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Article

Evaluation of the antibacterial synergism of two plant extracts belonging to Bignoniaceae family and development of a topical formulation

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Fridericia caudigera and *Cuspidaria convoluta* (Bignoniaceae) species, which grow in the northwest of Argentina, have shown antibacterial effect against strains isolated from skin infections, and each one displayed synergism with commercial antibiotics. The aims of this work were to evaluate the antibacterial activity and toxicity of the combination of these two plant species, and to design a stable gel for topical use including the blend of extracts. The combination of extracts was evaluated for synergistic effects (chequerboard assay), genotoxicity (Ames test) and cytotoxicity (*Artemia salina* test). A gel was subsequently formulated with the combination of extracts using carboxymethylcellulose as a polymer. The following physico-chemical characteristics of the gel formulation: pH, viscosity, spreadability and total phenol content, as well as resistance to severe temperature changes, biological activity (diffusion in agar), *in vitro* permeation (Franz cells) and primary dermal irritation (Draize test) were analyzed. The combination of extracts showed a synergistic effect on pathogenic bacteria and was not toxic in the *in vitro* tests. The gel was stable and retained the antimicrobial activity of the original extracts. The formulation proposed in this work could constitute an alternative for primary skin infections since it proved to be safe for topical administration.

Keywords: *Fridericia caudigera*. *Cuspidaria convoluta*. Genotoxicity. Franz cells. Topical gel.

INTRODUCTION

Herbal formulations have been recommended for the care of skin properties for a long time, and their effects are well accepted by modern society, which –in turn– results in good consumer acceptance (Gosh, Gaba, 2013). Plant extracts with aqueous or ethanolic solvent have been used to treat various skin ailments, such as wounds, psoriasis and inflammatory conditions. Since herbal remedies are more accepted worldwide for their fewer side effects and lower costs, the design of a proper formulation prepared from a concentrated extract of medicinal herbs could be beneficial either to prevent or reduce infectious skin diseases.

It is currently well-known that gel formulations are designed not only to deliver active polar ingredients efficiently but also to be less sticky, less oily, and easily washable. Gels are topical preparations that are readily applied to the skin and have an attractive physical appearance compared to other topical preparations. Thanks to their water content, they are refreshing, soothing, hydrating, easy to use and to penetrate into the skin. All these characteristics yield a faster healing effect

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which also depends on the polymers that are used for the formulation (Harahap, Nainggolan, Harahap, 2018).

Sodium carboxymethylcellulose (Na-CMC) is a representative derivative of cellulose, manufactured by the reaction of sodium monochloroacetate with cellulose in an alkaline medium. Na-CMC is a polysaccharide polymer with excellent bioadhesive properties, biodegradability and biocompatibility (Akalin, Pulat, 2018). In addition to these properties, it is used due to its easy availability, high viscosity, good water solubility, non-toxicity and low price.

The biological activities of species belonging to the Bignoniaceae family have been studied in detail. Previous research has shown the antibacterial effect and synergism of *Fridericia caudigera* and *Cuspidaria convoluta* ethanolic extracts with commercial antibiotics (Torres *et al.*, 2017). The extracts of both species showed not only antibacterial activity, mainly against skin microorganisms, but also anti-inflammatory activity (Torres *et al.*, 2017; Torres *et al.*, 2018).

In view of the above, the aims of this work were to evaluate the interaction effect on antibacterial activity and the toxicity of the combination of both extracts, and to design a stable gel for topical use including the blend of extracts.

MATERIAL AND METHODS

Plant material

Plants were collected in November 2016 and 2018 from the province of Misiones, Argentina. They were identified by specialists from the Herbarium of the *Instituto de Botánica del Nordeste* (IBONE-CONICET), in Corrientes province, Argentina, where the voucher specimens (*C. convoluta* AMG 104 and *F. caudigera* AMG 418) were deposited.

Extract preparation and standardization

Plant materials were dried at room temperature. Dry leaves were triturated using a mechanical mill (Dalvo, Argentina) until particle size ranged between 1.70 mm and 710 mm, as determined by ASTM sieves. Extracts were individually prepared by macerating 20 g of each powder in 100 mL of 80% ethanol for 7 days in a dark place at room temperature. All extracts were subsequently filtered through Whatman No. 1 filter paper and centrifuged at 1210g for 5 min. Then, the ethanol was evaporated at 40 °C, and the remains of water were cryodesiccated in a freeze-dryer (Rificor, model L-I-E 300-CRT, Argentina). The lyophilized powder was stored at -20 °C until its use.

Extracts were standardized by the total phenolic compound content (Singleton, Orthofer, Lamuela-Raventos, 1999). Results were expressed as gallic acid equivalent (GAE) per gram of dry extract (DE). Their HPLC characterization had already been done (Torres *et al.*, 2017; Torres *et al.*, 2018) and the main compounds found were: apigenin, luteolin and chrysin (*F. caudigera*), and coumaric and hydroxybenzoic acid derivatives and glycosylated and nonglycosylated flavones in *C. convoluta*, such as apigenin-O-pentoxyl-hexoside, luteolin and cirsiliol.

Antibacterial effects of the plant-combinations

Microorganisms and culture media

The microorganisms used were Gram-positive bacteria: *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, four methicillin-sensitive clinical isolates of *Staphylococcus aureus* (F13, F29, F32 and F33) and two methicillin-resistant clinical isolates of *S. aureus* (MRSA, F7 and F22), and Gram-negative bacteria, such as *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853 and antibiotic-resistant clinical isolates of *Enterobacter cloacae* (two strains, F302 and F338), *Klebsiella pneumoniae* (F364), *Pseudomonas aeruginosa* (F305), *Proteus mirabilis* (F304) and *Morganella morganii* (F339).

Bacterial strains were maintained in brain-heart infusion (BHI) medium containing 30% (v/v) glycerol at -20 °C. Before testing, the suspensions were transferred to Mueller-Hinton broth (Britania Laboratories, Argentina) and were aerobically grown overnight at 37 °C. Individual colonies were isolated and suspended in 5 mL of 0.9% NaCl solution. The inocula were prepared by adjusting suspension turbidity to match the 0.5 McFarland standards (1×10^8 CFU/mL).

Estimation of synergy between plant combinations

Synergy between the two plant extracts was assessed with the checkerboard assay which was performed in sterile 96-well microplates. To this end, the dry extracts were previously resuspended in dimethylsulfoxide (DMSO). Their combinations were transferred to each microplate well. The concentrations used in each extract combination were from 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250, 500 to 1000 µg of phenolic compounds/mL (µg GAE/mL). Inoculum (100 µL) with an approximate microbial load of 5 x 10^5 CFU/mL was added to each well. In parallel, a growth control containing bacterial cells, a DMSO control without extracts, and a blank control containing only growth medium were included.

Minimal inhibitory concentration (MIC) values were determined for each extract and their combinations to establish interaction effects. The Fractional Inhibitory Concentration (FIC) was calculated as follows (Schelz, Molnar, Hohmann, 2016):

$$FIC_{Fc} = MIC_{Fc} \text{ in combination with Cc}/MIC_{Fc} \quad (1)$$
$$FIC_{Cc} = MIC_{Cc} \text{ in combination with Fc}/MIC_{Cc} \quad (2)$$

where: MIC_{Fc} corresponds to the MIC of *F. caudigera* whereas MIC_{Cc} corresponds to the MIC of *C. convoluta*.

The FIC Index (FICI) was subsequently calculated according to the next equation (3).

$$FICI = FIC_{Fc} + FIC_{Cc} \quad (3)$$

Results were interpreted as either synergistic (≤ 0.5), additive (> 0.5 and ≤ 1), indifferent (> 1 and < 4) or antagonistic (≥ 4) (Schelz, Molnar, Hohmann, 2016). The assays were performed in triplicate and as independent tests. Results were expressed as mean values (n=9).

Toxicity of the plant-combinations

Genotoxicity assay

The mutagenicity assay using *Salmonella typhimurium* strains was performed as described by Maron and Ames (1983). The method employed was direct plate incorporation, using *S. typhimurium* TA98 and TA100. The bacterial strains were cultivated in nutritive broth (Laboratorios Britania, Argentina) at 37 °C during 16 to 18 h until reaching the stationary phase of growth. Different concentrations of each extract (1000, 500 and 250 µg GAE/plate) and concentrations of each of them combined in a mixture (*F. caudigera:C. convoluta;* 125:500, 125:250, 125:125 - 250:500, 250:250, 250:125 - 500:500, 500:250, 500:125 µg GAE/plate) were all dissolved in DMSO. The combined extracts were added to 2 mL of soft agar supplemented with 0.5 mM of L-Histidine and 0.5 mM of D-Biotin (Sigma Aldrich, United States) and mixed with 100 µL of bacterial suspension (1-2 x 10^8 cells/mL). These mixtures were then poured onto the surface of a plate containing minimal agar medium (no histidine). The plates were incubated at 37 °C for 48 h. The revertant colonies of each plate were counted manually.

The samples were tested in triplicate with two replicates. At the same time, a positive control with 10 µg/plate of 4-nitro-o-phenylenediamine (4-NPD) and a negative control (DMSO) were performed (100 µL/plate). The negative control makes it possible to evaluate the number of colonies that revert spontaneously. Results were expressed either as the number of revertants per plate or as the mutagenic index (MI) in accordance with the following equation:

$$MI = \frac{number\ of\ revertants\ in\ the\ sample}{number\ of\ spontaneous\ revertants}$$

An extract was considered mutagenic if the number of revertants per plate was more than twice the number of colonies produced on the solvent control plates (spontaneous revertant frequency) or the MI ≥ 2 (Maron, Ames, 1983).

Brine shrimp lethality bioassay

Brine shrimp (*Artemia salina*) lethality bioassay was carried out for preliminary assessment of extract toxicity. *Artemia salina* eggs were incubated in an incubation chamber with artificial seawater at 20-30 °C (Meyer *et al.*, 1982). The pH was adjusted to 9 using sodium carbonate to avoid the death of larvae by lowering the pH during incubation. After 24 h, larvae (nauplii) were extracted

and counted using a micropipette. Five concentrations of each extract individually and four concentrations of each extract in combination were tested to determine a dose-response relationship. A negative control with DMSO and a positive control with serial dilutions of potassium dichromate (3.75, 7.5, 15, 30 and 60 µg/mL) were also performed. The concentrations tested for each extract were 62.5, 125, 250, 500 and 1000 µg GAE/mL, while the concentrations for each extract in the combination were *F. caudigera: C. convoluta;* 125:500, 125:250, 125:125 - 250:500, 250:250, 250:125- 500:500, 500:250, 500:125. Each well containing the sample and ten *Artemia* larvae, together with the controls, were brought to a total volume of 100 µL with artificial seawater. After 24 h, and with the help of a stereo microscope, live larvae were counted and mean lethal concentration (LC_{50}) was calculated (González *et al.*, 2007). All tests were done in triplicate. Extracts with LC_{50} above 1000 µg/mL are non-toxic, LC_{50} of 500 - 1000 µg/mL are low toxic, extracts with LC_{50} of 100 - 500 µg/mL are medium toxic, whereas extracts with LC_{50} of 0 - 100 µg/mL are highly toxic (Clarkson *et al.*, 2004).

Topical phytopharmaceutical formulation (phytohydrogel)

Preliminary tests were carried out to analyze the behavior of lyophilized extracts with each auxiliary substance at concentrations adequate to achieve stable formulations and with desirable macroscopic characteristics. Carbopol® 934 and Carbopol® 940 at 1% (with triethanolamine, in sufficient quantity) and Na-CMC at 3% and 4% were the polymers used. The formula for the subsequent characterization study and stability monitoring was the following:

Components (%)	Herbal gel	Base gel
Na-CMC	4g	4g
Propylene Glycol	5mL	5mL
Ethanol 80°	10mL	10.84mL
***F. caudigera* extract**	0.27g	-
***C. convoluta* extract**	0.57g	-
Purified water in sufficient quantity for	100g	100g

Gels were prepared for topical use incorporating the combination of *F. caudigera* extract with *C. convoluta* extract as active ingredients. The quantities of each extract to be incorporated were firstly selected to ensure that 0.1 g of gel (each dose) contains 4 MIC values. To this end, the MIC values obtained on Gram-positive bacteria for the plant blend (15.625 µg GAE/mL for *F. caudigera* and 31.25 µg GAE/mL for *C. convoluta*) were taken into account. To prepare the gel, four grams of Na-CMC were moisturized in 80 mL of distilled water for 24 h with frequent agitation. A mixture of 10 mL of ethanol 80° and 5 mL of propylene glycol containing 0.27 g of *F. caudigera* (equivalent to 625 µg GAE/g gel) and 0.57 g of lyophilized powder of *C. convoluta* (equivalent to 1250 µg GAE/g gel) was subsequently added. At the same time, a negative control was prepared with the base gel containing 10.84 mL of ethanol 80° instead of the extracts. All preparations were kept at room temperature for 24 h to achieve equilibrium.

Formulation characterization and stability tests

In order to find out the physical stability of gels, accelerated storage testing was carried out using thermal stress tests including heating-cooling cycles.

Heating-cooling cycle test (thermal stress test)

This test evaluates storage capacity in areas exposed to marked and constant temperature changes. The formulations were subjected to two different temperatures, alternating two days at 4 °C and two days at 40 °C. Seven cycles of this exposure were performed during 28 days (Ali *et al.*, 2014). Once this time concluded, all the determinations described below for characterization and stability studies were carried out (*a-g*).

In addition, the long-term stability of the formulations was studied for 12 months. To this end, preparations were fractionated in 25 g packages and were kept at room temperature (25 °C ± 2). Both batches of gels (accelerated and long-term storage) were checked visually and characterized by physico-chemical and microbiological tests (*a-g*).

a- *Centrifugation assay.* Formulation stability was investigated against gravity by centrifugal device. This test was carried out weighing 1 g of each sample which was further centrifuged at 3000 rpm for 30 min. Each formulation was finally checked in terms of sedimentation.

b- *pH measurements.* One gram of each formulation was weighed and diluted with distilled water until 10 mL. After being homogenized, the pH of samples was measured with a pHmeter (HANNA® HI 9811-5). All measurements were made at room temperature.

c- *Microscopic study.* Formulations were checked in terms of uniformity, gel texture, and air bubble. To this end, a small amount of each sample was gently spread on a slide, covered by a cover slide and visualized through an Olympus microscope (40X).

d- *Spreadability.* 25 mg of each formulation were placed between two glass slides. Different weights were subsequently placed on the top plate at fixed intervals of time. The area (mm^2) was calculated and plotted as a function of weight.

e- *Viscosity.* Gel viscosity was determined using Brookfield viscometer (NDJ-1) at 25 °C with a spindle speed of the viscometer rotated at 10 rpm.

f- *Total phenol content.* 500 mg of each gel and 5 mL of 80° alcohol were placed in a test tube which was shaken and centrifuged at 3000 rpm for five minutes. An aliquot was subsequently taken to perform the determination according to Singleton, Orthofer and Lamuela-Raventos (1999). The determinations were carried out in triplicate and in parallel a control was carried out only with the base gel to discard oxide-reduction reactions of the other components of the gel.

g- *Microbiological control.* The number of viable aerobic microorganisms, Enterobacteriaceae, fungi, and yeast was determined in each preparation according to Farmacopea Argentina (2013). The presence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was also explored since the product is intended for topical application. All tests were performed in triplicate. In parallel, sterility tests were carried out on the culture media used. During the twelve months of the testing period (long-term stability), one of the packages with the preparation was opened on a daily basis and an operator inserted his finger to simulate daily use conditions.

Statistical analysis. All determinations were made in triplicate for each sample analyzed and mean values and standard deviations were subsequently reported. Data analysis was done using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test in SPSS 21.0. Differences between any two means could be accomplished using a Duncan's multiple range test (DMRT). The significance level was set at a P-value lower than 0.05. In the long-term stability study, these determinations were made within 24 h and after 1, 2, 3, 6, and 12 months of gel preparation.

Determination of the antimicrobial activity of the plant-formulations

Three strains, namely *S. aureus* (F32), *S. aureus* (F7) and *S. epidermidis* ATCC 12228, were used to determine antimicrobial activity using the agar diffusion technique (CLSI, 2006). Petri dishes with 20 mL of Mueller-Hinton agar were inoculated by swabbing with a bacterial suspension of 1 x 10^8 CFU/mL. Wells were made in the culture medium with a sterile punch (10 mm diameter). Each well was loaded with 100 mg of the gel after inoculation, and the plates were subsequently incubated at 37 °C for 18-20 h. Growth inhibition diameter around each well was measured (mm). Each experiment was carried out in triplicate, and mean diameter of the inhibition zone was recorded. Controls were performed within 24 h and after 3, 6 and 12 months of formulation preparation.

In vitro permeation assay

Assays were carried out in glass Franz diffusion cells with an absorption surface area of 2.66 cm^2. Pig ear skin was obtained from young animals sacrificed at a local slaughterhouse. The skin was initially cleaned with tap water and hairs and subcutaneous

fat tissue were subsequently removed. The membranes obtained were moistened, immersing the skin in the buffer solution for 30 min. The skin was subsequently mounted in a two-chamber glass Franz diffusion cell with the stratum corneum towards the donor chamber. The receptor chamber of 27 mL volume was filled with a solution of sodium phosphate buffer 0.1M pH 7 containing NaCl 0.5M. A hydrogel sample (0.25 g) was deposited in each donor chamber. The receptor chambers of the diffusion cells were surrounded with a water bath maintained throughout the experiment at 35 °C, corresponding to normal skin temperature. The receptor solution was continuously agitated with a magnetic stirrer. During the experiments, both donor compartments and sampling arms were occluded to prevent evaporation. Aliquots of fluid in the receptor chamber (1 mL) were removed and replaced by a new phosphate buffer solution at different periods (between 15 min until 12 h). Samples were stored at -18 °C before analysis (Lhez, Pappano, Debattista, 2010).

Skin retention study

At the end of the permeation assay, the skin was separated from Franz diffusion cells and the formulation remaining on the skin was removed. The skin was subsequently cut into small pieces and submerged in 5 mL ethanol 80° for 24 h to ensure effective extraction of the total phenolic content retained in the skin (Torky *et al*., 2018). These tests were carried out in triplicate.

Analysis of permeated compounds from phytopharmaceutical formulation

Aliquots (450 µL) of extracted samples in the permeation test and skin retention study (200 µL) were analyzed in the total phenolic content. The latter was determined by the Folin-Ciocalteu method (Singleton, Orthofer, Lamuela-Raventos, 1999). The permeated and retained quantities were calculated by interpolation in the calibration curve elaborated with gallic acid. Cumulative permeated percentage was also calculated.

Determination of Primary Skin Irritation

The experimental protocols for this study were approved by the Institutional Animal Care and Use Committee (Bioethics Committee of the Medicine School from the National University of Tucuman, Argentina). All animal care and use programs were carried out according to the Guide for the Care and Use of Laboratory Animals (NIH Publication 86 to 23, revised 1985).

Six albino rabbits (2.7 to 3.0 kg) were used for the present study. Approximately 24 h before the test, dorsal hairs of the trunk of the animals were shaved. Every animal was shaved in 4 (four) areas of 2.5 cm^2. Then, two parallel abrasions were made with a sterile injection needle, taking care of bleeding over the upper and lower areas on the right side of the animal (areas 2 and 4). The upper and lower shaved areas on the left side remained intact (areas 1 and 3). Following this procedure, animals were treated with 0.5 g of the extract-based topical gel. The formulation was applied to a small area, covered with sterile gauze and fixed with a hypoallergenic adhesive tape. After 24 h, the patches were removed, and the first evaluation was carried out. Control areas were compared with tested areas. A new reading was performed at 72 h. Animals were examined for signs of skin irritation according to Draize (1965). Edema formation was measured with a caliber. Finally, calculation of the primary dermal irritation index (PDII) was done using the following formula.

$$\mathrm{PDII} = \frac{M1+M2+M3+M4+M5+M6+M7+M8}{4}$$

Where M1 and M2 correspond to the arithmetic mean of the sum of the erythema and edema values at 24 h observed in intact skin, M3 and M4 correspond to the arithmetic mean of the sum of the erythema and edema values at 24 h observed in scraped skin, M5 and M6 correspond to the arithmetic mean of the sum of the erythema and edema values at 72 h observed in intact skin, and M7 and M8 correspond to the arithmetic mean of the sum of the erythema and edema values at 72 h observed in scraped skin. PDII values between 0-0.49 are considered non-irritant, 0.50-0.99 practically non-irritant,

1.00-1.99 minimally irritant, 2.00-5.99 moderately irritant and 6.00-8.00 highly irritant according to Draize (1965).

RESULTS AND DISCUSSION

Antibacterial effects of the plant-combinations

The total phenol content values of tinctures were 118.00 ± 8.30 mg GAE/g DE for *C. convoluta* and 131.69 ± 3.09 mg GAE/g DE for *F. caudigera*. The results of antibacterial interaction effects between *C. convoluta* and *F. caudigera* are shown in Table I. The extract mixture showed synergism against six Gram-negative bacteria (FICI 0.12 to 0.37). *Pseudomonas aeruginosa* strains were resistant to both the individual extracts and the combinations used. A decrease of up to 16 times in the value of MIC was observed in the cases of synergism, e.g. MIC from *F. caudigera* extract for *E. cloacae* (F338) was 2000 µg GAE/mL whereas the concentration used in the combination was 125 µg GAE/mL. It should be noted that *F. caudigera* extract failed to inhibit the growth of Gram-negative bacteria to values either equal to or lower than 2000 µg GAE/mL, whereas *C. convoluta* extract was only effective in *M. morganii* (F339) and *P. mirabilis* (F304) at MIC values of 500 µg GAE/mL, highlighting the importance of the results obtained by combining them. However, the most relevant results were those that occurred at the level of Gram-positive bacteria where a synergistic interaction was observed against all clinical isolates (FICI 0.25 to 0.500). In all of these cases, MIC values decreased 4-8 times, even against bacteria with stronger resistance profiles, such as F7 and F22 (MRSA).

The increased antimicrobial activity observed when extracts were combined could be explained by the fact that several bioactive constituents of plants affect various target sites and work in a synergistic way (Al-Bayati, 2008). The combination of natural drugs to treat complex diseases is a new strategy against bacterial resistance, which usually develops when single drugs are used (Gathirwa *et al.*, 2008; Zacchino *et al.*, 2017). This strategy, which is called "multidiana effects", refers to the use of herbs and drugs in a multi-target approach on account of the fact that multi-extract combinations affect not only a single target but also several ones, cooperating synergistically (Hemaiswarya, Kruthiventi, Doble, 2008; Chukwujekwu, van Staden, 2016). Previous research has demonstrated a synergistic antibacterial effect when testing mixtures of two or more plant extracts against multi-resistant bacteria (Ncube, Finnie, Van Staden, 2012; Chakraborty *et al.*, 2018). In addition, Torres *et al.* (2018, 2019) have demonstrated the synergistic effect of *F. caudigera* and *C. convoluta* or their metabolites, with commercial antibiotics. It seems likely that this synergistic effect is due to the action of some of the chemical compounds present in these extracts, such as luteolin, apigenin and flavone glycosides. Luteolin and apigenin were −in fact− observed to have the ability to enhance the effect of ampicillin and ceftriaxone against MRSA bacteria (Akilandeswari, Ruckmani, 2016; Amin *et al.*, 2016). Luteolin was also observed to have a synergistic effect with imipenem and methicillin. Regarding the mechanism of action of these flavonoids, Amin *et al.* (2015) reported that luteolin shows a good inhibitory effect against MRSA by influencing the efflux pump via potassium leakage. Moreover, Silva *et al.* (2016) demonstrated that apigenin and chrysin act against bacterial quorum sensing. Further studies have also indicated that the main targets of apigenin on bacteria could be the nucleic acid processing enzymes and cell wall/membrane (Wang *et al.*, 2017, 2019). This reflects, at least in part, the multiple targets of action of these flavonoids.

To improve the efficacy of natural product mixtures, bioactive mixtures should be comprehensively characterized and the concentrations and identities of the constituents contributing to biological activity (whether it be through additive, synergistic, or antagonistic means) should be determined. This will be further explored in future studies.

TABLE I - Effect of the combinations of *F. caudigera* and *C. convoluta* extracts on pathogenic bacteria

Strains	MIC Fc	MIC Cc	MIC combination	FICI
Gram positive				
S. aureus ATCC 29213	125	125	125/125	2.00
S. aureus (F13)	125	250	31.25/62.5	0.50
S. aureus (F29)	125	250	31.25/62.5	0.50
S. aureus (F32)	125	250	31.25/62.5	0.50
S. aureus (F33)	125	250	31.25/62.5	0.50
S. aureus (F7)*	125	250	15.62/31.25	0.25
S. aureus (F22)*	125	250	15.62/31.25	0.25
E. faecalis ATCC 29212	62.5	500	15.62/125	0.50
Gram negative				
E. coli ATCC 35218	2000	2000	250/250	0.25
E. cloacae (F302)	2000	2000	250/125	0.19
E. cloacae (F338)	2000	2000	125/125	0.12
K. pneumoniae (F364)	2000	2000	250/250	0.25
M. morganii (F339)	2000	500	250/125	0.37
P. mirabilis (F304)	2000	500	250/125	0.37
P. aeruginosa ATCC 27853	>2000	>2000	ND	ND
P. aeruginosa (F305)	>2000	>2000	ND	ND

* MRSA strains. ND: not detected in the range of used concentrations.

Toxicity of the plant-combinations

Table II shows the results collected from the genotoxicity studies against *S. typhimurium* TA98 and TA100 strains. The number of spontaneous revertants was 27 ± 1 for TA 98 strain and 141 ± 2 for TA100. Under the above-mentioned trial conditions, both extracts and all their combinations showed no mutagenic effect on any of the strains of *S. typhimurium* used because the MI was <2 in all cases. The genotoxicity tests therefore demonstrate that either the individual extract or the combinations herein analyzed do not induce any increase in the number of revertants since the values obtained were from 20 to 50 revertants for TA98 and 75 to 200 for TA100, respectively, in agreement with those reported by Mortelmans, Zeiger (2000). These results indicate the absence of compounds that induce frameshift-type mutations because TA98 responds to mutagens that cause a shift in DNA reading, which occurs preferably in DNA regions that have either repeated base sequences in tandem or mutations that produce substitutions of G-C base pairs (result demonstrated using TA100 strain) at the tested concentrations.

As regards the brine shrimp lethality bioassay, the LC_{50} values of both species were above their active concentrations (Table III), *C. convoluta* presented a LC_{50} of 446.77 µg GAE/mL (93 mg/mL) and *F. caudigera* presented a LC_{50} of 215.64 µg GAE/mL (200 mg/mL). These values are much higher than the concentrations at which the extracts show activity (MIC 31.25 µg GAE/

mL for *C. convoluta* and 15.625 µg GAE/mL for *F. caudigera*), which seems to indicate a wide therapeutic range for these extracts. In addition, taking into account the toxicity criteria proposed by Clarkson *et al.* (2004), who claim that the extracts with LC_{50} above 1000 µg/mL are non-toxic, it can be assumed that the extracts are non-toxic, which could −in turn− guarantee their safe use. It can be also taken into account that when the mixture of extracts was tested, toxicity was even lower and no negative effect on nauplii was observed, with a mortality rate of 0%. This could have occurred because the combination of extracts allows the use of lower concentrations of each of the extracts used individually maintaining their individual biological properties.

Lagarto Parra *et al.* (2001) performed a comparative study of the lethality trial in *A. salina* against the *in vivo* test in mice on the toxicity of crude extracts and found a good correlation between the two tests ($R = 0.85$ $p < 0.05$). This is the reason why they proposed this lethality assay as a useful tool to predict acute oral toxicity in mice. This prediction of results was also observed in previous studies by Velásquez (2010) and De Albuquerque Sarmento *et al.* (2014), who used both assays (brine shrimp lethality bioassay and toxicity in mice) for acute toxicity evaluation.

To our knowledge, no toxicity data on *C. convoluta* and *F. caudigera* have been collected to date, the present study being the first to be published in this respect. Only a few toxicity studies on species belonging to the genus *Fridericia* have been conducted, among them, Mafioleti *et al.* (2013)′s research concluding that the hydroalcoholic extracts of *F. chica* have low acute toxicity in animal testing and lack cytotoxicity. In turn, Dos Santos *et al.* (2013), working on the same species, demonstrated that its chloroform extract was neither mutagenic nor genotoxic. There are no records of tests carried out on species of the genus *Cuspidaria*. In this respect, the tests carried out in the present work are the first steps in the study on the safety of these species, and the results collected contribute to guaranteeing their safe use.

TABLE II - Mutagenicity testing of extracts and combinations

Sample	Concentration (µg GAE/plate)	*S. typhimurium* **TA98**		*S. typhimurium* **TA100**	
		Rev. N°/plate	**Mutagenic Index (MI)**	**Rev. N°/plate**	**Mutagenic Index (MI)**
Positive control		610 ± 21	22.59	590 ± 58	4.18
Negative control (DMSO)		27.0 ± 1	--	141 ± 2	--
F. caudigera	1000	20.0 ± 1	0.74	127 ± 2	0.90
	500	23.0 ± 2	0.85	118 ± 2	0.81
	250	22.5 ± 2	0.83	119 ± 1	0.84
C. convoluta	1000	23.5 ± 2	0.87	113 ± 2	0.80
	500	25.0 ± 1	0.92	117 ± 2	0.83
	250	22.5 ± 2	0.83	123 ± 1	0.87
F. caudigera: C. convoluta	500:500	21.0 ± 2	0.77	125 ± 3	0.89
	500:250	23.0 ± 1	0.85	127 ± 1	0.90
	500:125	22.5 ± 2	0.83	130 ± 2	0.92
	250:500	23.0 ± 2	0.85	124 ± 1	0.88
	250:250	25.0 ± 1	0.92	119 ± 2	0.84
	250:125	24.5 ± 1	0.91	128 ± 3	0.91
	125:500	23.0 ± 2	0.85	129 ± 1	0.91
	125:250	22.5 ± 1	0.83	115 ± 2	0.81
	125:125	24.0 ± 1	0.89	134 ± 1	0.95

Data correspond to the mean ± DE of three plates.

TABLE III - Brine shrimp lethality bioassay of extracts

Extract	Concentration (µg GAE/mL)	Mortality (%)	LC_{50} (µg GAE/mL)
F. caudigera	1000	100	215.64
	500	90	
	250	66	
	125	30	
	62.5	25	
C. convoluta	1000	90	446.77
	500	70	
	250	30	
	125	25	
	62.5	10	

Topical phytopharmaceutical formulation (phytohydrogel)

As regards the extract formulation design, Carbopol® gels showed a wide range of dispersed particle sizes whereas those made with CMC were the most uniform. The CMC gel containing 4% gelling agent evidenced better apparent consistency for skin application, and was the formulation chosen to continue the stability and permeation studies. The CMC gel was found to exhibit the following macroscopic characteristics: a dark green color typical of plant extract-based preparations, a smell typical of extracts, and tactile properties, such as good consistency and extensibility, and uniform homogeneity with absence of lumps. In the thermal stress tests, gels also showed a uniform appearance, unvarying dark green color, and nice smell typical of plant extracts.

Characterization of plant-formulations and stability tests

Table IV summarizes the results collected from the stability studies, the pH of the formulation was stable under the conditions evaluated (12 months at 25° C) and there were no significant differences ($p < 0.05$) between the values over time. In the gels subjected to thermal stress, pH was observed to have no variations with respect to the values obtained at room temperature. A decrease in pH was observed in the plant extract-based gel with respect to the base gel (pH value = 6.8 ± 0.08). The values slightly acidic and close to pH (5-5.5) ensure that these topical preparations produce no irritation at the time of application, which is also supported by skin irritation studies. These values generate less physical discomfort due to the compatibility of the formulation pH with skin pH. The facts that these values remained stable during the assay and are within the range allowed by Farmacopea Argentina (2013), which sets the limit between 5.5 and 7, are indicative of physical stability. The formulation was observed to have greater spreadability than the base gel and this was maintained over time (Figure 1). This is consistent with the results collected from the study on gel viscosity (Table IV) which showed that the base gel was more viscous than the plant extract-based preparations (9333 ± 841 cP). The extensibility of the gel after 12 months (Figure 1 B) decreased slightly with respect to that achieved at the initial time (Figure 1 A). Results from the thermal stress study revealed a slightly greater spreadability (data not shown) and viscosity values were indicative of gel low viscosity. The initial formulation was less viscous and had greater spreadability than the base gel. Based on these observations, it can be assumed that the extracts contribute to increasing gel fluidity. The low viscosity of the gel gives it the advantage of a more precise application due to better flow and pourability. A gel with these characteristics can be administered very accurately by a dropper or drip-type dispenser with respect to other commercial products which are thicker gels and therefore do not provide an accurate skin application.

TABLE IV - Stability studies of gel formulation in normal and accelerated storage conditions

Parameters	Long term stability (25° C and 60% relativity humidity) Months						Accelerated storage conditions Days	
	0	1	2	3	6	12	0	28
Color	No change in colour						No change in colour	
Odour	No change in odour						No change in odour	
Homogeneity	Uniform product						Uniform product	
pH	5.80 ± 0.00	5.80 ± 0.00	5.75 ± 0.07	5.65 ± 0.07	5.75 ± 0.07	5.65 ± 0.05	5.85 ± 0.05	5.80 ± 0.05
Viscosity (cP)	3500 ± 165	3658 ± 293	3789 ± 247	3856 ± 252	4820 ± 350	6000 ± 348	3520 ± 135	3790 ± 185
Microbiological control								
Aerobic bacteria	Not detected						Not detected	
Fungi and yeasts	Not detected						Not detected	
Enterobacteriaceae	Not detected						Not detected	
S. aureus	Not detected						Not detected	
P. aeruginosa	Not detected						Not detected	

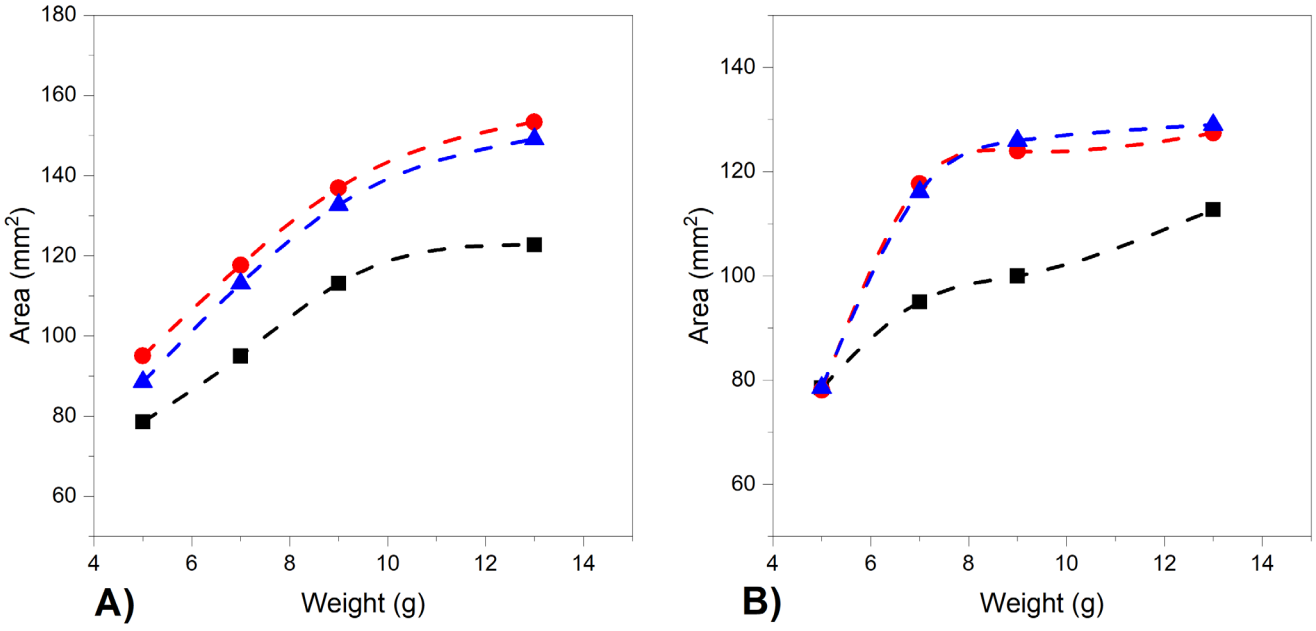

FIGURE 1 - Determination of the spreadability of the formulations with and without extract combinations **A)** at initial time, **B)** at 12 months. Formula base (■), Gel samples with combination of extracts stored on shelf (▲), Gel samples with combination of extracts for daily use (●).

Total phenol content represents the active principles in this formulation, and as can be seen in Table V, during the 12 months of the assay, it remained stable and within 90% of the initial content incorporated into the formulation. Under accelerated storage conditions, phenolic compound values decreased compared to those of the initial content (1908.15 μg/g ± 15.32 for the gel with the combination of extracts) but remained within 95% (1813.78 μg/g ± 8.89). The quantification of total phenols over the study time shows that the active principles are not altered and their pharmacological effect may not be affected so that gels may also remain chemically stable under these conditions. Thermodynamic stability confers long shelf life to the formulations (Ali *et al.*, 2014).

TABLE V - Total phenols content in the gels during the 12 months of storage expressed in μg/g

Initial	1 month	3 months	6 months	12 months
1958.35 ± 18.30*	1907.15 ± 2.31	1798.25 ± 5.66	1772.52 ± 4.78	1763.62 ± 16.84
1979.28 ± 14.65#	1912.36 ± 1.89	1792.44 ± 4.36	1781.35 ± 3.65	1776.44 ± 15.87

*Gel samples with combination of extracts stored on shelf, #Gel samples with combination of extracts for daily use.

As shown in Table IV, no microbiological contamination was detected in the plant extract-based products, neither at the beginning of the control nor during the trial. Furthermore, no bacterial growth was observed during microbiological control in the plant extract-based gels that were exposed to possible contamination through daily use. In contrast, the control gel did show microbial growth after the first 3 months. The absence of bacterial growth during the 12 months of the study seems to indicate that the extracts exert a preservative effect on the preparation, in addition to the therapeutic effect. A further strong support for this is the fact that the negative control (base gel) became contaminated over time. These results indicate that the gels produced stick to the microbiological quality specifications for non-sterile pharmaceutical products whose values −according to Farmacopea Argentina (2013)− must be ≤ 100 CFU of viable aerobic microorganisms/g of preparation and which must be free of *Enterobacteria*, fungi and yeasts, *P. aeruginosa* and *S. aureus*. Research on natural products with preservative properties is relevant on account of the fact that it may provide clues about the possibility of decreasing or substituting the concentration of synthetic preservatives applied to pharmaceutical and cosmetic formulations, thus securing greater safety for consumers. Previous studies have demonstrated the antimicrobial activity of herbal extracts and essential oils which have been proposed as natural preservatives, such as − among others− tea tree (*Melaleuca alternifolia*), lemon grass (*Cymbopogon citratus*), calamint or *lavender (Lavandula officinalis)* (Kunicka-Styczyńska, Sikora, Kalemba, 2011), *Santolina chamaecyparissus* (Kerdudo *et al.*, 2016), and *Silene vulgaris* (Boukhira *et al.*, 2017).

Determination of the antimicrobial activity of the plant-formulations

The plant extract-based gels showed inhibition of bacterial growth and this antimicrobial power was maintained during 12 months. The base gel used as a negative control did not show inhibition. The formulations showed inhibition halos against the two clinical isolates of *S. aureus* (F32, sensitive methicillin and F7, MRSA) and *S. epidermidis* ATCC 12228 with values between 15.5 and 18.5 mm. The inhibition halos were similar to those produced by the combination of extracts. This test was performed at 24 h of the gel preparations, once per month during 3 months and at 6 and 12 months, and it was observed that antimicrobial activity was maintained over time. This test demonstrates that gels retained the biological activity of the extracts incorporated as an active ingredient and that this activity was maintained over time.

In vitro permeation assay

Figure 2 shows the results derived from the *in vitro* cumulative permeation profile of polyphenols from gels. According to this figure, the amount of polyphenols detected in the receptor compartment was increased with time over the assay period. After 4 hours, the curve changed its slope, indicating a decrease in the flow rate. Between 60 min and 4 h the flow rate was 7.41 ± 0.84 μg/cm^2.h. Then, the flow rate decreased to 2.98 ± 0.16 μg/cm^2.h and was maintained until the end of the test.

At the end of the assay, an average of 10.85 ± 1.56% (54.25 ± 4.68 μg GAE/cm^2) of the initial amount loaded into the donor compartment was permeated and 26.88 ± 2.33% (134.58 ± 27 μg GAE/cm^2) was retained in the skin. The remaining percentage was not absorbed into the skin. The percentage of polyphenol permeation was low in agreement with findings from Torky *et al.* (2018) who claim that polyphenols have poor permeation. Many plant extracts and natural products can be deposited on the skin but cannot pass through it. This prevents them from being passed on to the receiving medium and could thus be useful for a local or topical application (Alalaiwe *et al.*, 2018). The amount of polyphenols retained in the skin is very close to 3 times the value of the MIC found for the mixture of extracts. Although the permeated percentage was low, this formulation achieved the expectation of retaining more polyphenols in the epidermis to meet local antimicrobial action for superficial wounds.

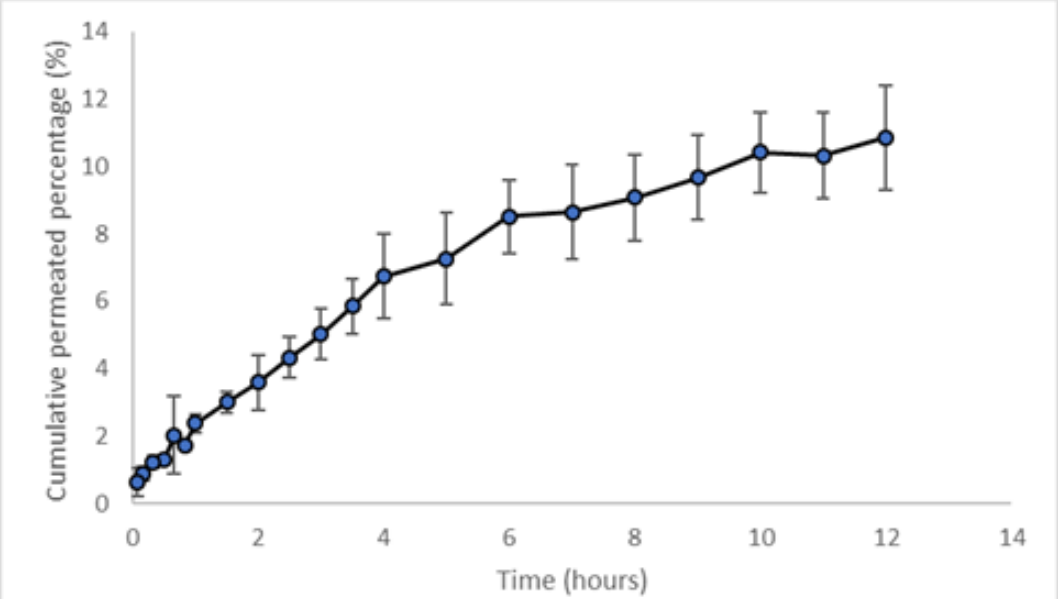

FIGURE 2 - Plot of cumulative percentage of permeated polyphenols vs time for formulation.

Determination of Primary Skin Irritation

As regards the irritation effect on the skin, the analysis showed that within Draize´s irritation scale, the PDII of the formulation ranged between 0-0.49 with a PDII value of 0.46. The formulation can be therefore categorized as non-irritant according to Draize´s classification. This confirms that the product is safe for application on the skin. Although previous studies report the potential allergenicity of certain phenolic compounds when reacting with the skin (Korkina, De Luca, Pastore, 2012; Christensen, 2014), no history of allergenicity was found in either *C. convoluta* or *F. caudigera* or in the polyphenols previously found in both plants (De Groot, 2013). It should also be noted that phenolic acids and flavonoids, which are present in these extracts, are the most studied natural substances known to have anti-inflammatory and anti-allergic potential (Juríková *et*

al., 2015). Polyphenols have been investigated for their anti-allergic effect in different disease models and in human clinical trials (Singh, Holvoet, Mercenier, 2011). Therefore, the risk of allergic reactions after continued use of this gel appears to be very low.

CONCLUSION

Summing up, the combination of *F. caudigera* and *C. convoluta* extracts efficiently inhibited the growth of pathogenic bacteria at concentrations lower than necessary when these extracts are used alone. This activity could be due to some of the chemical compounds previously found in these extracts, such as flavones. Both extracts and the combinations were non-genotoxic and non-toxic at therapeutic concentrations.

This is in support of the safe use of the mixture of these extracts.

The extracts could be incorporated into a pharmaceutical formulation. Gels showed good stability and correct formulation, preserving the antimicrobial activity of the original extracts. Taking into account that the parameters studied in the present work did not vary from those of the preparations stored at room temperature, it can be concluded that these hydrogels have good stability to abrupt temperature changes and this is also evidence of their stability under accelerated conditions. The product was safe for local application on the skin and could thus be an alternative for primary skin infections. In addition, the extract combination requires a lower quantity of each lyophilized extract, thus contributing to a cheaper and more efficient use of the extracts.

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