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Antifungal Activity of the Aqueous Extract of *Ilex paraguariensis* Against *Malassezia furfur*

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Malassezia furfur **is a lipodependent, dimorphic and saprophyte fungus which causes pityriasis versicolor, dandruff and seborrheic dermatitis in humans. The drugs available to treat this fungal infection are few. These drugs are highly toxic and are costly when used in prolonged treatments. For these reasons, it is necessary to find new compounds to treat these infections.** ***Ilex paraguariensis*** **St Hilaire is a plant that grows in Argentina, Brazil and Paraguay. The aim of this study was to evaluate the effect of the aqueous extract of** ***Ilex paraguariensis*** **on the growth of** ***M. furfur.*** **High performance liquid chromatography (HPLC) was employed to identify and isolate compounds of** ***I. paraguariensis*** **and the agar-well diffusion method was used to assess the antifungal activity of the extract. The fungicidal/fungistatic effect was evaluated by the modified Thompson assay. The results demonstrated that the aqueous extract of** ***Ilex paraguariensis*** **(1000 mg/ml) possesses inhibitory activity against** ***M. furfur*****. This anti*malassezial*** **activity was equivalent to 2.7 μg/ml of ketoconazole. Therefore, the topical use of** ***Ilex paraguariensis*** **extract as alternative antifungal agent can be suggested. Copyright © 2009 John Wiley & Sons, Ltd.**

Keywords: *Ilex paraguariensis*; *Malassezia furfur*; antifungal activity; pityriasis versicolor; psoriasis; dandruff.

INTRODUCTION

Antimicrobials of plant origin have enormous therapeutic potential (Al-Bakri and Afifi, 2007) as antifungal, antiprotozoal, antihelminthic and antiviral (Carson, 1987; Block, 1992; Bruneton, 1995; Hammer *et al.,* 2000; Davicino *et al.,* 2007). Some antimicrobial plants are employed as popular folk medicines, whereas others have gained popularity in the form of finished products. (Shams-Ghahfarokhi *et al.,* 2006)

Ilex paraguariensis St Hilaire (Aquifoliaceae) is a medium-to-large tree which grows naturally in north-eastern area of Argentina, southern Brazil and eastern Paraguay, where it is also cultivated (Giberti, 1989). *I. paraguariensis* has been demonstrated to have high levels of caffeine and theobromine (Filip *et al.,* 1998).

Malassezia furfur is a commensally lipodependent and dimorphic fungus belonging to the human skin microbiota (Silva *et al.,* 1997; Salah *et al.,* 2005; Morishita *et al.,* 2006). This fungus is found mainly in the seborrheic areas as the upper trunk and head (Roberts, 1969; Silva *et al.,* 1996). *M. furfur* has been associated with a variety of cutaneous (McGinley *et al.,* 1970; Ford *et al.,* 1982; Faergemann, 1990; Jensen Jarolim *et al.,* 1992; Squiquera *et al.,* 1994; Silva, 1995; Bulmer and Bulmer, 1999) and systemic diseases, such as, peritonitis, mixed bacterial-fungal septic arthritis, dacryocystitis, mastitis and chronic postoperative sinusitis among others (Marcon and Powell, 1992). There is a scarce-drugs spectrum in order to treat such illnesses, mainly, topical antifungal solutions containing 2% ketoconazole. These drugs are highly toxic (Martinez Fernandez *et al.,* 1998) and costly when used in prolonged treatments (Rodriguez Silva, 1990). Ketoconazole is a synthetic antifungal imidazolic drug (Hammer *et al.,* 2000) which is prescribed for skin infections (Urcuyo and Zaias, 1982; Savin, 1984; Ford *et al.,* 1984; Gupta *et al.*, 2004). Ketoconazole is very lipophilic, which accumulates in fatty tissues leading to important side effects when administered orally. The undesired effects include severe toxic hepatitis, acquired cutaneous adherence (Svedhem, 1984; Knight *et al.*, 1991; Polsen *et al.*, 1995), vomiting, loss of appetite, rash, pruritus, menstrual irregularities, gynecomastia and decreased libido. This drug is not recommended during pregnancy or lactation (Flores, 2004). When used in the topical form, Di Fonzo *et al.* (2008) has found an urticant effect of this antifungal compound (Di Fonzo *et al.,* 2008). Other topical antifungal agents such as clotrimazole, miconazole or terbinafine are less recommended due to recurrence (Giusiano, 2006).

Taking these data into account, the aim of this study was to evaluate the effect of the aqueous extract of *Ilex paraguariensis* on the growth of *M. furfur.*

MATERIAL AND METHODS

Plant material. Leaves of *I. paraguariensis* before industrial processing were used. The sample was obtained directly from a commercial company which cultivates and industrializes yerba mate in the province of Corrientes, Argentina. For the identification, the sample was compared with a voucher-specimen from herbarium standards.

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Preparation of plant extract. Dried leaves were ground to fine powder. The Decoction extract (Dec) was prepared according to the Farmacopea Nacional Argentina (1978) to obtain a similar preparation to those used by local people. The vegetal product (10 g) was boiled with 200 ml of water for 20 min and left to cool at room temperature to 40–45 º C. After filtration though a Nº 1 Whatman filter paper, the extract was lyophilized using a flexi-dry FTS Systems, yielding an aqueous crude extracts residue of 3.22 g.

High performance liquid chromatography (HPLC). Validated methods for the determination of methylxanthines (Filip *et al.,* 1998), caffeoyl derivative compounds and flavonoids (Filip *et al.,* 2001) were applied. The identification of compounds was carried out using a photodiode array UV detector.

Standard compounds. Carl Roth caffeine, theobromine, chlorogenic acid, caffeic acid, 1,5-dicaffeoylquinic acid (cynarin) and rutin (Sigma, San Diego, CA, USA) were employed as standards. The amount of 3,4-dicaffeoylquinic, 3,5-dicaffeoylquinic and 4,5-dicaffeoylquinic isomer acids were calculated and expressed as cynarin.

***Malassezia* culture and identification.** *Malassezia* was obtained from the Micology Departament, National Institute of Infectiuos diseases (INEI), National Administration of Laboratories and Health Institutes Dr Carlos Malbrán (ANLIS), Av. Vélez Sarsfiedl 563, Buenos Aires, Argentina. The *Malassezia furfur* strain Nº 8 was used. This strain has been isolated from patients with pytiriasis versicolor and identified by Lic. María Eugenia Bosco Borgeat. The strain was cultured in modified Dixon's agar (3.6% malt extract agar, 0.6% peptone, 2% ox bile, 1.0% Tween 40, 0.5% glycerol and 2.0% oleic acid) at 32ºC. Identification was performed according to the method of Guillot *et al.* (1996).

Determination of the antifungal activity. To assess the antifungal activity of Dec, the agar-well difusion method was used (Anesini and Perez, 1993). Modified Dixon's agar was utilized as a culture medium. Fourteen milliliters of Dixon medium were inoculated with 1 ml of a suspension of *M. furfur* (10^5 CFU/ml in phosphate-buffered saline (PBS) 1 X) in Petri dishes. Inoculums suspensions were previously adjusted by spectrophotometry (Microplate Reader, Model 450, BioRad, Hercules, CA, USA) to an absorbance of 0.1 at 530 nm. Wells of 10 mm in diameter were punched into the agar and filled with Dec (125 mg/ml, 250 mg/ml, 500 mg/ml, and 1000 mg/ml). Solvent blank (PBS 1 X) or standard solutions of Ketoconazole (0.08 µg/ml, 0.32 µg/ml, 1.28 µg/ml, 5.12 µg/ml and 20 µg/ml) were used. The antibiotic standard was obtained from Jansen-Cilag Farmaceutica S.A, Buenos Aires, Argentina.

The antifungal activity was evaluated by measuring the inhibition-zone diameter observed **after** 48 h **of incubation**. The mean values were interpolated **in** a reference concentration-response curve of ketoconazole.

Treatment of *Malassezia furfur* with Dec. Fourteen milliliters of Dixon's agar were inoculated with 1 ml of a suspension of *M. furfur* (10^5 CFU/ml) in Petri dishes. Wells of 10 mm in diameter were punched into the agar. After 48 h incubation (Fig. 2A), wells were filled with Dec (1000 mg/ml), PBS 1 X or ketoconazole (5.12 µg/ml) once each 24 h over 9 days.

Fungal toxicity. To detect the fungicidal/fungistatic nature of Dec, the technique described by Thompson (1989) was employed with slight modifications. Briefly, treated, untreated and ketoconazole-treated fungal colonies were reinoculated each 48 h, onto the fresh medium and the revival of fungal growth was recorded. After 48 h of each reinoculation the growth of fungi was measured and the percentage (%) of inhibition was computed after comparison with the controls (PBS 1 X and ketoconazole).

Statistical analysis. Data were analyzed by the Student's *t*-test, one way ANOVA and Dunnett`s test. Differences were considered significant when $p \leq 0.05$.

RESULTS

Reference curve

A linear regression analysis was applied to each experiment. A good fitting of data to a linear function (antimicrobial activity vs. logarithm of the concentration) could be achieved in the case of ketoconazole (slope: 1.43 ± 0.140 mm/log ug/ml; origin ordinate: 1.18 ± 0.120 mm; correlation coefficient: 0.927–0.995). Therefore, these curves were used to interpolate values of the samples (Fig. 1A).

Determination of antifungal activity

Antifungal activity of Dec was found when the extract was assayed at 1000 mg/ml (Table 1A and1B and Fig. 2B). Table 1A and Fig. 1B show the inhibitory effects of ketoconazol on *M. furfur*. The results of the treatment of *M. furfur* with 1000 mg/ml of extract are summarized in Table 2A and Fig. 3. Ketoconazole was used at 5.12 µg/ml for comparison with the extract, because the inhibitory activity of the extract was equivalent to 2.7 µg/ml of ketoconazole.

HPLC analysis

The results of HPLC analysis are summarized in Table 2B.

DISCUSSION

The increasing number of in micro-organisms that have developed resistance to currently available antimicrobial agents has become a major cause of the spread of infections. A major problem in the management of infections caused by such organisms is the paucity of new drugs (Finch and Hunter, 2006). Reports indicating that drug resistance is increasing worldwide have presented a scientific challenge and an economic opportunity for the pharmaceutical industry to develop new antimicrobial agents (Liss and Batchelor, 1987). The

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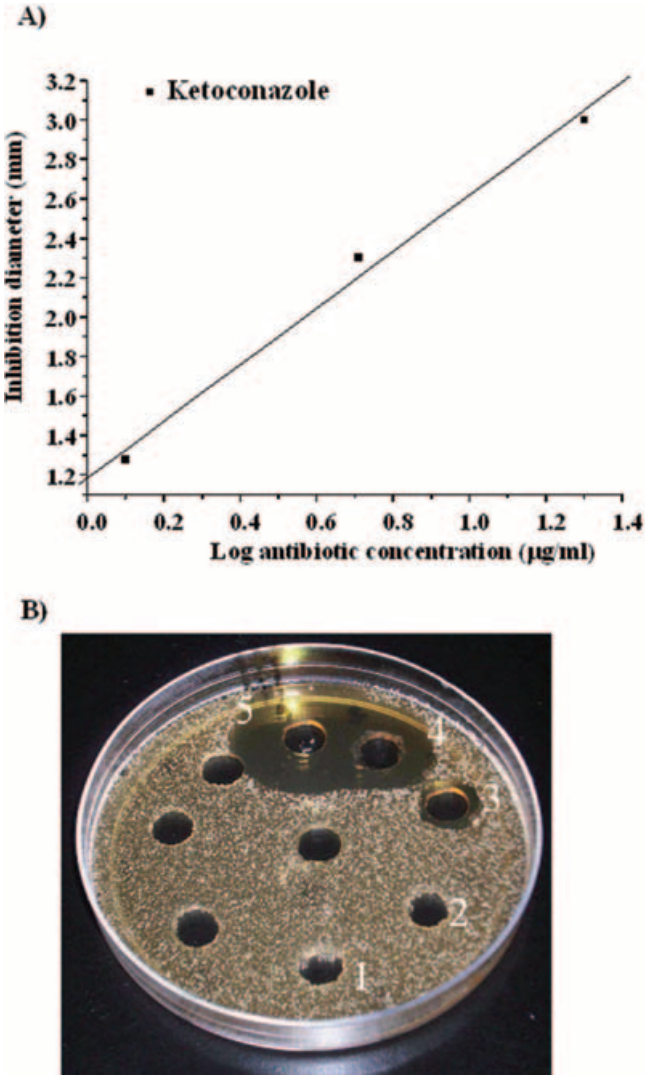

Figure 1. (A) Inhibition curve of the reference antibiotic (ketoconazole). Inhibition diameter is expressed in millimeters. Data represent the means of two experiments, each performed by duplicate. (B) Diameter of inhibition zones of ketoconazole at different concentrations (1) 0.08 µg/ml, (2) 0.32 µg/ml, (3) 1.28 µg/ml, (4) 5.12 µg/ml and (5) 20 µg/ml) on *M. furfur* growth. Results are a representative of two separate experiments.

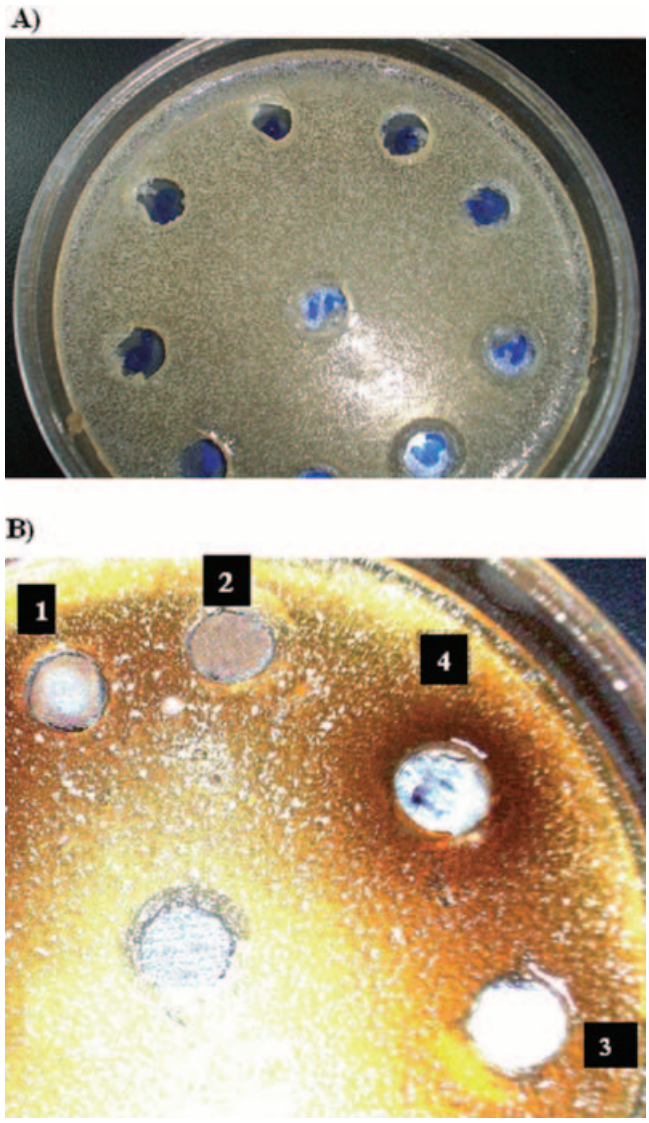

Figure 2. (A) *M. furfur* (10^5 CFU/ml) incubated during 48 h in Petri dishes without any treatment. (B) Diameter of inhibition zones of Dec at different concentrations (1) 125 mg/ml, (2) 250 mg/ml, (3) 500 mg/ml, (4) 1000 mg/ml. (5) PBS on *M. furfur* growth. Wells of 10 mm in diameter were punched into the agar. Results are representative of two separate experiments.

Table 1. A. Antimicrobial activity of Dec and ketoconazol against *M. furfur* (diamater of inhibition zone in mm)*

	Dec (mg/ml)				Ketoconazole (µg/ml)				
	125	250	500	1000	0.08	0.32	1.28	5.12	20
M. furfur	–	–	–	18.5 ± 2	–	–	13 ± 2	22.9 ± 2.6	29.9 ± 3.2

*The values are the mean of two experiments ± S.E. M. – No inhibition zone.

B. Antimicrobial equivalence of Dec and ketoconazol*

Diamater of inhibition zone (mm) of Dec (1000 mg/ml)	Antimicrobial equivalence with respect to ketoconazole (µg/ml)
18.5 ± 2.1	2.7 ± 0.5

*Data are means ± SEM calculated from two experiments. **Equivalence** was calculated by interpolation **in** the **corresponding** curve **depicted** in Fig. 1A.

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Table 2. A. Fungicidal/Fungistatic activity of Dec agaisnt *M. Furfur

Fungal toxicity of Dec (% of growth inhibition with respect to control)					
Days	1	3	5	7	9
PBS	0	0	0	0	0
Ketoconazole	87.7 ± 8	89 ± 8.2	87 ± 7.2	82.5 ± 8.8	98.84 ± 9.3
Dec	50.4 ± 4.3**	52.2 ± 6**	50.2 ± 5.3**	40.5 ± 3.9**	86.25 ± 8.7

* Data are means ± SEM calculated from two separate experiments.

** $p < 0.05$ Dec vs ketoconazol.

B. Cafeoyl derivatives compounds, metilxanthine and rutin content in *I. paraguariensis*

Compound %	Green leaves
Chlorogenic acid	1,84 ± 0,04
Caffeic acid	0,033 ± 0,002
3, 4–DCQ*	0,71 ± 0,01
3, 5 DCQ*	1,57 ± 0,01
4, 5 DCQ*	1,86 ± 0,02
Caffeine	0,91 ± 0,03
Theobromine	0,40 ± 0,01
Rutin	0,98 ± 0,05

The results shown represent the mean ± SEM of three independent experiments carried out by duplicate and are expressed as equivalents of compound (g/100 g dried plant material). DCQ: dicafeoylquinic acid. * calculated and expressed as cynarin.

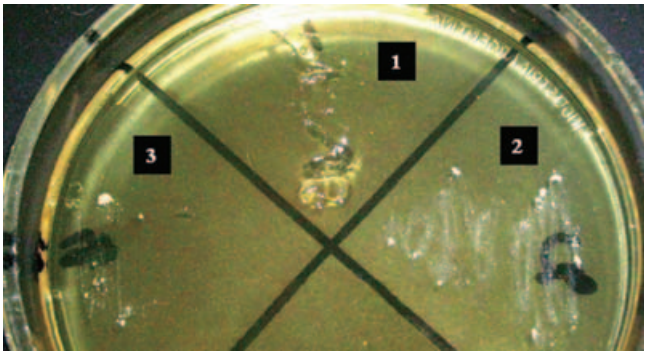

Figure 3. *M. furfur* growth after treatment with: (1) ketoconazole at 5.12 μg/ml, (2) PBS and (3) Dec (1000 mg/ml) for 9 days. Results are a representative of two separate experiments.

use of standard antifungal therapies is limited because of their toxicity, low efficacy, and drug resistance (Liss and Batchelor, 1987). These antifungals are costly when an extended treatment time is necessary; therefore, many patients leave therapy before being cured (Rodriguez Silva, 1990). One way to prevent antibiotic resistance of pathogenic species is to use new compounds with chemical structures that are not based on existing synthetic antimicrobial agents (Shah, 2005).

The processed *Ilex paraguariensis* ('mate' or 'yerba mate') is a typical beverage prepared as a hot infusion of the dried and minced leaves. The habit of drinking mate, which has centuries of usage and has remained unchanged, is widely extended all over Argentina, Paraguay, Uruguay and Brazil. This plant is also used in popular medicine and employed in commercial herbal preparations as a natural medicine for arthritis, headache, constipation, rheumatism, haemorrhoids, obesity, fatigue, fluid retention, hypertension, slow digestion and hepatic disorders, among others. Pharmacological studies have reported stimulant, diuretic (Gonzalez *et al.,* 1993) and antioxidant effects (Gugliucci, 1996). Externally and topically, this plant is used to cure conjunctivitis, various skin ulcers and as a cicatrizing on gangrenous wounds (Bajaj, 1993). *I. paraguariensis* has no significant toxic effects and has been included for their nutritional and medicinal value in important national food codes such as the Argentine Food Code, the LatinAmerican Food Code and Pharmacopoeias such as Martindale, British Herbal Pharmacopoeia, and German Commission E Monographies (Anesini *et al.,* 2006). In this work, we demonstrated that the aqueous extract of *Ilex paraguariensis* had inhibitory activity against *M. furfur* at 1000 mg/ml. This activity was equivalent to 2.7 μg/ml of ketoconazole (Fig. 2B and Table 1). These results showed that after 9 days of treatment *I. paraguariensis* was able to induce an inhibition comparable to ketoconazol (Fig. 3 and Table 2A). To date, there are no works about the antimicrobial activity of *Ilex paraguariensis* on *M. furfur*. Vanessa Müller *et al.* (2007) have demonstrated antiviral activity of *Ilex paraguariensis* against HSV-1.

We believe that the anti*malassezial* activity observed is due to some compound present in the extract (Table 2B). The treatment period against *Malassezia* recommended ranges from 5 to 30 days (Janssen-Cilag, S.A., Lab. Grupo Ferrer). For this reason we treated the colonies for 9 days. The results obtained so far seem to demonstrate that the aqueous extract of *I. paraguariensis* has a marked antifungal activity against *M. furfur* and that it may be used to treat (Fig. 3 and Table 2A) and to prevent *M. furfur* infections. Finally, we can postulate the topical use of *Ilex paraguariensis* extract at least 1000 mg/ml in the present form or as a pharmaceutical preparation against *M. furfur*, or as a possible alternative therapeutic agent destined to prevent and treat illnesses, such as pytiriasis versicolor, seborrheic dermatitis, dandruff, folliculitis, atopic dermatitis, psoriasis and catheter-related systemic infections in humans and in animals.

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