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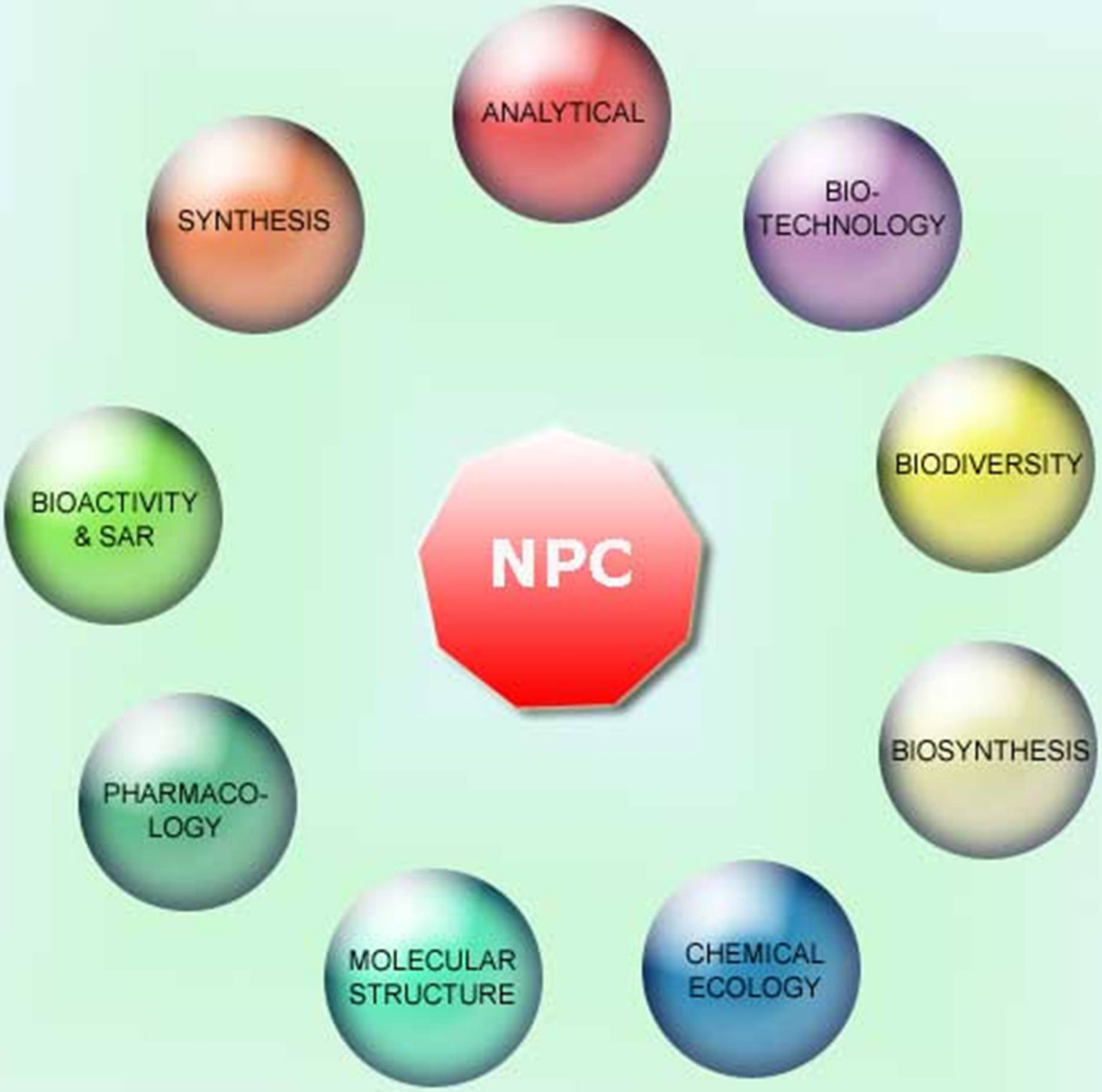

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Effect of Seasonality on Chemical Composition and Antibacterial and Anticandida Activities of Argentine Propolis. Design of a Topical Formulation

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The effect of seasonality on Argentine propolis collected during one year on its phenolic and flavonoid content and on the growth of Gram-positive and Gram-negative antibiotic resistant bacteria and *Candida* species was evaluated. Extracts of propolis samples collected in the summer and spring showed higher phenolic and flavonoid contents than the samples collected in other seasons (5.86 to 6.06 mg GAE/mL and 3.77 to 4.23 mg QE/mL, respectively). The propolis collected in summer and autumn showed higher antibacterial activity (30 µg/mL) than the other samples (MIC values between 30 and 120 µg/mL). No antibacterial activity was detected against Gram-negative bacteria. Also, these extracts were able to inhibit the development of five *Candida* species, with MFC values of 15-120 µg/mL. Pharmaceutical formulations containing the more active propolis extract were prepared. The hydrogel of acrylic acid polymer containing summer propolis extract as an antimicrobial agent showed microbiological, physical and functional stability during storage for 180 days. The pharmaceutical preparation, as well as the propolis extracts, was active against *Candida* sp. and antibiotic-multi-resistant Gram-positive bacteria. These results reveal that propolis samples collected by scraping in four seasons, especially in summer in Calingasta, San Juan, Argentina, can be used to obtain tinctures and hydrogels with antibacterial and antimycotic potential for topical use.

Keywords: Argentinean propolis, Seasonality, Hydrogel, Antibacterial activity, Antifungal activity.

Propolis is a complex resinous material produced by honeybees from plant exudates, beeswax, and bee secretions. Once collected, this material is enriched with salivary and enzymatic secretions and is used by bees to cover hive walls, fill cracks or gaps and embalm killed invader insects, thus being responsible for the safety of honeycombs, especially against microorganisms [1]. Several studies on the chemistry and biological activities of Argentine propolis have previously been reported [2a-4e]. It is increasingly being used as a dietary supplement and was incorporated into the Argentine Food Code (Secretaría de Agricultura, Ganadería, Pesca y Alimentación, Ministerio de Salud de la Nación) [4g].

Since the different geographical regions of Argentina are covered with a conspicuous diversity of plant species, and considering that some factors (e.g., rainfall and variations in temperature) could affect the chemical composition of propolis, investigations on the seasonal variations of both its chemical composition and biological properties are very important for the chemical and biological standardization of a particular type of propolis. Also, detailed knowledge of the phenolic content during the year may provide the basis for choosing the most appropriate timing for propolis harvesting in terms of high levels of these compounds.

Recently, we evaluated the effect of seasonal variations and harvesting on chemical parameters and antioxidant capacity of propolis from hives located in the Cuyo region, Argentina [3c]. It was concluded that the propolis from Cuyo region was a good source of antioxidant phenolic compounds and that the best harvesting time for propolis samples was from October to December [3c]. Six main flavonoids, the flavanones 7-hydroxy-8-methoxyflavanone, pinocembrin, and pinobanksin, the flavones chrysin and tectochrysin, and the flavonol galangin, have been isolated [3c,4f]. In a previous report, it was demonstrated that *Baccharis* species could be one of the plant species from which the bees collect the resins to produce San Juan propolis [3c]. The goal of this work was to evaluate the effect of seasonality on the antimicrobial activity (antibacterial and antifungal) of Argentine propolis, since there are no data in the literature concerning this, and to design an active, topical, pharmaceutical formulation using the propolis extract.

To obtain standardized propolis extracts, it is necessary to select a good raw material to develop the extraction and formulation process and to analyze different batches of the final product, both fresh and during storage because the chemical and biological parameters may vary with time. For these reasons, the phenolic and flavonoid content and antimicrobial activity were determined in fresh propolis ethanolic extracts and stored extracts during three years at 4°C.

The antimicrobial activities of Argentine propolis against human pathogenic bacteria, phytopathogenic bacteria and phytopathogenic fungi were reported previously [2a,3b,4a-4e]. In this work we analyzed for the first time the antimicrobial activity of Argentinean propolis from the Cuyo region (San Juan province) collected in

Table 1: Content of phenolic compounds and flavonoids in propolis extracts grouped according to the collection season.

Seasons/ year	Collection months by scraping	Phenolic compounds (%)		Flavone and flavonol (%)		Flavanone and dihydroflavanone (%)	
		FE[1]	PE[2]	FE[1]	PE[2]	FE[1]	PE[2]
Autumn/ 2000	April	27.0^{c}	27.0^{c}	16.9^{d}	17.5^{d}	0.4^{a}	0.3^{a}
	May	27.0^{c}	26.0^{c}	17.0^{d}	17.4^{d}	0.7^{c}	0.6^{c}
	June	26.5^{c}	26.0^{c}	14.0^{c}	14.7^{c}	0.5^{b}	0.5^{b}
Winter/ 2000	July	23.9^{a}	22.0^{a}	18.0^{e}	18.5^{d}	0.2^{a}	0.2^{a}
	August	23.7^{a}	23.0^{a}	11.0^{a}	10.0^{a}	0.3^{a}	0.4^{a}
	September	25.1^{b}	25.0^{b}	14.0^{c}	13.0^{b}	0.3^{a}	0.3^{a}
Spring/ 2000	October	29.5^{c}	30.5^{c}	17.0^{d}	17.5^{d}	0.4^{a}	0.3^{a}
	November	28.0^{d}	28.5^{d}	13.0^{b}	13.0^{b}	0.4^{a}	0.3^{a}
	December	35.0^{f}	36.0^{f}	18.0^{e}	18.0^{d}	0.6^{b}	0.6^{b}
Summer/ 2001	January	36.0^{f}	35.5^{f}	24.0^{i}	24.0^{e}	1.0^{d}	0.9^{d}
	February	36.0^{f}	37.0^{f}	23.0^{f}	23.0^{e}	0.7^{c}	0.7^{c}
	March	24.0^{b}	24.8^{b}	16.0^{d}	16.0^{d}	0.5^{b}	0.4^{b}

1. Fresh extract; 2. Extract preserved at 4°C for 3 years. Data are means of three determinations of two separate experiments. Means with the same letter are not significantly different (p<0.05)

different seasons of the year. The Cuyo propolis samples were grouped according to their collection time in summer, autumn, winter and spring.

In order to compare the chemical composition of different seasonal collections of Cuyo propolis, we evaluated their phenolic and flavonoid contents (Table 1). All the samples showed higher phenolic and flavone/flavonol contents than flavanone and dihydroflavanone. The quantities of these compounds were not modified greatly by season, but propolis collected in summer and spring showed higher contents of phenols and flavonoids than the autumn and winter samples. Similar results were found in the analyzed samples from 2009 [3c], demonstrating that the phenolic and flavonoid contents are stable over several years. Previous studies demonstrated that Argentinean propolis from the arid and tropical mountainous areas (San Juan, Tucumán and Catamarca province) showed high contents of phenolic compounds (nearly 35%), and there is evidence that there is a positive correlation between phenolic content and bioactivities [2a-2b,3b-3c,4d-4e]. The antibacterial and antifungal activity of fresh extracts and those conserved for three years were evaluated. The propolis extracts were more active against Gram-positive than Gram-negative bacteria. The MIC values obtained for the propolis samples collected in different seasons varied between 30 and 120 µg/mL for Gram-positive bacteria, while no antibacterial activity was detected against Gram-negative bacteria (Table 2). The summer and autumn samples were the most active (MIC 30 µg/mL). These results are in agreement with those reported by Castro *et al*. [5a] for Brazilian propolis. The absence of activity of the Argentine propolis on Gram-negative bacteria could be ascribed to the morphological and chemical differences in the cell wall of these microorganisms. Our results are in agreement with those of Grange and Davey [5b], who observed a marked action of propolis against Gram-positive bacteria and limited activity against Gram-negative bacteria.

Other reports [2a,6a] present similar results, supporting the hypothesis that propolis is active mainly against Gram-positive bacteria. Bioautographic assays revealed at least two bands with antibacterial activity (R*f* 0.61 and 0.78), coincident with the flavonoids (revealed by NP/PEG). These were detected in all propolis samples, indicating that the antibacterial components are present in all months of the year, but probably in different quantities.

Previously, 7-hydroxy-8-methoxyflavanone, pinocembrin, pinobanksin, chrysin, tectochrysin, and galangin have been isolated from San Juan propolis [3c, 4f]. In propolis from other countries, the antimicrobial activity was attributed to the flavonoids, in particular to galangin and pinocembrin [6a-b]. On the other hand,

Table 2: Antimicrobial activity (MICs) of propolis extracts from different seasons against growth of human pathogenic bacteria.

Microorganisms	Propolis samples: Winter	Autumn	Spring	Summer	Phenotype of *clinical isolate
	MIC values (µg/mL)				
Staphylococcus aureus					
SAMS F16	30	30	30	30	Met^{s} Oxa^{s}Gen^{s}
SAMR F7	30	30	120	30	Met^{r} Oxa^{r}Gen^{r}
SCNMS F30	30	30	60	30	Met^{r} Oxa^{s}Gen^{s}
ATCC 29213	30	30	30	30	Control strain
Enterococcus faecalis					
F201	60	30	30	30	Gen^{s} Str^{s} Van^{s} Amp^{s}
F 203	60	30	30	30	Gen^{r} Str^{r} Van^{s} Amp^{s}
F 208	60	30	30	30	Gen^{r} Str^{r} Van^{s} Amp^{r}
ATCC 29212	60	30	30	30	Control strain

*The antibiotics were used as positive control

the antifungal properties of propolis from San Juan against *Candida* species were similar to the antibacterial activity, with MFCs ranging from 15 to 120 µg/mL (Table 3). These values were similar to or lower than those reported for other Argentinean propolis samples with antifungal activity [4c]. The summer PEE was more active against *Candida parasilopsis* and *C. glabrata*, followed by *C. tropicalis* and *C. albicans*. No difference of antibacterial and anticandidal activity was observed between the fresh extracts and those conserved at 4°C over 3 years.

In the pharmaceutical and cosmetic fields, polyphenols appear particularly promising because of their antimicrobial effects and that they are characterized by an absorption spectrum which can filter UV-radiation, thereby reducing the penetration of radiation into the skin and consequently lowering inflammation, oxidative stress and DNA damaging effects [7a-b]. Different hydrogel types were prepared using a hydrogel-forming polyacrylic acid polymer (Carbopol® 934) and PEE, corresponding to summer propolis samples, without preservatives. Stability studies were performed on the formulations. Measurement of pH of the formulations is necessary to detect alterations during storage, ensuring that the pH value is compatible with the components of the formulation and with the application site, avoiding irritation.

Table 3: Minimal fungicide concentrations, according to season, against *Candida* species.

Fungal strain	MFC (µg GAE/mL)			
	Winter	Spring	Summer	Autumn
F_{01}	60	60	60	60
F_{02}	30	30	30	30
F_{03}	120	120	60	120
F_{04}	60	15	30	60
F_{05}	120	60	60	120
F_{06}	15	15	15	15
F_{07}	15	15	15	15

Fungal strains assayed: *Candida albicans* (F_{01} and F_{02}), *C. tropicalis* (F_{03} and F_{04}), *C. krusei* (F_{05}), *C. parasilopsis* (F_{06}), *C. glabrata* (F_{07}). The miconazol nitrate was used as positive control.

The pH values of the preparation were stable over 6 months at room temperature. Macroscopic and microscopic examinations indicated that the hydrogel was homogeneous during this period. The centrifugation test is of major interest since it provides rapid information about the comparable stability properties of different emulsions. In the centrifugation study, all the formulations were stable. The preparation containing propolis ethanolic extracts was microbiologically stable for 6 months at room temperature, as well as the hydrogel with a commercial antibiotic/antifungal agent. The susceptibility test, using the agar well diffusion method, indicated that preparations were active against antibiotic-multi-resistant Gram-positive bacteria and *Candida albicans*. The inhibition values obtained with the hydrogel containing the commercial antibiotic were similar to those obtained with propolis extract against the F7 and F16 clinical isolated.

The present study reveals that propolis extracts from San Juan in the Cuyo region of Argentina collected in the autumn and summer are good antifungal and antibacterial agents. Other assays are being carried out in order to evaluate their effect *in vivo* in order to have a complete understanding of this action mechanism.

Experimental

Propolis collection: The samples tested were collected from beehives in Calingasta, San Juan, Argentina from April to December in 2000 and January to March in 2001. The samples were hand-gathered by scraping from hives of Red de Ensayos del INTA-PROAPI (Programa Argentino de Apicultura). They were stored at -20°C. Sample collection dates and environmental conditions in Calingasta have been described previously [3c].

Preparation of propolis ethanolic extract (PEE): Once frozen at -20°C, the propolis samples were ground and extracted with *n*-hexane first and then with ethanol (2 g of each sample in 100 mL of each solvent) [8a]. The fresh extracts and those maintained at 4°C for 3 years were standardized for total phenolic compound content [8b], flavones and flavonol content [8c] and flavanone and dihydroflavanone [8d]. The results were expressed as gallic acid equivalents (GAE) per mL, quercetin equivalent (QE) per mL, and naringenin equivalent (NE) per mL, respectively.

Antimicrobial activity

Microorganisms: Clinical isolates of *Staphylococcus aureus* methicillin resistant (F7), methicillin sensitive (F16) and negative coagulase methicillin resistant (F30), *Enterococcus faecalis* (F201, F203, F208), *Escherichia coli* (F301 and 331), *Klebsiella pneumoniae* (F364), *Proteus mirabilis* (F304), *Enterobacter cloacae* (F302), *Morganella morganii* (F339) and *Pseudomonas aeruginosa* (F305) were obtained from clinical samples from Hospital Dr Nicolás Avellaneda, San Miguel de Tucumán, Tucumán, Argentina. The following reference strains were included in the study: *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 35218, and *Klebsiella pneumoniae* ATCC 700603. Five clinical fungal isolates, *Candida albicans* (F_0, F_1), *C. glabrata* (F_2), *C. tropicalis* (F_3), *C. krusei* (F_4), and *C. parasilopsis* (F_5), were collected from the Hospital del Niño Jesús, San Miguel de Tucumán, Argentina. Bacterial strains were identified by their biochemical profiles [9a]. All organisms were maintained in brain–heart infusion (BHI medium, Britania) containing 30%, v/v, glycerol at -20°C. *Candida* strains were maintained at 4°C in Sabouraud dextrose agar (Merck). For the assays, individual colonies were isolated and suspended in 5 mL of 0.9% NaCl solution. The inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland standards and diluted in cation-adjusted Müller–Hinton broth (CAMHB) for bacteria and Sabouraud dextrose broth (SDB) for yeast, in order to achieve adequate inocula in each case.

Bioautographic method: PEE (2.5 µg GAE) were seeded in spots on TLC plates or the components were separated using toluene: chloroform: acetone (4.0: 2.5: 3.5, v/v/v) as development solvents. Then, the plates were dried overnight in a sterile room and either visualized at UV-254 nm or sprayed with NP/PEG and visualized with UV-365 nm. Other plates were developed, then dried and covered with 6 mL of soft medium (BHI with 0.6% agar) containing $1x10^5$ colony forming units (CFU) of *S. aureus* (F7 and F16) and incubated at 35°C for 16-20 h. Then, the plates were sprayed with a 2.5 mg/mL MTT solution (3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) in PBS (10 mM sodium phosphate buffer, pH 7, with 0.15 M NaCl) to determine the cellular viability. Plates were incubated at 35°C for 1 h in the dark for color development [2a].

Agar macrodilution methods

Minimal inhibitory concentration (MIC) for bacteria: MIC values were determined by the serial agar macrodilution method [9b]. The same volume (0.5 mL) of dilutions of each PEE (15 to 480 µg GAE/mL) was added to 4.5 mL of MHA (Müller-Hinton agar) medium. After cooling, the plates were inoculated in spots with 2 µL of each bacterial cell suspension ($1x10^5$ CFU/mL) and incubated aerobically for 16-20 h at 35°C. A growth control of each tested strain was included. Controls of ethanol were carried out. MIC_{100} was defined as the lowest concentration of PEE at which no colony was observed after incubation. MIC values were also determined for different commercial antibiotics. Resistance was defined for each case: levofloxacine (Lvx, MIC ≥8 µg/mL), piperacillin/tazobactam (Tzp, ≥ 128 µg/mL), imipenem (Ipm, MIC >16 µg/mL), meropenem (Mem, MIC >16 µg/mL), ceftriaxone (Cro, MIC >128 µg/mL), cefotaxime (Ctx, MIC >128 µg/mL), ceftacidime (Caz, MIC >32 µg/mL), cefuroxime (Cxm, MIC ≥32 µg/mL), cefepime (Fep, MIC ≥32 µg/mL), amikacin (Amk, MIC >16 µg/mL) and ampicillin/sulbactam (Sam, MIC ≥32 µg/mL) for Gram-negative bacteria and oxacillin (Oxa, MIC >16 µg/mL), streptomycin (Str, MIC ≥300 µg/mL), ampicillin (Amp, MIC > 64 µg/mL), methicillin (Met, MIC >16 µg/mL), gentamicin (Gen, MIC >100 µg/mL) and vancomycin (Van, MIC > 6 µg/mL) for Gram-positive bacteria.

Agar macrodilution methods for fungi: Minimum inhibitory concentration of PEE was determined in Sabouraud Dextrose Agar (SDA). The same volume (0.5 mL) of dilutions of each PEE (15 to 480 µg GAE/mL) or miconazol nitrate (2%) was added to 4.5 mL of SDA. The plates were inoculated in spots with 2 µL of standardized suspension of test organism (10^5 CFU/mL) and incubated at 28°C for 2 days. MFC was the lowest concentration that did not show any growth of the *Candida* sp. after macroscopic evaluation [9b].

Topical formulation preparation (hydrogel): An emulsion stabilized by an anionic hydrophilic colloid (Carbopol® 934, 0.47% w/w) was prepared [10a-c]. An appropriate amount of carbopol powder was slowly added to water with constant stirring using a helix agitator (Decalab®) at room temperature and was then neutralized with triethanolamine (0.27%, w/w). Erythromycin (0.1%) or miconazol nitrate (2%) or PEE (final concentration of 0.15 and 0.30%) was added to the gel preparations and mixed thoroughly until the gel was uniform. The extract concentration was determined according to the MIC values. Good Manufacturing Laboratory Practices (GMLP), Farmacopea Argentina VI Edition and Food and Drugs Administration Norms were applied.

Stability studies

Physical stability evaluation: Physical stability was evaluated by submitting the formulation to storage at room temperature (25-40°C with 70% relative humidity) for a period of 3 months. Samples from each formulation were evaluated at the initial time and after 1, 7, 15, 30, 60, 90,120 and 180 days by the following methods:

pH measurements: Each formulation (1 g) was diluted with distilled water to 10 mL. After homogenization, the pH of the samples was measured with a pH meter. All measurements were made in triplicate at room temperature.

Centrifugation assays: Each sample (1 g) was centrifuged at 3000 r.p.m. for 30 min.

Loss of water: The weight of each formulation maintained at different temperatures for 6 months was determined.

Microscopic assays: An aliquot of each sample was gently spread on a plate, covered with a tiny plate and the formulation homogeneity visualized by Olympus Microscopy (40X).

Extensibility: Five g of each formulation was placed between 2 acrylic plates. Then, different weights were placed on the top plate at fixed time intervals. The area (mm^2) was calculated and plotted as a function of weight.

Microbiological stability assays: (a) The number of viable aerobic microorganisms (CFU), coliforms, fungi and yeast was determined in each preparation kept at room temperature for 3 months using peptone-agar, casein-glucose-yeast extract, lauryl sulfate broth, maltose-glucose agar (Sabouraud). (b) Freshly made samples of hydrogel preparations (1 g) were inoculated with either 10^6 CFU of *S. aureus*, methicillin-resistant (F7) or *Candida albicans* (F1). Aliquots were taken at different times. The number of CFU was determined every 15 days for 6 months [10e].

Evaluation of antimicrobial activity of pharmaceutical formulation: *Agar-well diffusion method*: Petri dishes (9 cm in diameter) were prepared with 10 mL of a base layer of MHA medium and a top layer (3 mL) of 0.2% BHI agar medium inoculated with 30 μL of each bacterial suspension (10^4 UFC/mL) or 5×10^4 UFC/mL (levaduriform fungi). After drying, 0.1 g of each hydrogel preparation, 10 μL of extract or 40 μg of gentamicin sulfate or miconazole nitrate were placed in each well. Antibacterial assay plates were incubated at 35°C for 18 h, while antifungal ones underwent the same process at 28°C for 48 h. The growth inhibition diameter around each well was measured. Each experiment was carried out in triplicate, and the mean diameter of the inhibition zone was recorded.

Statistical analyses: All tests were conducted in triplicate. Values are expressed as mean ± standard deviation. A one-way ANOVA followed by post-hoc analysis (Tukey) were performed in order to evaluate the difference between the groups. p values of < 0.05 were regarded as significant.

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Contents

Continued inside backcover