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Design and quality control of a pharmaceutical formulation containing natural products with antibacterial, antifungal and antioxidant properties

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ABSTRACT

The aims of the present study were to determine the antibacterial and antifungal activity as well as mutagenicity of *Sechium edule* fluid extract and to obtain a pharmaceutical formulation with them. The extract exhibited antimicrobial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter cloacae*, *Serratia marcescens*, *Morganella morganii*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Candida* spp. and *Aspergillus* spp. isolated from clinical samples from two hospitals of Tucuman, Argentina. Non-toxicity and mutagenicity on both *Salmonella typhimurium* TA98 and TA 100 strains until 100 μg/plate were observed. A hydrogel with carbopol acrylic acid polymer containing *S. edule* fluid extract as antibacterial, antimycotic and antioxidant agent was obtained. Microbiological, physical and functional stability of pharmaceutical formulation conserved at room temperature for 1 year were determined. Addition of antioxidant preservatives to store the pharmaceutical formulation was not necessary. The semisolid system showed antimicrobial activity against all Gram positive and Gram negative bacteria and fungi assayed. The minimal inhibitory concentration (MIC) values ranged from 20 to 800 μg/mL. Its activity was compared with a pharmaceutical formulation containing commercial antibiotic and antifungal. A pseudoplastic behavior and positive thixotropy were observed. Our current finding shows an antimicrobial activity of hydrogel containing *S. edule* extract on a large range of Gram negative and Gram positive multi-resistant bacteria and fungi. This topical formulation may be used as antimycotic and as antibacterial in cutaneous infections.

1. Introduction

Sechium edule Swartz (Cucurbitaceae), known under the popular name *chayote*, is a native species from tropical America and it is a traditional crop of Mexico (Yang and Walters, 1992). In Argentina it is not grown yet, but it is known as an exotic species, mainly in the northwest.

Chayote fruits and roots, have been important elements in the diet of people in many parts of Latin America and other areas of the world. Leaves and stems of *S. edule* are used as daily vegetables (Booth et al., 1992). The diuretic, antihypertensive, cardiovascular and antiinflammatory properties of the leaves, fruits and seeds of chayote have been confirmed by pharmacological studies (Bueno et al., 1970; Lozoya, 1980; Salama et al., 1986; Gordon et al., 2000). A protein isolated from seed aqueous extracts of *S. edule* called Sechiumin have ribosomal inactivation properties and is a potential chemotherapeutic agent (Wu et al., 1998; Yen et al., 2000). In a previous work we reported the antimicrobial activity of aqueous and ethanolic extracts of different parts of *S. edule* against Gram positive bacteria isolated from skin infections and soft parts (hurt of surgical site, end of catheter) of patients at Hospital Nicolás Avellaneda, Tucumán, Argentina (Ordoñez et al., 2003). Based on MIC values, leaf liquid extracts proved to be potent inhibitors against different Gram positive species, including those resistant to many antimicrobial agents. Fluid extracts have also shown antioxidant and free radical scavenging activity (Ordoñez et al., 2006). Stored (1 year at 7 °C) and recently prepared extracts showed similar antibacterial and antioxidant activities. From the biological properties found in *S. edule* extracts, the antimicrobial and antioxidant ones deserve special interest since they suggest *S. edule* extract could be successfully applied topically to prevent and treat skin damage and infection. When modern or traditional drugs are swabbed on the skin, an active agent must be released from the carrier (vehicle) before it contacts the epidermal surface and it should be available for penetration into the stratum corneum and lower layers of the

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skin. In the case of phytopharmaceuticals, they may be released from the formulation. Different strategies have been proposed to achieve efficient drug delivery systems and in the last few years hydrogels and gel in general have been considered good candidates for oral, rectal, ocular, cutaneous and subcutaneous administration. Hydrogels in particular have been widely utilized in the medical and pharmaceutical field for their biocompatibility and their similarity to a natural tissue (Ratner and Hoffman, 1976; Peppas et al., 2000). The purpose of the present study was to determine the antimicrobial activity against yeast and antibiotic-resistant Gram negative bacteria and genotoxicity of standardized extract of *S. edule* and to prepare a pharmaceutical formulation containing this extract as antibacterial, antimycotic and antioxidant agent.

2. Material and methods

2.1. Plant material

The plants were collected from September to March in San Miguel de Tucumán, Tucumán, Argentina. Voucher specimens were deposited in the Herbarium of the Instituto de Estudios Vegetales (IEV 2024), Facultad de Bioquímica, Química y Farmacia, UNT (Tucumán, Argentina). The part used was leaves.

2.2. Preparation of leaves extract

Fluid extract: lixiviated (LS): ground, air-dried plant material was extracted with 80% ethanol (v/v) solvent, using a percolator apparatus at room temperature until achieving total extraction (Farmacopea Argentina VI Ed. 1978). The drug was filtered through Whatman No. 1 filter paper, dried under reduced pressure at 40 °C and weighed. Prepared extract were stored at 4 °C in the dark.

2.3. Determination of total phenolic compounds content in *Sechium edule* extract

Total phenolic compounds content was determined by the Folin–Ciocalteau method (Singleton et al., 1999). Results were expressed as gallic acid equivalents.

Total flavonoids were estimated using the method of Woisky and Salatino (1998). Concentrations were ascertained spectrophotometrically at 420 nm with quercetin as standard.

2.4. HPLC analysis

HPLC analysis was performed using the method described by Siciliano et al. (2004) with minor modification. The *S. edule* extract was filtered through Whatman No. 1 filter paper and used for cromatographic analysis. Analyses were performed using a 250 mm, 4.6 mm i.d., 5 μm, Phenomenex C-18 column. The eluent was a mixture of acetonitrile (solvent A) and 0.05% acetic acid (solvent B). The solvent gradient was as follows: 0–2 min from 0 (A) to 15% (A); 2–25 min from 15 (A) to 20% (A); 25–28 min maintained at 20% (A). Elution was perfomed at flow rate of 1.0 mL/min. The total running time was 40 min, and compounds detection was carried out at 280 nm and 340 nm and the identification by comparison with standards dissolved in methanol (96%, v/v) for its retention time.

2.5. Microbiological assays

2.5.1. Microorganisms and media used in this study

Clinical isolates of the following organisms: methicillin resistant *Staphylococcus aureus* ($n = 1$), *Escherichia coli* ($n = 12$), *Klebsiella pneumoniae* ($n = 6$), *Proteus mirabilis* ($n = 6$), *Enterobacter cloacae* ($n = 9$), *Serratia marcenscens* ($n = 2$), *Morganella morganii* ($n = 2$), *Acinetobacter baumannii* ($n = 2$), *Pseudomonas aeruginosa* ($n = 7$), *Stenotrophomonas maltophilia* ($n = 3$) and *Providencia stuartii* ($n = 1$) were recovered between January 1999 and December 2000 from clinical samples obtained from the Hospital Nicolás Avellaneda, San Miguel de Tucumán, Tucumán, Argentina. The following reference strains were included in the study: *S. aureus* ATCC 29213, *E. coli* ATCC 35218, ATCC 25922 and *P. aeruginosa* ATCC 27853. The strains were identified by using biochemical profiles according to the recommendations of the Manual of Clinical Microbiology (Murray et al., 1999). Six clinical fungi isolates, *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Candida parasilopsis* and *Aspergillus* spp., were collected at Hospital del Niño Jesús, San Miguel de Tucumán, Tucumán, Argentina. The stock culture was kept in Sabouraud Dextrose Agar medium at 4 °C. The strains were identified according to CLSI, 2006, Clinical Laboratory Standards Institute.

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 trypticase soy agar supplemented with 5% sheep blood (Difco) and aerobically grown overnight at 35 °C. Individual colonies were isolated and suspended in 5 mL of 0.9% NaCl. The inocula were prepared by adjusting suspension turbidity to match the 0.5 Mac Farland standards (10^8 CFU/mL) and diluted in CAMHB (cation-adjusted Müller–Hinton broth). The cell number in CAMHB was estimated using a serial dilution technique (CLSI, 2006) for each assay.

The mycelial fungi were cultured on Sabouraud Dextrose Agar (SDA) and incubated at 28 °C for 7–14 days. The growth was aseptically crushed and macerated thoroughly in sterile distilled water and the fungal suspension was spectrophotometrically standardized to an absorbance of 0.50 at 450 nm (10^6 spore/mL) according to CLSI, 2006.

2.5.2. Minimum inhibitory concentration (MIC) and minimal bactericidal or fungicidal concentration (MBC or MFC)

In all cases the antibacterial and antifungal activity was evaluated by the two-fold serial dilution method (*Sechium* extracts concentration 10–800 μg/mL).

2.5.3. Agar macrodilution methods

2.5.3.1. Fungi and yeast. Minimun inhibitory concentration of *Sechium* extracts was determined in Sabouraud Dextrose Agar (SDA). The plates were inoculated in spots with 2 μL of standardized suspension of test organism (10^4 CFU). The plates were incubated at 35 °C for 48 h (yeast) and 28 °C for 7–14 days (mycelial fungi). MIC was the lowest concentration that did not show any growth of the tested organism after a macroscopic evaluation (CLSI, 2006).

2.5.3.2. Bacteria. The test was performed in Müller Hinton Agar medium (MHA, Laboratorio Britania, Argentina). Plates were inoculated in spots of 2 μL with each bacterial cell suspension (10^4 CFU) and aerobically incubated for 18 h at 35 °C. A growth of each tested strain was included.

MIC was defined as the lowest concentration of soluble principle or antibiotics where no colony was observed after incubation.

2.5.4. Broth microdilution method

This test was performed in sterile 96-well microplates. The extract was transferred to each well in order to obtain a two-fold serial dilution. The inocula (100 μL) containing 5×10^4 CFU or 5×10^4 spore of each microorganisms (in CAMHB or Sabouraud Dextrose Broth) were added to each well. A number of wells were reserved in each plate for sterility control (no inoculum added), inoculum viability (no extract added) and ethanol inhibitory effect. Plates were aerobically incubated at 35 °C. After incubation for 18 h, microbial growth was assayed by absorbance measurement

at 625 nm. MIC was defined as the lowest concentration of phenolic compounds able to restrict growth to a level <0.05 at 625 nm (no macroscopically visible growth). To establish MBC or MFC, 10 μL of each culture medium without visible growth was used to inoculate SDA or MHA plates. After 16–20 h of aerobic incubation at 35 °C the number of surviving organisms was determined. MBC or MFC was defined as the lowest extract concentration at which 99.9% of the bacteria/fungi strains were killed. Each experiment was carried out three times and twice-repeated. The MIC values were also determined Lvx, levofloxacin; Tzp, piperazilin/tazobactam; Ipm, imipenem; Cro, ceftriaxone; Ctx, cefotaxime; Caz, ceftacidime; Cxm, cefuroxime; Fep, cefepime; Amk, amikacine, Sam, ampicillin/sulbactam; Mem, meropenem for Gram negative bacteria and methicillin, vancomycin, oxaciline, gentamicin, ampicillin and streptomycin for Gram positive bacteria (F7). Each experiment was carried out three times and twice-repeated. The antimicrobial agents were supplied by Sigma Chemical Co. (USA) and Britania Laboratory S.A. Argentina.

2.6. Mutagenicity assay

2.6.1. Ames test

2.6.1.1. Toxicity assay. To examine the toxic effects on *Salmonella typhimurium* strains TA98 and TA100, a diluted extract (0.01–100 μg/plate) were added to overnight-cultured *S. typhimurium* strains TA98 or TA100 (0.1 mL) and S9 mix (0.5 mL) or 0.1 M phosphate buffer, pH 7 (0.5 mL) instead of S9 mix. The mixture was preincubated at 37 °C for 5 min before it was diluted with phosphate buffer and the mixture was then poured onto nutrient agar plates. The plates were incubated at 37 °C for 2 days and the number of colonies was counted. The *Sechium* extract was then tested for their mutagenic potency in the non-toxic concentration range.

2.6.1.2. Mutagenicity assay. The mutagenic effects of extract were assayed according to the Ames test (Maron and Ames, 1983) using *S. typhimurium* strains TA98 and TA100 with and without metabolic activation (S9 mix fraction). The tested strains were cultured overnight in Oxoid Nutrient Broth for 12 h. Different concentrations of extract (10–50 μg/plate) were added to 2 mL of soft agar containing L-hystidin (0.05 mM) and D-biotin (0.5 mM) and 0.1 mL of bacterial culture and then poured onto a plate containing minimum agar (Oxoid No. 2). The plates were incubated at 37 °C for 48 h and the His+ revertant colonies were manually counted. The influence of metabolic activation was tested by adding 500 μL of mixture prepared with S9 fraction obtained from liver of Sprague-Dawley rats pre-treated with a polychlorinated biphenyl mixture.

All experiments were analyzed in triplicate with at least two replicates. A sample was considered to be mutagenic when the number of revertant colonies was at least twice the negative control yield (MUI ≥ 2) and showed a significant response in analysis of variance. The mutagens used as positive controls were 4-nitro-*o*-phenylenediamine (NPD, 5 μg/plate) which is a direct acting mutagen, and isoquinoline (IQ, 0.1 μg/plate for TA 98 and 0.5 μg/plate for TA100), which required S9 mix for metabolic activation.

2.7. Topical formulation preparation (hydrogel)

An emulsion stabilized by an anionic hydrophilic colloid (Carbopol® 934, 0.47% w/w) was prepared (Vila Jato, 1997; Farmacopea Argentina VI Ed., 1978). An appropriate amount of carbopol powder was slowly added to water under constant stirring with a helix agitator at room temperature. Gel preparations were added gentamicin sulphate (0.1%) or miconazol nitrate (2%) or fluid extract of *S. edule* leaves (0.24%). The fluid extract concentration used in these preparations was determined according to the MIC values and the MBC values. After the mixture had been kept at room temperature for 24 h, a small amount of 0.5% (w/w) triethanolamine was added and mixed throughly until the gel was formed (Table 5).

Good Manufacturing Laboratory Practices (GMLP), Farmacopea Argentina VI Ed. and Food and Drugs Administration Norms were applied.

2.8. Stability studies

2.8.1. Physical stability evaluation

Physical stability was evaluated by submitting the formulation to storage at 5, 25 and at 40 °C with 70% RH (relative humidity) for a period of 1 year. Samples from each formulation container were evaluated at the initial time and after 1, 7, 15, 30, 60, 90, 120, 180 and 360 days in the following methods:

2.8.1.1. pH measurements. One gram of each formulation was weighted and diluted with distilled water until 10 mL. After homogenized, the pH of the samples was measured with pH meter. All measurements were made at room temperature in triplicate for each analyzed sample.

2.8.1.2. Centrifugation assays. One gram of each sample was centrifuged at 3000 r.p.m. during 30 min.

2.8.1.3. Loss water. The weigth of each formulation maintained at different temperature during 1 year was determined.

2.8.1.4. Microscopic assays. A small amount of each sample was gently spread on a plate, covered by a tiny plate and visualized by Olympus Microscopy (40×).

2.8.1.5. Viscosity. Minimum apparent viscosity and rheological behavior were determined using a cone and plate Brookfield Digital Viscometer, equipped with a recirculating water-bath for control of sample-container temperature. Viscosity determination was done in 0.5 mL of sample, sequentially increasing and decreasing values of shear rate in the range 0.05–20 r.p.m. to obtain the ascending curve and the procedure was repeated in reverse with progressively slower rates (20–0.05 r.p.m.) to obtain the descending curve. Temperature was kept constant at 26 ± 0.5 °C and all samples were equilibrated at run temperature on the plate for 5 min prior to viscosity measurement. The material was monitored as a function of stored time (Sheth, 2007).

2.8.2. 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 1 year using MHA, Lauryl Tryptosa Broth plus 4-methylumbelliferyl- β-D-glucuronide (MUG) with Durham tubes, violet Red Bile Agar with MUG (DIFCO cod:229100), Plate count Agar (Merck), Levin and Sabouraud Dextrose Agar.

(b) Freshly made samples of hydrogel preparations (1 g) were inoculated with 10^6 CFU of *S. aureus*, methicillin-resistant (F7) or *P. aeruginosa* (F353). Aliquots were taken at different times. Then, the number of CFU was determined every 15 days for 1 year (Farmacopea Española, 1999).

2.9. Evaluation of antimicrobial activity of pharmaceutical formulation

a) *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 bacteria/mL) or 5×10^4 spores/mL (mycelial fungi). After drying, 0.1 g of each hydrogel preparation, 10 μL of fluid extract or 40 μg of gentamicin sulphate or miconazol 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 in the case of yeast or 7–14 days for mycelial fungi. 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.

b) The minimal inhibitory concentration (MIC) was determined by agar macrodilution method and broth microdilution method (CLSI, 2006).

2.10. Antioxidant activity of pharmaceutical formulation

Antioxidant activity of pharmaceutical formulations containing fluid extract was measured and compared with the activity of fluid extract and BHT.

The rate of β-carotene-bleaching and DPPH free radical scavenging activity were determined according to Ordoñez et al., 2006. Each experiment was carried out three times and twice-repeated.

3. Results

S. edule fluid extract showed a high content of phenolic compounds (0.94 mg/g of dry plant material) and mainly flavonoids (0.75 mg/g dry plant material). In order to standardize the *S. edule* leaves extracts, HPLC fingerprint analysis was carried out, the peak 4 was identified as luteolin-7-*O*-glucoside (Fig. 1). The present study was conducted to investigate antimicrobial activity of *S. edule* fluid extract (chayote), and to use this extract to make a semisolid pharmaceutical system (hydrogel) with antimicrobial and antioxidant properties. The microorganisms used in this study were antibiotic multi-resistant Gram negative clinical isolated, strains of yeast and mycelial fungi (Table 1).

The MIC and MFC values of the *S. edule* extract were determined by agar macrodilution and broth microdilution assays. The MIC values obtained confirm the existence of activity against the Gram negative bacterial strains tested in our study, with MIC values

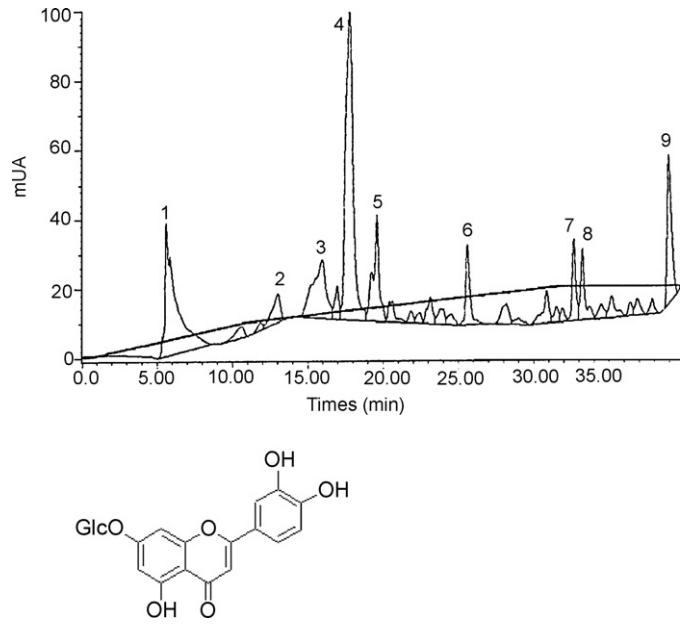

Fig. 1. Fingerprint by HPLC of standardized extract of *Sechium edule* leaves. The peak 4 was identified as luteolin-7-*O*-glucoside.

Table 1
Fenotype of clinical isolates.

Bacterial strain	Fenotype of clinical isolates
E. coli	
347	Lvx^s Cro^s Ctx^s Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^s Fep^r Amk^r
348	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^s
350	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^r
301	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^r
306	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^s Fep^r Amk^s
324	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^r Amk^r
330	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^r
331	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^r
333	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^r Amk^r
335	Lvx^r Cro^r Ctx^s Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^s
337	Lvx^s Cro^s Ctx^s Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^s Amk^r
345	Lvx^r Cro^r Ctx^s Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^r
E. cloacae	
302	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^s Amk^r
308	Lvx Cro Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^r
326	Lvx^s Cro^s Ctx^s Cxm^r Sam^s Mem^r Tzp^s Ipm^s Caz^s Fep^s Amk^s
349	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tz p^r Ipm^s Caz^r Fep^r Amk^r
307	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^r
309	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^r
327	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^s Amk^r
328	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^s Amk^s
338	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^r Amk^r
P. mirabilis	
304	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^r Amk^r
319	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^s Fep^r Amk^r
332	Lvx^s Cro^s Ctx^s Cxm^s Sam^s Mem^s Tzp^s Ipm^s Caz^s Fep^s Amk^r
342	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^s Fep^r Amk^r
359	Lvx^r Cxm^r Sam^r
361	Clinical $isolate^s$
K. pneumoniae	
310	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^r Amk^r
321	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^r
325	Lvx^s Cro^s Ctx^s Cxm^s Sam^s Mem^s Tzp^s Ipm^s Caz^s Fep^s Amk^s
336	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^r Amk^r
364	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^s Fep^r Amk^r
365	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^r Amk^r
M. morganii	
320	Lvx^s Cro^s Ctx^s Cxm^s Sam^r Mem^r Tzp^s Ipm^s Caz^s Fep^s Amk^r
347	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^s Amk^s
S. marcenscens	
313	Lvx^r Cro^s Ctx^s Cxm^s Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^s Amk^r
356	Lvx^s Cro^s $Ctx^s Cxm^r$ Sam^s Mem^s Tzp^s Ipm^s Caz^s Fep^s Amk^r
P. stuartii	
343	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^s Fep^r Amk^s
S. maltophilia	
351	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^r Amk^r
357	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^r Caz^r Fep^r Amk^r
362	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^r Caz^r Fep^r Amk^r
P. aeruginosa	
305	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^r Caz^r Fep^r Amk^r
352	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^r Amk^r
353	Lvx^r Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^r Fep^r Amk^s
354	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^s Ipm^s Caz^s Fep^s Amk^s
A. baumanii	
355	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^s Caz^r Fep^r Amk^r
P. stuartii	
375	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^r Caz^r Fep^r Amk^r
P. mirabilis	
304	Lvx^s Cro^r Ctx^r Cxm^r Sam^r Mem^r Tzp^r Ipm^r Caz^r Fep^r Amk^r

Lvx, levofloxacin; Tzp, piperazilin/tazobactam; Ipm, imipenem; Cro, ceftriaxone; Ctx, cefotaxime; Caz, ceftacidime; Cxm, cefuroxime; Fep, cefepime; Amk, amikacine, Sam, ampicillin/sulbactam; Mem, meropenem.

Table 2
MIC, MBC and MFC (μg/mL) of *Sechium* extracts against Gram negative pathogenic bacteria and fungi isolated from skin, blood or respiratory tract infections and reference strains.

		Strains			
Bacteria	*n*	MIC (T_0)	MIC (T_{12}) (μg/mL)	MBC/MFC (T_0)	MBC/MFC (T_{12})
E. coli	12	20–40	20–40	40–80	80–160
K. pneumoniae	6	20–40	40	40–80	40– 80
P. mirabilis	6	20–40	20–40	40–80	40– 80
E. cloacae	9	20–40	20–40	40–80	40 –160
S. marcenscens	2	20–40	40	40–80	80–160
M. morganii	2	20–40	40	40–80	40– 80
A. baumannii	2	20–40	40	40– 80	40– 80
P. aeruginosa	7	20–40	20–40	40–80	80–160
S. maltophilia	3	20–40	20–40	40–80	40–80
P. stuartii	1	20–40	20–40	40– 80	40– 80
E. coli ATCC 35218	1	20	20	40	40
E. coli ATCC 25922	1	20	20	40	40
P. aeruginosa ATCC27853	1	20	20	40	40
Fungi					
Candida albicans	1	20	40	40	20–40
Candida glabrata	1	320	320	640	320–640
Candida parasilopsis	1	40	40	80	40–80
Candida tropicalis	1	800	800	800	800
Candida krusei	1	80	80	160	80–160
Aspergillus spp.	1	40–80	40	80	40–80

n: Strain number, each experiment was carried out three times and twice-repeated.
The MIC and MBC or MFC were determined at different time during 1 year. T_0: MIC values at time zero; T_{12}: MIC values at 11 months of conservation of extract.

Table 3
Toxicity of *Sechium* extract towards *S. typhimurium* TA100 and TA98.

Dose (μg/plate)	Toxicity	
	Colony number (mean)	
	TA98	TA100
100	950	2195
10	1000	2153
1	1050	2100
0.1	1100	2000
Negative control	1100	2591

ranging from 20 to 40 μg/mL (Table 2) similar to those previously found against Gram positive bacteria (MIC values of 20–40 μg/mL, Ordoñez et al., 2003). The extract showed inhibitory activities on bacterial growth in a dose dependent fashion. In all cases, the MBC was always found to be similar or twofold higher than MIC values. Furthermore, the Chayote leaf extract was effective against *C. albicans*, *C. glabrata*, *C. krusei*, *C. parasilopsis*, *C. tropicalis* and *Aspergillus* spp. with MFC values of 40–800 μg/mL. *C. tropicalis* was the most resistant to *S. edule* extract (Table 2). The extract exhibited non-toxicity on both *S. typhimurium* TA98 and TA 100 strains at a dose 100 μg/plate (Table 3). Otherwise, for the testing doses, no mutagenicity was found in any samples with and without S9 mix (Table 4).

Table 4
Mutagenicity of *Sechium edule* extract towards *S. typhimurium* TA98 and TA100.

Dose (μg/plate)	Mutagenicity relation: His^+ revertant per plate[a]/His^+ spontaneous revertant[b]	
	TA98	TA100
50	0.62 ± 0.01	0.81 ± 0.03
40	0.68 ± 0.02	0.92 ± 0.02
30	0.75 ± 0.02	0.98 ± 0.03
20	0.84 ± 0.03	0.99 ± 0.04
10	0.98 ± 0.01	1.07 ± 0.03

[a] Extracts and TA98 or TA100 were preincubated at 37 °C for 20 min with or without S9 mix. Data are means ± S.D. of three plates.
[b] The number of spontaneous revertants was determined in assays without extract. The number of spontaneous revertants obtained was 41 ± 5. NPD and IQ were used as positive control. Revertants induced by IQ (0.1 μg/plate) and NPD (5 μg/plate) were 2540 ± 30 and 2343 ± 20, respectively.

Different hydrogel types were prepared using an optimal stabilizer combination of hydrogel-forming poly(acrylic acid) polymer (Carbopol® 934) and antimicrobial agent (fluid extract of *S. edule* or gentamicin sulphate or miconazol nitrate) without preservatives (Table 5). Carbopol is commonly used in cosmetic and pharmaceutical products because of its high stability, compatibility and low toxicity. The average pH was about 6 for the preparation with the active substances and was maintaining during 1 year of conservation at room temperature (Table 6). Macroscopical and microscopical examination indicated that the hydrogel formed was homogeneous in aspect during 1 year. Fig. 1 shows changes in viscosity with increasing shear rate for the hydrogel containing leaves fluid extract. Apparent viscosity was stabled maintained for 1 year. A pseudoplastic behavior and positive thixotropy were observed.

Control of microbial contamination in each pharmaceutical formulation was performed. The preparation containing leaves fluid extract was microbiologically stable for 1 year at room temperature as well as the hydrogel with commercial antibiotic/antifungal. The susceptibility test using the agar well diffusion method indicated that preparations were as active against antibiotic-multi-resistant Gram negative and Gram positive bacteria and fungi as the fluid extract of *Sechium* leaves (Fig. 2). The inhibition obtained with hydrogel containing gentamicin sulphate is similar to those obtained with chayote fluid extract against F7 and F353 clinical isolated (Fig. 2A and B). The gels containing fluid extract or miconazol

Table 5
Composition of the hydrogel added or not with the different active substances.

Components	Formulations			
	F1 (%)	F2 (%)	F3 (%)	F4 (%)
Carbopol® 934	0.47	0.47	0.47	0.47
Triethanolamine 99%	0.7	0.7	0.7	0.7
Fluid extract of *S. edule* leaves	0.24	–	–	–
Gentamicin sulphate	–	0.1	–	–
Miconazol nitrate	–	–	2	–

F1 is hydrogel containing fluid extract of *Sechium edule*, F2 hydrogel containing gentamicin sulphate, F3 hydrogel containing miconazol nitrate, F4 base hydrogel.
Each experiment was carried out three times and twice-repeated.

Table 6
Physical properties of pharmaceutical formulations at time zero (T_0) and after 1 year of conservation at 30 °C (T_{12}).

Physical properties	Formulation							
	F1		F2		F3		F4	
	T_0	T_{12}	T_0	T_{12}	T_0	T_{12}	T_0	T_{12}
pH	6.0 ± 0.5	6.0 ± 0.5	6.0 ± 0.4	6.0 ± 0.5	6.0 ± 0.2	6.0 ± 0.5	6.0 ± 0.2	6.0 ± 0.5
Temperature resistant	Unstable	Unstable	Stable	Stable	Stable	Stable	Stable	Stable
Centrifugation stability	Stable	Unstable	Stable	Stable	Stable	Stable	Stable	Stable
Extensibility (cm^2)	715.14	700.00	720.12	740.00	720.09	740.09	718.02	758.02
Viscosity (mPa s)	20113	19500	13200	13200	17560	17060	16956	16000
Loss of water	–	1.80%	0.10%	0.10%	0.35%	0.35%	0.32%	0.32%

F1 is hydrogel base, F2 hydrogel gel containing fluid extract, F3 hydrogel containing gentamicin sulphate, F4 hydrogel containing miconazol nitrate. Each experiment was carried out three times and twice-repeated.

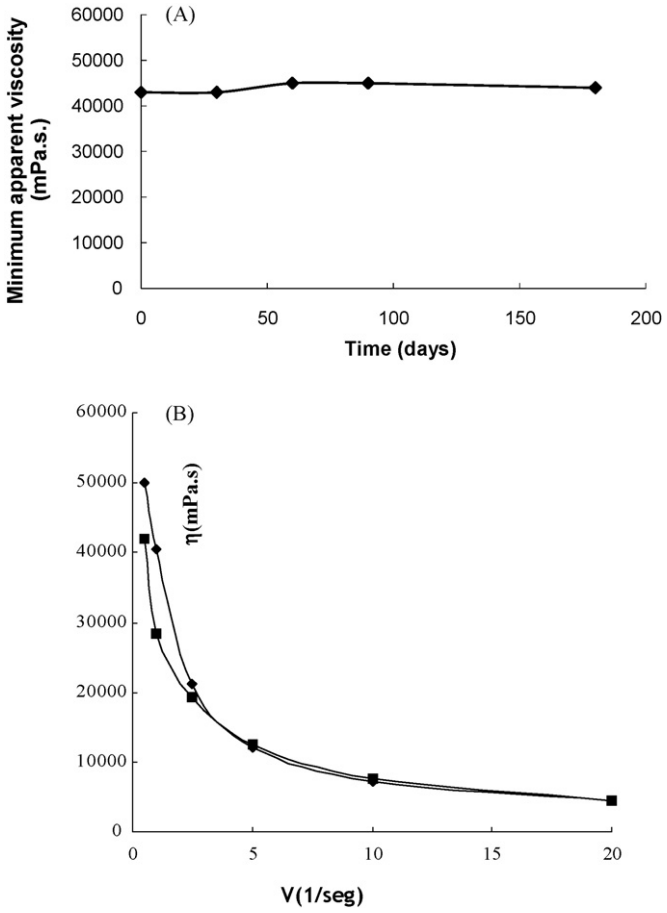

Fig. 2. Apparent viscosity values of hydrogel containing *Sechium* fluid extract stored at 25 °C during six months (A), at different shear rate, in the range 0.05–20 r.p.m. to obtain the ascending curve and 20–0.05 rpm to obtain the descending curve (B).

nitrate inhibited *C. albicans* and *Aspergillus* spp. growth at the same concentration as that of the soluble principles (Fig. 2C and D).

The MIC values obtained for the *Sechium* hydrogel were similar to those previously found for the *Sechium* extract fresh (40–80 μg/mL).

The *S. edule* hydrogel preparation inoculated with methicillin resistant *Staphylococcus aureus* (F7) and *P. aeruginosa* multiresistant (F353) clinical isolate inhibited the growth of them in 24 h (Table 7). The microbial stability was conserved until 1 year.

The antioxidant capacity of the topical formulation containing *S. edule* fluid extract was similar to the one determined for fluid extract (Ordoñez et al., 2006) (Figs. 3 and 4). The pharmaceutical formulation has showed inhibitory activity of lipoperoxidation at 20 μg/mL and scavenging activity of DPPH with scavenging concentration of 50% free radical (SC_{50}) values of 4 μg/mL (Fig. 5).

4. Discussion

In a previous work we reported antioxidant properties (Ordoñez et al., 2006) and antibacterial activity of *S. edule* extracts against antibiotic resistant Gram positive cocci species (Ordoñez et al., 2003). Most antibacterial medicinal plants attack Gram positive strains while few are active against Gram negative bacteria and fungi (Herrera et al., 1996; Meng et al., 2000; Scrinivasan et al., 2001, Arias et al., 2004, Zampini et al., 2005). Interestingly, our current finding shows a remarkable antimicrobial activity of *S. edule* on a large range of Gram negative antibiotic-resistant bacteria and fungi clinical isolates. Antimicrobial action was highly reproducible and the responsible compounds appeared to be chemically stable since the crude extract was tested for 1 year without loss of activity. The quantitative analysis of phenolic compounds demonstrated that the flavonoids represent a high percentage of this type of compounds. Recently, by LC-PDA-MS studies, Siciliano et al. (2004) has isolated eight flavonoids (vicenin-2, apigenin-6-C-β-D-glucopyranosyl-8-C-β-D-apiofuranoside, vitexin, luteolin-7-*O*-rutinoside, luteolin-7-*O*-β-D-glucopyranoside, apigenin-7-*O*-rutinoside, chrysoeriol-7-*O*-rutinoside, and diosmetin-7-*O*-rutinoside) in aerial parts of *S. edule*. In this work, we found that extracts are rich in flavonoids

Table 7
Antimicrobial activity of hydrogels against *Staphylococcus aureus* (F7) and *Pseudomonas aeruginosa* (F353).

												CFU/g
	0 h		24 h		48 h		72 *h*		96 *h*		120 *h*	
	F7[a]	F353[a]	F7[a]	F353[a]	F7[a]	F353[a]	F7[a]	F353[a]	F7[a]	F353[a]	F7[a]	F353[a]
Formulation 1	573	552	1320	1452	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^5$	10^5
Formulation 2	571	551	80	65	0	0	0	0	0	0	0	0
Formulation 3	0	0	0	0	0	0	0	0	0	0	0	0

Hydrogel preparations (1 g) were inoculated with 10^6 CFU of *Staphylococcus aureus* (clinical isolate Met^r Oxa^r Gen^r, F7) or *Pseudomonas aeruginosa* (isolate clinical meropenem resistant, F353). Aliquots were taken at different times. Then, the number of CFU was determined every 15 days for 1 year (Farmacopea Española, 1999). F1 is hydrogel base; F2 hydrogel containing fluid extract; F3 hydrogel containing gentamicin sulphate. Each experiment was carried out three times and twice-repeated.

[a] Microorganisms.

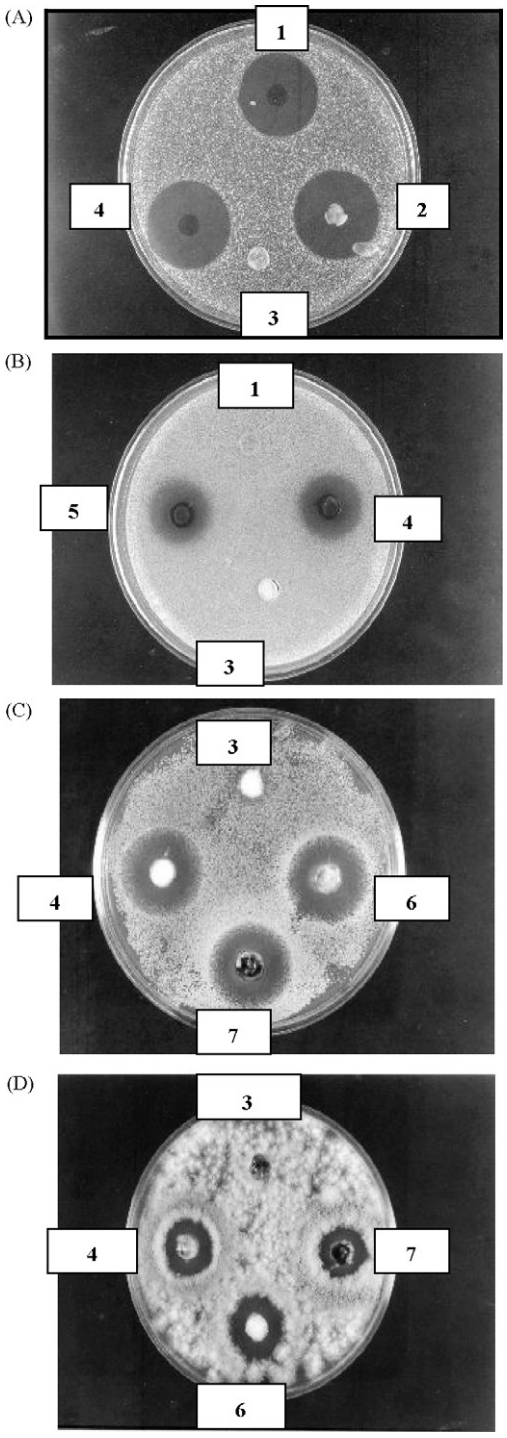

Fig. 3. Antimicrobial activity of *Sechium edule* fluid extract and gel formulation (A) *Staphylococcus aureus* (1) gentamicin sulphate solution, (2) hydrogel containing gentamicin sulphate, (3) hydrogel base, and (4) hydrogel containing fluid extract; (B) *Pseudomonas aeruginosa* (1) hydrogel containing gentamicin sulphate, (4) hydrogel containing fluid extract, (3) hydrogel base, (5) fluid extract (C) *Candida albicans* (3) hydrogel base, (6) hydrogel containing miconazol nitrate, (4) hydrogel containing fluid extract, (7) miconazol nitrate solution; (D) *Aspergillus* spp.; (3) hydrogel base, (4) hydrogel containing fluid extract, (6) hydrogel containing miconazol nitrate, (7) miconazol nitrate solution.

and demonstrated that 20 μg of total flavonoid may be sufficient to produce an inhibitory effect on the growth of pathogenic agents. For its antimicrobial activity and antioxidant properties, *S. edule* fluid extract could be used to prepare a pharmaceutical formulation against skin infections. The hydrogel containing

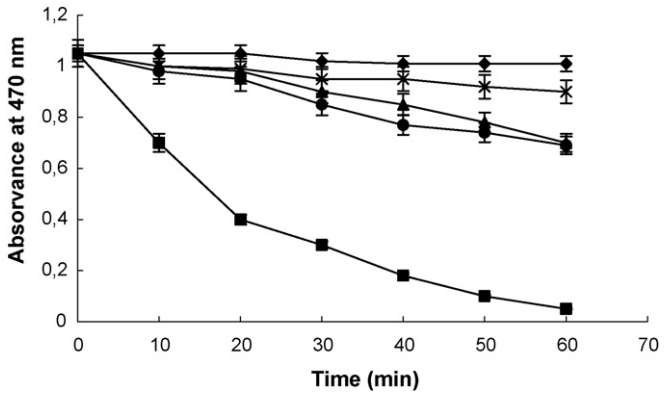

Fig. 4. Antioxidant activity of leaf fluid extract of *Sechium edule* in the β-carotene-linoleic acid system at different incubation times (*), gel formulation containing fluid extract recently prepared (▲) and after six month maintained at room temperature (●). BHT was used at the final concentration of 50 μg/mL (♦), negative control (■). Measurements were carried out in triplicate.

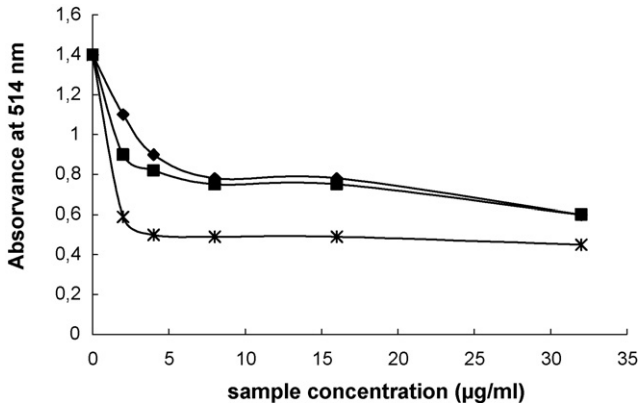

Fig. 5. DPPH radical scavenging activities of *Sechium edule* extract and gel formulation. Fluid extract (*), hydrogel formulation containing fluid extract recently prepared (■) and hydrogel formulation containing fluid extract after six month maintained at room temperature (♦). Measurements were carried out in triplicate.

Sechium leaves fluid extract showed antibacterial and antifungal effect on strains isolated from clinical samples from hospitals of Tucumán, Argentina. The activity was similar to the effect observed when the extract was assayed without bioadhesive polymers in the formulation. Microbiological, physical and functional stability of the formulation were observed for 1 year at room temperature. Addition of antioxidant preservatives to store the pharmaceutical formulation was not necessary. The results obtained show that antioxidant and antimicrobial compounds could be released from the hydrogel. The semisolid formulation is a promising topical antibacterial and antimycotic agent. Actually, other assays are being carried out in order to evaluate their effect *in vivo* and have a complete understanding of this action mechanism.

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