cav. (Fabaceae) is used for many years in traditional medicine in Argentina (Cabrera, 1971) as antimicrobial and anti-inflammatory (Toursarkissian, 1980). Antioxidant properties (Moran Vieyra et al., 2009), antifungal activity (Svetaz et al., 2004, 2007; Agüero et al., 2010; Nuño et al., 2014; Moreno et al., 2015), antibacterial activity against antibiotic resistant Gram-negative bacteria (Zampini et al., 2005) and *Streptococcus pneumoniae* (Zampini et al., 2012), activity on P-glycoprotein, a membrane ABC transporter (Chieli et al., 2012), antigenotoxic properties (Zampini et al., 2008), and antiulcer activity (De la Rocha et al., 2003) were reported for *Z. punctata* ethanolic extracts. Some bioactive phenolic constituents and major components of *Z. punctata* ethanolic extracts were also reported (Svetaz et al., 2004; Zampini et al., 2005; Agüero et al., 2010; Nuño et al., 2014).

The aim of the present work was to evaluate the effect of *Z. punctata* extracts and their major chemical constituents on *S. aureus* growth and virulence factor production as well as pro-inflammatory enzyme inhibition in order to validate its popular use. The toxicity (mutagenic and acute toxicity) was also evaluated.

2. Experimental

2.1. Extraction, isolation and compounds identification

The aerial parts of *Z. punctata* Cav. (voucher specimen N° 605,935/LIL, Herbarium of "Fundación Miguel Lillo") was collected from Monte region of Argentina. The plant samples, were dried at 40 °C grounded, macerated and extracted with dichloromethane (DCM) (800 mL) to obtain a non-polar extract of *Z. punctata* (ZpE). Then, ZpE was dried by rotary evaporator to obtain the yield in dry weight. The extract was resuspended in methanol and subjected to bio-guided fractionation by Silica gel, Sephadex LH-20 and C18-column. The fractions with antibiotic activity were analyzed with HPLC–ESI-MS/MS for compounds identification (Nuño et al., 2014). The major compounds identified in these fractions were: 2′,4′-dihydroxy-3′-methoxychalcone (DHMC), 2′,4′-dihydroxy chalcone (DHC), 7-hydroxyflavanone (7-HF) and 3,7-dihydroxyflavone (DHF). These compounds were also quantified. A calibration curve was prepared using commercial standards to determine the relationship between the peak area and concentration. The compounds concentration was expressed as μg/mg of ZpE. Three replicates were used for all samples.

The antibacterial activity of each fraction was tested by bioautographic and broth microdilution assays (Zampini et al., 2009; CLSI, 2006).

2.2. Effect of Zp extract and major components on planktonic cells growth and biofilm formation

2.2.1. Microorganisms

Seven clinical isolates of *S. aureus* (F2, F5, F7, F8, F22, F23 and F31) with different antibiotic resistance profile obtained from patients of the Hospital Nicolás Avellaneda, San Miguel de Tucumán, Tucumán, Argentina were used. A strain of American Type Culture Collection (ATCC 10231) was also used. The inocula were prepared by adjusting the suspension turbidity to match the 0.5 McFarland standards (10^8 CFU/mL).

2.2.2. Activity against planktonic cells

The activity of ZpE and pure compounds on *S. aureus* was analyzed by a broth microdilution method according to the CLSI reference M100S17 (CLSI, 2006). Assayed natural products were dissolved in 1% dimethyl sulfoxide (DMSO) to obtain final concentrations between 125 to 500 μg of dry weight per mL (μg/mL) for the ZpE and between 25 to 400 μg/mL for each fraction and pure compound. The inoculum (100 μL of Mueller Hinton broth containing 1×10^7 CFU/mL) was added to each well. Plates were incubated at 37 °C for 24 h. Aliquots of 10 μL of each well without visible growth were inoculated onto the surface of Mueller Hinton agar. The MIC and MBC values were determined.

2.2.3. Bioautographic assay

The *Z. punctata* aerial parts extracts were spotted on silica gel TLC and developed using $CHCl_3$:MeOH (9:1) as the mobile phase. Then, the plates were covered with soft medium (BHI with 0.6% agar) containing 10^5 CFU of a methicillin resistant *S. aureus* (F7), incubated at 37 °C for 16–20 h and revealed with MTT solution (2.5 mg/mL) in PBS according to Zampini et al. (2009).

2.2.4. Effect of natural product on biofilm formation and metabolic activity

Z. punctata extract or isolated compounds (20 μL of 1/2 MIC) were added to *S. aureus* (ATCC 25923 and F7 strains) suspension (180 μL of 1×10^8 cells/mL). The plates were incubated at 37 °C during 24 h and then, the medium was aspirated, and non-adherent cells were removed by washing with sterile PBS. Ciprofloxacin (50 μg/mL) was used as positive control.

Finally, 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium hydroxide (XTT) or crystal violet was added to determine the effect of natural product on metabolic activity and biofilm formation, respectively (Gilabert et al., 2015).

2.2.5. Electron microscopy

S. aureus suspension (100 μl of 1×10^8 cells of F7 strain/mL) was added to 100 μL of PBS or DHC at the MIC concentration. All tubes were incubated 24 h with stirring (500 rpm). Then, were submitted to centrifugation for 5 min at 5000*g* and the pellets were re-suspended in PBS.

2.2.5.1. Scanning electron microscopy (SEM). Untreated cells and cells exposed to DHC were processed for electron microscopy according to Thein et al. (2006) using 4% (v/v) formaldehyde and 2% (v/v) glutaraldehyde in PBS, washed

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thoroughly in the same buffer, post-fixed in 1% (w/v) osmium tetroxide and dehydrated in alcohol (70% and 100%). Then, the cells were treated with hexamethyldisilazane for 5 min. The dehydrated samples were critical point-dried and then coated with gold/palladium (40%/60%), before viewing using a Zeiss-supra 55VP scanning electron microscope operated at high vacuum at 15 kV.

2.2.5.2. Transmission electron microscopy (TEM). The treated and non treated cells were placed in contact with 0.3% terpinen-4-ol and then were fixed over night with 2.5% of glutaraldehyde, and dehydrated in alcohol, propylene oxide and araldite. The dehydrated samples were embedded in resin, sectioned and stained according to Carson et al. (2002) before examination using a Zeiss-EM 109 transmission electron microscope operated at an accelerating voltage of 80 kV.

2.3. Effect of ZpE and selected major constituents upon pro-inflammatory enzymes

2.3.1. Cyclooxygenase (COX) inhibition assay

The inhibitory activity of the samples was determined using the COX inhibitor assay screening kit (Cayman Chemical Co., Ann Arbor, MI, USA). Prostaglandins (PGs), which are produced in the COX-2 reaction, were quantified via enzyme immunoassay with a specific antibody that binds to major PG compounds (D'Almeida et al., 2013). The natural products at various concentrations in DMSO were tested. DMSO was used in the control of 100% COX-2 activity. Nimesulide and indomethacin were used as positive controls.

2.3.2. Lipoxygenase (LOX) inhibition assay

The assay was carried out by monitoring the LOX-mediated transformation of linoleic acid into lipidic hydroperoxide at 234 nm. The reaction mixtures contained the following reagents: soybean LOX-1 (0.9 mM), in borate buffer 0.2 M, pH 9.0, tested natural products at various concentrations in DMSO (25–200 μg/mL), and linoleic acid (50 μM). The increase in absorbance was monitored for 4 min (D'Almeida et al., 2013). The negative control was DMSO, and the positive controls were naproxen and caffeic acid.

2.3.3. Phospholipase ($sPLA_2$) inhibition assay

The substrate for $sPLA_2$ were micelles of 1,2-di (heptanoylthio) glycerophosphocholine (1.25 mM) with Triton X-100 (0.3 mM) (D'Almeida et al., 2013). The substrate was dissolved in 10 mM Tris–HCl (pH 8) containing 10 mM $CaCl_2$ and 100 mM KCl. Ten microlitre of $sPLA_2$ (1 μg/mL) was added to 50 μL of the buffer, 10 μL of 5,5′- dithio-*bis*-2-nitrobenzoic acid (DTNB) 10 mM and 5 μL of natural products in DMSO (10 μg/mL). The reaction was initiated with 150 μL of substrate and measuring absorbance at 415 nm for 20 min at 25 °C. The negative control was DMSO, and the positive control was naproxen.

2.4. Toxicity assay

2.4.1. General toxicity assay

The acute toxicity levels of ZpE and isolated compounds were studied using *Artemia salina* as test organism (Svensson et al., 2005). Different concentrations of DHC (12.5–50 μg/mL), DHMC, HF, DHF (50–200 μg/mL) or ZpE (125–500 μg/mL) were used.

2.4.2. Mutagenicity test

The assays were performed according to Maron and Ames (1983), with the *Salmonella typhimurium* TA98 and TA100 strain using different concentrations for ZpE and isolated compounds (until 250 μg per plate). Negative and positive controls were used simultaneously in each experiment. The positive control was 4-nitro-o-fenilendiamine (10 μl per plate, 1 mg/mL solution) for TA98 and TA100, and the negative control was DMSO (100 μl per plate). His + revertants were counted after 48 h of incubation at 37 °C. The test substance was considered mutagenic if it reproducibly induced a twofold increase in the number of mutants with at least one concentration, compared to the corresponding negative control (mutagenicity relationship).

2.5. Statistical analysis

The data determined were expressed as the mean of three replicate determinations and the values of relative standard deviation (RSD) $< 10\%$ were considered to meet the demands for bioanalytical assay. The IC_{50} values were estimated by linear/non-linear regression.

3. Results and discussion

3.1. Antimicrobial activity

In this paper we analyzed the antibiotic potency of *Z. punctata* non-polar extract and major compounds previously isolated (Nuño et al., 2014), against seven *S. aureus* strains isolated from infections of patients from an hospital of Tucumán, Argentina, and a collection strain.

The HPLC analysis showed that the contents of DHMC, DHC, DHF and HF were 92.61, 109.4, 22.53 and 28.93 μg/mg of ZpE, respectively; corresponding to 9.3, 10.9, 2.3, 2.9% of the extract weight.

It was shown that all assayed natural products affect the normal growth of all clinical isolates of *S. aureus* and the ATCC strains. The MIC values for ZpE were between 125 and 500 μg/mL and MBC values indicated that the extract was active on 60% of strains. For the isolated compounds, the MIC values were between 25 and 400 μg/mL and the MBC values were between 50 and >400 μg/mL against all strains assayed (Table 1).

The ZpE showed similar or greater potency as compared to aqueous and hydroalcoholic extracts of the same and other xerophyte plant species collected in arid environments of Northwest Argentina against Gram positive and Gram negative bacteria (Zampini et al., 2005, 2009, 2012). Isolated compounds showed greater potency than the extract, the order of reactivity for these latter was DHC > DHF = HF > DHMC. Previously other authors reported that OH groups of chalcones induce and enhance the antimicrobial activity, while the methoxy groups in the structure of chalcones decrease or eliminate the antimicrobial activity (Hatano et al., 2000). Moreover, the unsaturated chain of

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Table 1 Antibacterial activity of *Z. punctata* dichloromethanic extract (ZpE) and the major isolated compounds against antibiotic sensible and resistant *Staphylococcus aureus*.

N° strains	ZpE	DHC	DHMC	HF	DHF	Fenotype of clinical isolate
	MIC/MBC (μg/mL)					
2	250/R*	25/50	200/400	50/R*	25/400	Met^r Oxa^r Gen^r Van^s
5	125/500	25/100	200/200	200/R*	200/R*	Met^r Oxa^r
7	500/R*	25/100	200/200	200/200	200/R*	Met^r Oxa^r Gen^r
8	125/500	25/100	200/200	25/R*	25/400	Met^r Oxa^r
22	250/R*	25/400	200/R*	25/R*	25/R*	Met^r Oxa^r Gen^r Van^s
23	125/250	25/200	100/200	200/R*	200/R*	Met^r Oxa^r
31	125/250	100/200	200/200	200/R*	200/R*	Met^r Oxa^r Van^s
ATCC 29213	125/250	50/50	200/200	200/R*	400/R*	Control strain

ZpE: *Z. punctata* dichloromethanic extract. DHC: 2′,4′-dihydroxychalcone; DHMC: 2′,4′-dihydroxy-3′-methoxychalcone. HF: 3-hydroxyflavanone; DHF: 3,7-dihydroxyflavone. R*: resistent until 500 μg/mL of extract.
^{r}Resistant, ^{s}Susceptible; methicillin (Met), oxacillin (OXA), gentamycin (Gen), vancomycin (Van).

DHC between rings A and B, was not a determinant of *S. aureus* antimicrobial activity since the flavone and flavanone exhibit similar antimicrobial activity. The compound DHC showed a similar or higher inhibitory effect against *S. aureus* growth than others hydroxylated chalcones on the ring B and flavones previously reports (2′,4,6′-trihydroxy-4′- metoxychalcone, 3,5-dihydroxy-6,7,8-trimetoxyflavona, apigenin and luteolin) (Gibbons, 2004; Süzgeç-Selçuk and Birteksöz, 2011; Yi et al., 2008).

S. aureus treated with DHC was observed by electron microscopy (SEM and TEM) to confirm the antimicrobial efficacy of the chalcone along with the morphological changes in the cell appearance. Fig. 1 showed that the treated bacteria with MIC values of DHC produce visible cellular aggregation, blebs on the cellular surface and cytoplasmic coagulation. The formation of blebs is an indicator of membrane damage because the total leakage of cytoplasmic material was not observed. The compound DHC produced cytoplasmic coagulation of *S. aureus* cell, evidenced by dark areas in the cells, as exemplified by Fig. 1IV (Codling et al., 2005). Stapleton et al. (2007) reported that a flavonol (epicatechin gallate) promoted staphylococcal cell aggregation and increases cell-wall thickness.

3.2. Biofilm inhibition

Pathogenicity of microorganisms depends on the virulence factors developed to produce adhesion and invasion of host. Not all antibiotics are able to inhibit these virulence factors, hence the importance of finding alternative treatments. Therefore, biofilm formed by methicillin-resistant *S. aureus* have become resistant to most available antimicrobial agents, including the β-lactams (Lewis, 2005). For these reasons, it is interesting to

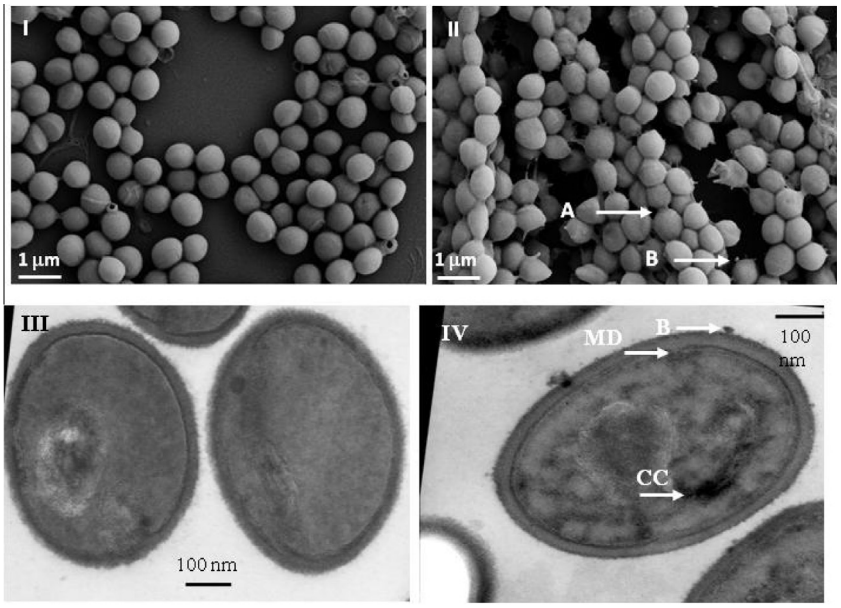

Figure 1 Electron microscopy of *Staphylococcus aureus* cells (I, III) untreated (control) and (II, IV) treatment with 50 mg/mL 2′,4′-dihydroxychalcone. I and II SEM. III and IV TEM. Key to arrows: A, aggregation; B, blebbing; CC, cytoplasmic coagulation; MD, membrane damage.

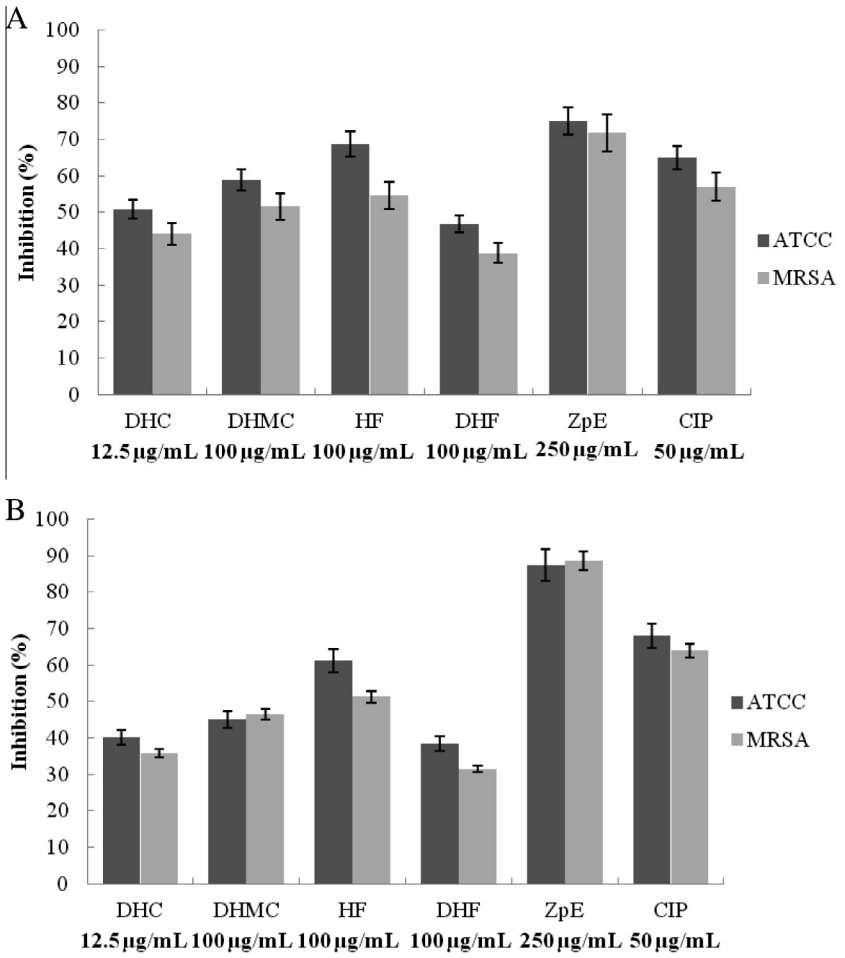

Figure 2 The percent of biofilm formation inhibition (A) and metabolism decrease (B) by *Zuccagnia punctata* (ZpE) and pure metabolites. Methicillin resistant *Staphylococcus aureus* (MRSA) F7 strain and ATCC 25923 strain. DHC: 2′,4′-dihydroxychalcone; DHMC: 2′,4′-dihydroxy-3′-methoxychalcone. HF: 3-hydroxyflavanone; DHF: 3,7-dihydroxyflavone; ZpE: *Z. punctata* extract; CIP: Ciprofloxacin. Data are presented as mean values ± SD ($p < 0.05$).

study the effect of ZpE and isolated compounds on biofilm formation by *S. aureus*. The effect of the samples was evaluated on biofilm formation by two strains of *S. aureus* a clinical isolate (F7 strain) and a collection strain (ATCC 25923), which showed increased adherence to microplates. Fig. 2 shows that the ZpE and the isolated compounds inhibit biofilm formation and reduce the metabolism of cells embedded in it. The DHC was the most active pure compound in inhibiting *S. aureus* biofilm formation. Cushnie and Lamb (2011) reported inhibition of biofilm formation by flavonoids. While De Lima Pimenta et al. (2013) demonstrated biofilm activity for other natural and synthesized chalcones against Gram positive bacteria.

Furthermore, the present study is the first report of medicinal species extracts from arid environments of Argentina that inhibit biofilm formation of *S. aureus*. Vegetal extracts of *Quercus cerris* L. (Fagaceae) showed similar *S. aureus* biofilm inhibition at the concentrations of 200 µg/mL (Hobby et al., 2012).

3.3. Anti-inflammatory properties of ZpE and selected major constituents

In the inflammatory process, the membrane phospholipids are hydrolyzed to produce arachidonic acid by phospholipase enzymes (PLA_s) and then, this acid is metabolized through the enzymes cyclooxygenases (COXs), and lipoxygenases (LOXs) by different enzymatic pathways to produce prostaglandins, thromboxanes and leucotrienes (D'Almeida et al., 2013).

In this work we investigated the inhibitory effect of ZpE and isolated metabolites on pro-inflammatory enzymes ($sPLA_2$, LOX and COX-2).

For the secretory phospholipase A_2 ($sPLA_2$), DHC was the most active compound follows by ZpE and DHMC, HF and DHF (Table 2). The low inhibitory activity of flavanones could be due to the absence of the double bond between C2-C3 in the ring B (Kim et al., 2004). The pure compounds were more potent than the commercial anti-inflammatory drug, naproxen.

Lipoxygenases are the enzymes responsible for generating hydroxy acids and leucotrienes from arachidonic acid. The compounds DHF and DHMC showed the highest activity with IC_{50} values of 57.8 and 63.4 µg/mL, respectively. The potency of both compounds was similar to the caffeic acid used as control. *Z. punctata* extract also showed important inhibitory activity. The flavanone HF was the less active compound; this is in agreement with Ribeiro et al. (2014) who reported that the double bond between C2-C3 in the ring C is important for the LOX inhibitory activity.

Table 2 Inhibitory activity of *Z. punctata* extracts (ZpE) and the major isolated compounds against three pro-inflammatory enzymes.

Samples	Inhibition (%)	IC_{50} (μg/mL)	
	$sPLA_2$ (10 μg/mL)	LOX	COX-2
ZpE	40.47 ± 2.00	69.30 ± 3.50	293.00 ± 20.50
DHC	60.50 ± 2.90	99.50 ± 3.00	1.72 ± 0.10
DHMC	38.18 ± 1.80	63.40 ± 3.10	–
HF	8.42 ± 0.40	155.00 ± 3.10	35.00 ± 2.40
DHF	2.70 ± 0.20	57.80 ± 2.90	25.50 ± 1.90
Naproxen	6.00 ± 0.40	8.14 ± 0.40	–
Nimesulide	–	–	0.39 ± 0.01
Indomethacin	–	–	0.28 ± 0.01
Caffeic acid	–	57.5 ± 1.1	–

ZpE: *Z. punctata* dichloromethanic extract. DHC: 2′,4′-dihydroxychalcone; DHMC: 2′,4′-dihydroxy-3′-methoxychalcone.; HF: 3-hydroxyflavanone; DHF: 3,7-dihydroxyflavone. Data are presented as mean values ± SD ($n = 3$, $p < 0.05$).

Cyclooxygenase-2 is a pivotal enzyme in inflammation, and inhibitors of COX-2 are being continuously developed to obtain safer anti-inflammatory drugs. As present in Table 2, within the examined flavonoids DHC showed the highest COX-2 inhibitory activity with IC_{50} value of 1.72 μg/mL followed by DHF, HF and ZpE. The DHMC was not active against COX-2 at the tested concentrations (10–150 μg/mL). The DHC showed similar activity to the synthetic anti-inflammatory drugs assayed (indomethacin and nimesulide).

To our knowledge the compounds studied in this work were for the first time found as inhibitors of COX-2, LOX and sPLA2 enzymes. However others chalcones, flavones and flavanones were found to be inhibitors of these pro-inflammatory enzymes (Kim et al., 2004).

Chalcone derivatives such as 3,4-dihydroxychalcone, 3,4,2′-trihydroxychalcone, 3,4,4′-trihydroxychalcone and 3,4,2′4′-tetrahydroxychalcone, inhibited LOX and COX activities, being more active on LOX (Kim et al., 2004). In regard to the chalcones evaluated in this work, DHMC was more active on LOX, while DHC was more potent on COX-2. However, weak or no activity was observed on COX by Likhitwitayawuid et al. (2002) for 4,4′-dihydroxy-2,6-dimethoxydihydrochalcone, 2,4′-dihydroxy-4,6-dimethoxydihydrochalcone, 4′-hydroxy-2,4,6-trimethoxydihydrochalcone,4,6,4′-trihydroxy-2-methoxydihydrochalcone.

A fraction of *Parastrephia lucida* enriched in methoxy flavones strongly inhibited sPLA2 activity (D'Almeida et al., 2013). The flavone DHF at the concentration assayed in our work was not active. On the other hand, Takano-Ishikawaa et al. (2006) suggested that the 4-oxo functional group of the C-ring is essential for the higher COX inhibition activities and that the C2-C3 double-bond enhances the activity; this could be the reason why DHF was more active than the HF.

Won et al. (2005) suggested that 2′,4-dihydroxychalcone; 2′,5′-dihydroxy-indol-3-yl-chalcone; 2′-hydroxy-2-thienylchalcone; 2′-hydroxy-3-tienylchalcone, 2′-hydroxy-3,4-dichloro chalcone and 3,5-di-ter-butyl-2′,4,5′-trihydroxychalcone were potential anti-inflammatory agents.

3.4. Evaluation of acute toxicity

A. salina test is based on the possibility of a toxic compound to kill *A. salina* larvae grown in the laboratory. None of the pure compounds showed toxicity against *A. salina* at the concentrations tested. *Z. punctata* extract showed LC_{50} (concentration that kills 50% of the *A. salina* larvae) value of 250 μg/mL, while the positive control (sodium dichromate) showed a LC_{50} value of 30 μg/mL.

3.5. Mutagenicity assay

In this study, the mutagenicity relationship resulted less than 1.5 for both the ZpE and the isolated compounds; indicating that they were not mutagenic agents. Zampini et al. (2008) did not find genotoxicity of *Z. punctata* hidroalcoholic extract and DHC using the *in vitro* comet assay test on human hepatoma HepG2 cells.

4. Conclusions

In summary, the ZpE (phytocomplex) and the pure compounds were active against the *S. aureus* clinical isolated and on three pro-inflammatory enzymes ($sPLA_2$, LOX and COX), so arachidonic acid metabolism could be inhibited at different levels. The results showed that these natural products could be a good alternative therapy for infective and inflammatory processes but *in vivo* assays are necessary to determine the effectiveness of its use. The mutagenic effect absence is a positive advance for determination of safety use of this medicinal plant species.

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