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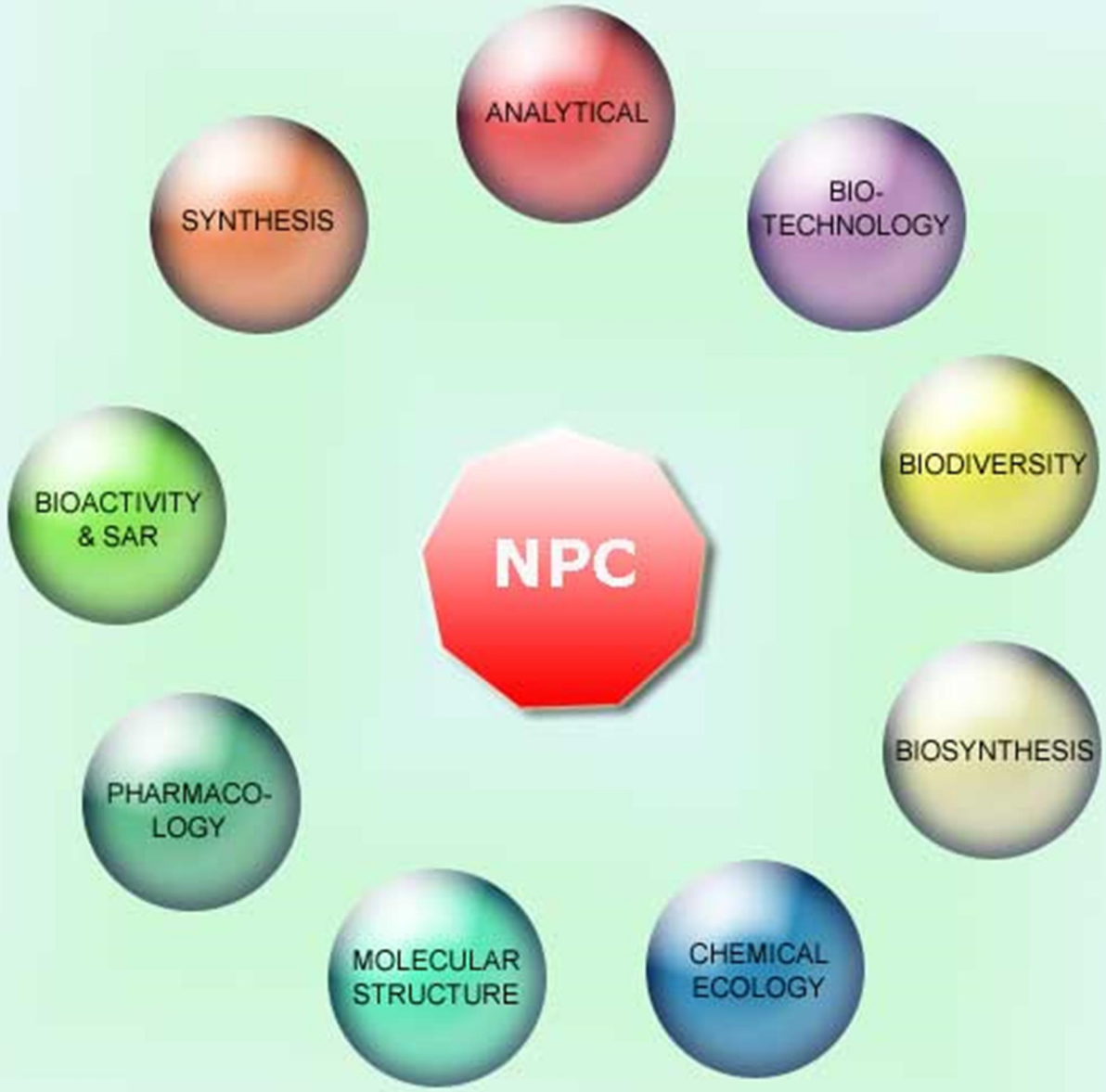

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Dr. Pawan K. Agrawal
On the Occasion of his 60th Birthday

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Anti-inflammatory, Antioxidant and Antimicrobial Activity Characterization and Toxicity Studies of Flowers of "Jarilla", a Medicinal Shrub from Argentina

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Zuccagnia punctata Cav. (Fabaceae) is an Argentine medicinal aromatic shrub (jarilla pispito, puspus, lata and jarilla macho). The chalcones were identified as pigments responsible for the yellow color of the flowers. Hydroethanolic extracts were obtained both from fresh flowers and from flowers dried by lyophilization. The extracts were standardized by their phenolic and flavonoids content. Their fingerprints by HPLC-DAD indicated the presence of two chalcones as major compounds (2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3′-methoxychalcone**)**. Both extracts showed the same total phenolic, non-flavonoid phenolic and flavonoid phenolic content and their phenolic profiles were similar. The polyphenolic extracts exhibited antioxidant (free radical scavenging and inhibitory activity on lipoperoxidation) and anti-inflammatory (inhibition of lipoxygenase and cyclooxygenase enzymes) activities. The flower extracts were active against six *Candida* species with MIC values between 60 and 120 µg GAE.mL^{-1} and were also active on methicillin-resistant *Staphylococcus aureus* (MIC: 250 µg GAE.mL^{-1}) and *Enterococcus faecalis* (MIC: 500 µg GAE.mL^{-1}). The extracts were neither toxic (*Artemia salina* test) nor mutagenic (Ames test). Jarilla flowers could be considered as a new dietary supplement that could help to prevent pathologies associated with oxidative stress and the polyphenolic extract obtained from them could be considered as a standardized phytotherapeutic product with antimicrobial, antioxidant and anti-inflammatory activities.

Keywords: *Zuccagnia punctata*, Flowers, Functional food, Phytotherapeutic product, Bioactive chalcones.

Zuccagnia punctata Cav. (Fabaceae) is a monotypic species, commonly known as jarilla pispito, puspus, lata and jarilla macho. It is an Argentine medicinal endemic shrub widely distributed in the biogeographic region of Monte de Sierras y Bolsones from western Argentina (provinces of Jujuy, Salta, Tucumán, Catamarca, La Rioja, San Juan, Mendoza and San Luis) [1a] and it shows resistance to diseases and pests. It is a glutinous and aromatic shrub with pinnate resinous leaves of 3-5 cm. The plants bloom from August to March. The yellow flowers, born in erect racemes, have a funnel-shaped calyx, with 5 sepals, corolla with 5 free petals and 10 free stamens [1a-1c]. The aerial parts of *Z. punctata* with and without flowers or fruits have been used extensively as a traditional medicine in Argentina for the treatment of bacterial and fungal infections, asthma, arthritis and rheumatism [2a,2b].

Previous reports showed the biological activities (antioxidant, antibacterial, antifungal, antiulcer, antigenotoxic, chemopreventive) and the chemical composition (flavanones, flavones, chalcones and caffeoyl esters) of the aerial parts (fruit, stem and leaves) of *Z. punctata* [3a-3k]. However, the biological activities and chemical composition of *Z. punctata* flowers have not yet been analyzed.

The aim of this work was to determine the pigments responsible for the yellow color of the flowers of *Z. punctata* and to evaluate the functional properties of the polyphenolic extract of the flowers. The toxicity (*Artemia salina*) and mutagenic activity (Ames test) of the extract were also evaluated.

Table 1: Total phenolic, non-flavonoid and flavonoid phenolic content of aerial parts (Ap), fresh and dried flowers (FF and DF) of *Z. punctata*.

Z. p.	Total phenolic compounds (TP)	Non flavonoid phenolics (NFP)	Flavonoid phenolics (FP)	Flavone and flavonol
	mg GAE.g^{-1} plant material			mg QE. g^{-1}
A.p.	168.0±2.0^a	34.4±1.0^a	133.6±2.0^a	16.0 ± 1.0^a
F.F	166.0±2.0^a	30.8±2.0^a	135.2±2.0^a	9.0±0.9^b
D.F.	166.7±2.0^a	31.0±2.0^a	135.7±2.0^a	8.8±0.9^b

Values are reported as mean ± standard deviation of triplicates. Similar letters (a, b) in the same column show no significant differences between different samples according to Tukey's test ($p \leq 0.05$).

The flowers were used either directly after harvesting or as dried material by lyophilization. Hydroethanolic extracts were obtained from fresh and dried flowers. The content of phenolic compounds (166.7±15 mg GAE.g^{-1} flowers) did not show a significant difference between them and was similar to the content of phenolic compounds in the aerial parts of *Zuccagnia punctata* (168.0±15 mg GAE.g^{-1} aerial parts). Both fresh and dried flowers contained principally flavonoid phenolics (Table 1). The content of flavones and flavonols was higher in aerial parts (16.0 ± 1.0 mg QE.g^{-1}) than in flowers (9.0±0.9 mg QE. g^{-1} flowers) (Table 1).

HPLC-DAD analysis of both flower hydroethanolic extracts showed the presence of cinnamic acid (**C1**), galangin (**C2**), crysin (**C3**), 2′,4′-dihydroxychalcone (**C4**) and 2′,4′-dihydroxy-3′-methoxychalcone (**C5**). These results were confirmed by standard compound retention time, UV spectral analysis and by co-injection (standard plus flower extract). 2′,4′-Dihydroxy-3′-methoxy-chalcone (**C5**) was the major component of flower extracts.

Carotenoid pigments were not detected in flowers by qualitative and quantitative methodology. Consequently, the yellow pigmentation of *Z. punctata* flowers could be mainly due to chalcones. These pigments confer a striking yellow color to the flowers of different species such as *Bidens*, *Coreopsis*, *Cosmos*, *Dahlia*, *Acacia* and *Dianthus* and are useful as attractants of pollinators [4a,4b].

Both samples (fresh and dried) exhibited $ABTS^{\bullet+}$ reducing capacity, with similar SC_{50} values (3.8±0.2 μgGAE.mL^{-1}). A dose-response relationship between the antioxidant activity percentage and phenolic compound content was observed (R^2=0.98). The scavenging activity was higher than the commercial natural and synthetic antioxidants used by the food industry such as quercetin (SC_{50}= 6.7±0.3 μg.mL^{-1}) and BHT (SC_{50}= 7.7±0.4 μg.mL^{-1}). The autographical assays showed that both chalcones should be responsible for the antioxidant activity. This clearly suggests that antioxidant mechanisms by both chalcones could be related to their direct antioxidant properties (reducing capacity).

In addition, the flower extracts were able to protect lipids from oxidation, showing an IC_{50} value of 14.7±0.2 μgGAE.mL^{-1}. A dose-response relationship between antioxidant activity percentage and phenolic compound content was observed (R^2= 0.95). Nevertheless, standards such as quercetin (IC_{50}=7.3±0.3 μg.mL^{-1}) and BHT (IC_{50} = 4.0±0.2 μg.mL^{-1}) inhibited the lipid oxidation more efficiently in a concentration-dependent manner.

It is known that many anti-inflammatory drugs have various and severe adverse effects. Therefore, natural agents with very little side-effects are required to substitute chemical therapeutics. The *in vitro* inhibitory activity of two enzymes, cyclooxygenase (COX-2) and lipoxygenase (LOX), responsible for the biosynthesis of inflammatory mediators, was used as an indication of the anti-inflammatory activity of the samples. COX products are well known to play an important role in pain and inflammation and LOX products are also involved in inflammatory processes, being important mediators in bronchial constriction and in hypersensitivity reactions. The phenolic extracts obtained from *Z. punctata* flowers were able to inhibit COX-2 and LOX in an equipotent manner (Table 2) behaving as dual inhibitors that block both routes of biosynthesis of mediators of the inflammatory process. Nevertheless, 2′,4′-dihydroxychalcone exhibited an inhibitory effect on LOX activity, but did not show any effect on COX-2 activity until a concentration of 150 μg.mL^{-1}. 2′,4′-dihydroxy-3′-methoxychalcone was able to inhibit PG biosynthesis (IC_{50} value for COX-2 of 1.72 μg.mL^{-1}), being 58 fold more active on COX-2 than on LOX enzyme. According to our results, *Z. punctata* flower extracts and metabolites isolated from them may reduce the risk of inflammation-related diseases via either inhibition of COX-2 and LOX pathways and by scavenging free radicals and suppressing lipid peroxidation.

Candida species are opportunistic pathogens that produce mucosal to systemic infections such as oral candidiasis, vulvovaginitis and candidemia [5a,b]. In recent years, the number of infections worldwide by *Candida* species has increased considerably and resistance to traditional antifungal therapies is also rising. The current therapeutic options appear to be highly toxic and there are a lot of drug interactions. Furthermore, Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococus faecalis* have developed resistance mechanisms against several commercial antimicrobial drugs [5c]. For this reason natural products could be a new alternative therapy.

Table 2: Effect of *Zuccagnia punctata* flower extract and pure compounds on the pro-inflammatory enzymes, lipoxygenase (LOX) and cyclooxygenase-2 (COX-2).

Samples	LOX	COX-2
	IC_{50} (μg.mL^{-1})	
Flower extract	49.6 ± 2.5	59.7 ± 3.1
DHC	99.5 ± 5.0	1.7 ± 0.01
DHMC	63.4 ± 3.3	ND
Naproxen	14.0 ± 0.7	-
Nimesulide	-	0.39 ± 0.03

DHC: 2′,4′-dihydroxychalcone; DHMC: 2′,4′-dihydroxy-3′-methoxychalcone.
Values are reported as mean ± standard deviation of triplicates.
ND: IC_{50} not determined. 50% inhibition was not observed until 150 μg.mL^{-1}.

Table 3: *In vitro* determination of antifungal activity (MIC values) of flower extract against clinical strains of *Candida* species.

Yeast	Voucher number	MIC (μg GAE.mL^{-1})
C. albicans	F-100	60±10
C. albicans	F-101	60±10
C. tropicalis	F-300	60±10
C. tropicalis	F-301	120±20
C. krusei	F-400	60±10
C. parasilopsis	F-500	120±10
C. glabrata	F-200	60±10
C. guilliermondii	F-600	60±10
C. parapsilosis	ATCC 22019	60±10
C. albicans	ATCC10231	60±10

Values are mean ± standard deviation of triplicates.

Table 4: Minimal inhibitory concentration (MIC) values of flowers extract against antibiotic- resistant Gram-positive bacteria.

Microorganism	Voucher number/strain	Phenotype	MIC (μg GAE.mL^{-1})
Staphylococcus aureus	F2/ MRSA	Met^r, Oxa^r, Gen^r	250±50
	F7/ MRSA	Met^r, Oxa^r, Gen^r	250±50
	F22/ MRSCN	Met^r Oxa^r Gen^r Van^s	250±50
	F24/ MRSCN	Met^r Oxa^r Gen^r Van^s	250±25
	ATCC 29213	Control strain	125±25
Enterococcus faecalis	F201	Van^s Amp^s Gen^s Str^s	500±50
	F202	Gen^r Str^r Van^s Amp^s	>500
	F203	Gen^r Str^r Van^s Amp^s	>500
	F204	Van^s Amp^s Gen^s Str^s	500±50
	ATCC 29212	Control strain	250±25

^{r}Resistant, ^{s}Susceptible; Vancomycin (Van), Ampicillin (Amp), Gentamycin (Gen), Streptomycin (Str), Methicillin (Met), Oxacillin (Oxa). Values are mean ± standard deviation of triplicates.

Flower extracts were active on six *Candida* species (MIC values between 60 to 120 μg GAE.mL^{-1};Table 3) and on clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin resistant *S. aureus* coagulase negative (MRSCN) (MIC values of 250 μg GAE.mL^{-1}; Table 4). The MIC values against *Enterococcus faecalis* were two-fold higher (MIC values: 500 μg GAE.mL^{-1}) than those against *S. aureus*. The bioautographical assay of *Z. punctata* flower extract on MRSA showed that the extract had two bands with antibacterial and anti-*Candida* activity coincident with the Rfs of **C4** and **C5**.

The flower extracts showed no toxic effect on *Artemia salina.* None of the doses was mutagenic to TA98 or TA100 strains under the conditions used in this assay. This result indicates the absence of mutagens that cause base pair substitution (detected in TA100) and frame-shift (detected in TA98) mutations. The absence of mutagenicity of the extract studied in the test against *Salmonella* strains indicates that DNA does not seem to be a relevant target.

Conclusions: The present study indicates that *Z. punctata* flowers could be a significant source of natural compounds with beneficial health effects (antifungal, antibacterial, antioxidant and anti-inflammatory) and may be useful as either a dietary supplement or for phytotherapy.

Experimental

Plant material: *Zuccagnia punctata* flowers were collected in December 2013 at 2000 m above sea level (masl) in Amaicha del

Valle, Tucumán, Argentina. Voucher specimens (IML 605935) were deposited at the Herbarium of Fundación Miguel Lillo, Tucumán, Argentina. *Z. punctata* was authenticated by Dra Soledad Cuello, INQUINOA (CONICET). The flowers were used either immediately after harvesting or dried by lyophilization.

Sample preparation: The flowers (10 g), fresh or dried, were extracted with 300 mL of ethanol: H_2O (60:40, v:v) for 1 h with sonication. Then, the extracts were filtered through Whatman N° 1 filter paper. The filtrate was evaporated to dryness and then lyophilized.

Total polyphenols, non-flavonoids and flavonoids: Total polyphenols (TP) of the extracts were determined by Folin–Ciocalteau's reagent [6a]. Non-flavonoid phenols (NFP) were measured by determination of total phenols content remaining after precipitation of the flavonoids with acidic formaldehyde [6b]. Flavonoid phenolics (FP) was determined as TP-NFP=FP. In all cases the results are expressed in mg gallic equivalents (mgGAE) per g of flowers. Flavones and flavonols were determined with aluminum chloride ($AlCl_3$) according to Lamaison and Carnet [6c]. Flavonoid content was expressed as mg quercetin equivalents (mg QE) per g of flowers.

Total carotenoids: The samples (1 g of flowers) were extracted with 6 mL of light petroleum: acetone (50:50, v/v). The total carotenoid content was calculated according to Rodríguez-Amaya [6d].

HPLC fingerprints of flower extracts: The HPLC system used consisted of a Waters 1525 Binary HPLC Pumps system with a 1500 Series Column Heater, a manual injection valve with a 20 µL loop (Rheodyne Inc., Cotati, CA), a Waters 2998 photodiode array detector (PDA) and a XBridge™ C18 column (4.6 x 150 mm, 5 µm; Waters Corporation, Milford, MA). The solvent system for the separation of components from extracts was composed of solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in methanol) (conditions: 10–57% B from 0 to 45 min and kept at 100% B from 45 to 60 min). The flow rate was set at 0.5 $mL.min^{-1}$. A solution of 4 mg $DW.mL^{-1}$ was used. Data collection was carried out with Empower™ 2 software. The identification of phenolic compounds present in the extract was carried out by comparing the retention times and spectral data (220–600 nm) of each peak with those of standards from Sigma-Aldrich (MO, USA), Fluka Chemical Corp. (USA) and Indofine Chemical Company, Inc.

Measurement of antioxidant capacity

ABTS free radical scavenging activity: The antioxidant capacity assay was carried out by the $ABTS^{\bullet+}$ spectrophotometric method as described by Re *et al*. [6e] and the autographic assay as described by Zampini *et al*. [6f]. Commercial antioxidants (BHT and quercetin) were used as positive controls. SC_{50} values denote the µg $GAE.mL^{-1}$ required to scavenge 50% ABTS free radicals.

β-Carotene bleaching assay: Antioxidant activity was determined according to the β-carotene bleaching method following the procedure described by Ordoñez *et al.* [6g]. Quercetin and BHT were used as standards.

Measurement of anti-inflammatory activity

Cyclooxygenase inhibition assay: The ability of the extracts to inhibit the conversion of arachidonic acid to prostaglandin (PG) by human recombinant cyclooxygenase 2 (COX-2) was determined using a COX inhibitor screening assay kit (No. 560131; Cayman Chemical, Ann Arbor, MI, USA). PG produced was measured by enzyme immunoassay (EIA). The inhibitory assays were performed in the presence of 25 to 150 $\mu g.mL^{-1}$ of *Z. punctata* extract, 1 to 4 $\mu g.mL^{-1}$ of 2′,4′-dihydroxychalcone and 10 to 150 $\mu g.mL^{-1}$ of 2′,4′-dihydroxy-3′-methoxychalcone. The commercial anti-inflammatory drug nimesulide was used as reference.

Lipoxygenase inhibition assay: Lipoxygenase (LOX) activity was determined using a continuous spectrophotometric method, based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxide [6h].The inhibitory assays were performed in the presence of either *Z. punctata* extract (50-150 $\mu g.mL^{-1}$) or pure compounds (25-150 $\mu g.mL^{-1}$). The anti-inflammatory effect of test compounds was evaluated by calculating the percentage inhibition of hydroperoxide production from the ΔOD values at 234 nm at the end of incubation. The anti-inflammatory drug naproxen was employed as reference compound.

Antimicrobial assays

Microorganisms: The following reference microorganisms were obtained from the American Type Culture Collection (ATCC): *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Candida parapsilosis* ATCC 22019 and *C. albicans* ATCC10231. Clinical isolates of antibiotic methicillin-resistant *Staphylococcus aureus* (n=2, MRSA), *S. aureus* coagulase negative (n=2, MRSCN) and *Enterococcus faecalis* (n=2) were obtained from Hospital Dr Nicolás Avellaneda, San Miguel de Tucumán, Tucumán, Argentina. The yeasts *Candida albicans*, *C. tropicalis*, *C. glabrata*, *C. parasilopsis* and *C. guilliermondii* were obtained from clinical samples from Hospital del Niño Jesús, San Miguel de Tucumán, Tucumán, Argentina. Bacterial strains were identified by the biochemical profiles according to [6i].

Minimal inhibitory concentration: This was determined by the agar macrodilution technique according to the guidelines of the Clinical and Laboratory Standard Institute for yeasts and bacteria [6j, 6k]. Concentrations were tested up to 500 µg $GAE.mL^{-1}$. The inoculum (2 µL) containing 5×10^5 $CFU.mL^{-1}$ (bacteria) and 5×10^3 $CFU.mL^{-1}$ (yeasts) was added to each medium. Plates were aerobically incubated at 37°C for bacteria and 35°C for yeasts for 24 h and 48 h, respectively. MIC was defined as the lowest extract concentration without macroscopically visible growth. As references, several antibiotics were used.

Bioautographic assays: The assay was realized on TLC developed with toluene: acetone: chloroform (4.5:3.5:2.5; v/v/v) by the method described by Zampini *et al*. [6i]. The R_f values of growth inhibition areas were compared with the R_f values of the related spots on the TLC plate observed under UV light at 365 nm before and after spraying with Neu's reagent, 2-aminoethyl-diphenylborinate (Sigma) [6l].

Mutagenicity assay: The mutagenicity assay with *S. typhimurium* was performed as described by Maron and Ames [6m]. The extract was considered mutagenic if the number of revertants per plate was more than two-fold the number of colonies produced on the solvent control plates (spontaneous revertant frequency). Different concentrations (25-500 µg/plate) were tested in duplicate with two replicates. DMSO was used as the negative control, 100 µL/plate, and the positive control employed was 4-nitro-o-phenylenediamine (4-NPD), 10 µg/plate.

General toxicity using Artemia salina: Ten nauplii were added to a well of a 24-well plate containing an extract dilution (between 125 and 500 $\mu g.mL^{-1}$). After 24 h incubation under light, the number of dead and surviving brine shrimps in each well was counted. The

LC_{50} value was defined as the amount of phenolic compound that caused the death of 50% of nauplii.

Statistical analysis: Sampling and analyses were performed in triplicate, and the data are presented as mean ± standard deviation (S.D.). The Pearson test correlation coefficient with 95% confidence was used. Statistical analysis was performed by one way ANOVA followed by Tukey's multiple comparison test (p<0.05). All statistical analyses were carried out using Infostat software.

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