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Comparison of antioxidant activities and total polyphenolic and methylxanthine contents between the unripe fruit and leaves of *Ilex paraguariensis* A. St. Hil.

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Ilex paraguariensis is used in Brazil as a stimulating beverage called "mate". Leaves and immature fruit extracts of *Ilex paraguariensis* were evaluated for their radical scavenging capacity, total methylxanthine and polyphenol contents. Antimicrobial activity of two enriched saponin fractions obtained from the fruits were also evaluated. The radical scavenging activity of the fractioned extracts was determined spectrophotometrically using 1,1-diphenylpicrylhydrazyl free radical (DPPH). The IC_{50} of L-ascorbic acid, ethyl acetate and *n*-butanol fractions from the leaves and ethyl acetate fraction from the fruits were 6.48 μg/mL, 13.26 μg/mL, 27.22 μg/mL, and 285.78 μg/mL, respectively. Total methylxanthine content was 1.16 ± 0.06 mg/g dry weight in the fruits and 8.78 ± 0.01 mg/g in the leaves. Total polyphenol content varied from $86.82 \pm 3 \times 10^{-4}$ to $199.91 \pm 3 \times 10^{-3}$ mg/g in leaf fractions and from $54.25 \pm 1 \times 10^{-3}$ to $110.36 \pm 4 \times 10^{-4}$ mg/g in fruit fractions. Enriched saponin fractions from the fruits showed no antimicrobial activity. To our knowledge, this are the first data available on the antioxidant/antimicrobial activities and polyphenol/methylxanthine contents of *Ilex paraguariensis* fruits.

1. Introduction

Ilex paraguariensis A. St. Hil (Aquifoliaceae), a perennial tree that grows in Southern Brazil, Paraguay and Argentina, is the source of a stimulating drink called "mate", prepared as a hot infusion of the dried leaves and stemlets. Infusions prepared with cold water are called "tererê". *Ilex paraguariensis* is also cultivated in Argentina, Brazil and Paraguay and is an important agricultural product. The stimulating properties of the beverage are due to the presence of the methylxanthines, caffeine and theobromine (Reginatto et al. 1999; Athayde et al. 2000; Coelho et al. 2001), caffeine being the most important xanthine in respect to the stimulating effect on the Central Nervous System (Sawynok 1995). Besides methylxanthines, *Ilex paraguariensis* leaves also contain high amounts of triterpene saponins with ursolic and oleanolic aglicones (Gosmann et al. 1995; Kraemer et al. 1996; Schenkel et al. 1996) polyphenols, such as chlorogenic acids (Clifford and Ramirez-Martinez 1990), flavonoids (Filip et al. 2001), and several other vitamins such as B_1, B_2 riboflavin, ascorbic and pantotenic acids, and β-carotene. *Ilex paraguariensis* leaves are also used in popular medicine in South America for the treatment of slow digestion, liver disease, arthritis, rheumatism and obesity. Choleretic, hypocholesterolemic and bitter taste properties of "mate" could be attributed to the constituents of the leaves, mainly polyphenols, methylxanthines and saponins. Polyphenols belong to the category of natural antioxidants and are the most abundant antioxidants in our diet. Saponins have been investigated with respect to their biological activities, including hypocholesterolemic (Ferreira et al. 1997), antifungal and antimicrobial (Pistelli et al. 2002) among other important properties. In Europe, the extract of *Ilex paraguariensis* leaves is also used in dietetic food supplements, such as stimulants and as weight loss treatments. Nowadays, there is a great interest in the biological properties of "mate", including the antioxidant effects (Gugliucci 1996; Schinella et al. 2000). *Ilex paraguariensis* fruits are found from December to February (summer in Brazil). The fruit is small and spherical with an average diameter of 5 mm and it can be green, red or wine colour depending on its maturation stage. Unripe or immature fruits are pale green and they are also called "white fruits" by the farmers, whereas mature or ripe fruits are red/wine colored. Brazilian legislation forbids the inclusion of the fruits in the commercial "mate" product (Ministério da Saúde 2005) However, during summer, a significant amount of fruits will be processed together with the leaves. This procedure modifies not only the chemical composition of the drink, but also alters the taste, causing as a result a negative effect on consumer acceptance. It is important to note that there are few studies concerning the fruit composition (Athayde 2000; Taketa et al. 2004) and

biological properties. Recently, Taketa et al. (2004) isolated 6 triterpenes and triterpenoidal saponins from the fruits of *I. paraguariensis* and the bitterness of two compounds was evaluated and compared with the saponins from leaves of *Ilex paraguariensis* and other *Ilex* species. From the legislative point of view, it is prohibited to include the fruits in the commercial product. Thus, the fruits must be considered as a waste in the process of obtaining the genuine "mate" product. Alternatively, it would be very significant to find a destination for this material, other than its use for seeds, to increase commercial value of *Ilex* cultivation for the farmers. In the present study, we report the investigation of antimicrobial activity of two saponin enriched fractions obtained from the unripe fruits and the assessment of antioxidant activities and polyphenol content of leaf and fruit fractions. Considering that the fruits are rich in triterpenoidal saponins, we hypothesize that the capacity of saponins to lyse cell membranes may be relevant as a defence mechanism of the fruit against fungi and bacteria. In order to evaluate the possible contribution of the fruits to the methylxanthine content of commercial "mate", we measured the methylxanthine content in fruits and leaves, separately. Antioxidant activity was chiefly focused on due to the currently growing demand from the pharmaceutical industry for natural antiaging and anticarcinogenic bioactive compounds that possess health benefits. Moreover, plants are known to offer excellent perspectives for the discovery of new therapeutic products, including antimicrobial agents. To our knowledge, there is no information available regarding the antimicrobial activity of the saponins fractions from the fruits or the antioxidant activities and polyphenol content of fractioned extracts of *Ilex paraguariensis* leaves and fruits.

2. Investigations, results and discussion

The free radical scavenging effect of the extracts was assessed by the decoloration of the ethanolic solution of 1,1-diphenylpicrylhydrazyl free radical (DPPH) according to a slightly modified method from that described by Choi et al. (2002). DPPH solutions show a strong absorption band at 518 nm appearing as a deep violet colour. In the presence of an active radical scavenger, the absorption vanishes and the resulting decolourization is stoichiometric at a selected range with respect to the degree of reduction. The remaining DPPH, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant, i.e., the degree of decolouration indicates the free radical scavenging efficiency of the fractions (Kulisic et al. 2004). An ethanolic solution of DPPH served as a control and a calibration curve made with L-ascorbic acid was used to compare the activities, as a positive control, since ascorbic acid antioxidant activity is well established. The chloroformic fraction of the leaves and butanolic fraction of the immature fruits showed very weak antioxidant activities and therefore, the IC_{50} for these fractions could not be calculated under our test conditions. This fact was somewhat expected, since the chromatographic analysis of these fractions indicated the lack of phenolic compounds and, therefore, these fractions were considered a poor source of antioxidants (TLC analysis, data not shown). The ethyl acetate fraction from the fruits showed weak antioxidant activity: $IC_{50} = 285.78$ μg/mL. In contrast, the values of the IC_{50} of L-ascorbic acid, the ethyl acetate fraction and the butanolic fraction from the leaves were 6.48 μg/mL ($y = 2.1225x + 36.234$, $r = 0.89$), 13.26 μg/mL ($y = 1.7482x + 26.806$, $r = 0.90$) and 27.22 μg/mL ($y = 2.2036x - 9.9838$, $r = 0.99$), respectively. These low IC_{50} values indicate that strongly antioxidant compounds were present in the ethyl acetate fraction followed by the butanolic fraction from the leaves. Values of IP% obtained at the different concentrations are shown in the Fig. The ethyl acetate fraction possessed a strong DPPH free radical scavenging activity similar to ascorbic acid activity. At the concentration of 250 μg/mL, ascorbic acid exhibited free radical-scavenging activity of 98.30 % and the ethyl acetate fraction afforded 93.83% at the same concentration. The butanolic fraction showed good activity (IP = 78.34%) when compared with the ethyl acetate fraction or L-ascorbic acid at the same concentration. Similar antioxidant activities were also obtained by Turkmen et al. (2006) in "mate" tea. IC_{50} and IP values are considered to be a good measure of the antioxidant efficiency of pure compounds and extracts, but it is not clear what criteria are used by numerous authors to assign the free radical scavenger activities as high, good, moderate, weak or poor, based on comparison with known radical scavengers profiles. Concerning *Ilex paraguariensis*, the ethyl acetate and *n*-butanol fractions from the leaves were the most appropriate sources of antioxidant compounds as shown by their inhibition concentration (IC_{50}) and inhibition percentage (IP%) values. However, none of the fractions were more effective at scavenging the DPPH radical in this assay than the positive control ascorbic acid at the same concentrations. The IC_{50} and IP values characterize the antioxidant capacity of pure compounds, but for plant extracts these parameters should be used to indicate extracts which are more suitable as sources of pure antioxidant compounds and may be used to guide further purification and isolation. Considerable interest has arisen in finding dietary sources of antioxidants, resulting in an increase in the number of studies pursuing natural antioxidants. The antioxidant activity of aqueous extracts of *Ilex paraguariensis* leaves or commercial products (prepared in the way they are usually brewed and drunk) are well established (Filip et al. 2000; Gugliucci 1996; Schinella et al. 2000; Turkmen et al. 2006; Bastos et al. 2006). The effects of extraction solvents on the concentration and antioxidant activity of "mate" tea polyphenols were determined by Turkmen et al. (2006) and a peroxidase-based biosensor to evaluate the antioxidant capacity of "mate" tea was developed by

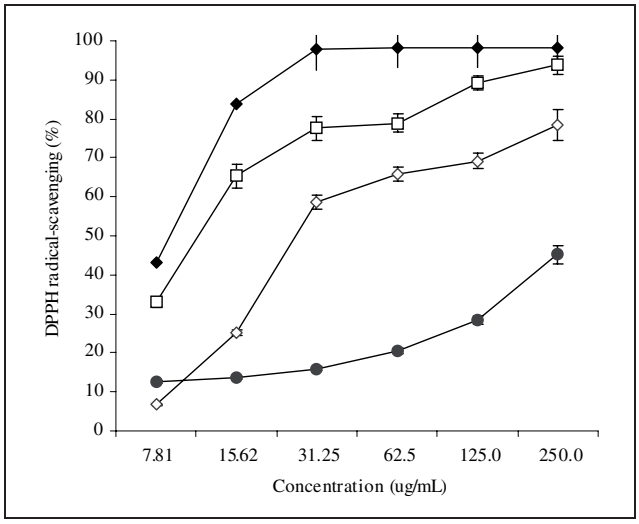

Fig.: Free-radical scavenging activity of leaf and fruit fractions measured by the DPPH assay: (◆) Ascorbic acid. (□) Ethyl acetate fraction from leaves. (◇) Butanolic fraction from leaves (●). Ethyl acetate fraction from fruits. Each value is mean ± S.D. of triplicate analysis.

Table: Total polyphenol contents in *Ilex paraguariensis* leaf and fruit fractions

Fractions	Leaves		Fruits	
	mg/mL[a]	mg/g DF[b]	mg/mL	mg/g DF
Chloroform	$0.013 \pm 5 \times 10^{-5}$[c]	$86.82 \pm 3 \times 10^{-4}$	ND	ND
Ethyl acetate	$0.030 \pm 4 \times 10^{-4}$[d]	$199.91 \pm 3 \times 10^{-3}$	$0.016 \pm 6 \times 10^{-5}$[e]	$110.36 \pm 4 \times 10^{-4}$
Butanolic	$0.027 \pm 2 \times 10^{-5}$ [f]	$183.24 \pm 2 \times 10^{-4}$	$0.008 \pm 2 \times 10^{-4}$[g]	$54.25 \pm 1 \times 10^{-3}$

Three analytical replicates ($n = 3$) were carried out on each fraction. Measurements were averaged, and results are given as mean ± standard deviation. Abbreviations: DF, dry fraction; ND, not determined. [a]Concentration of phenolic compounds expressed as milligram equivalents of pyrogallol per milliliter of aqueous extract; [b]Concentration of phenolic compounds expressed as milligram equivalents of pyrogallol per gram of dry fraction (DF).[c–g] Different superscripted letters indicate statistical differences ($p < 0.05$).

Mello et al. (2005). However, little is known about the antioxidant and polyphenol contents in studies with fractionated extracts of *Ilex paraguariensis* leaves and fruits. In order to establish the relationship between the total phenolic content and antioxidant activity, total phenolics were quantified in all the fractions used in this study. Regarding *Ilex paraguariensis* fruits, no previous studies have been found concerning the antioxidant activity and total polyphenol quantification. The Table presents the total polyphenol content of leaf and fruit fractions of *Ilex paraguariensis*. Values are expressed both as milligram equivalent per mL of pyrogallol and as milligrams per grams of dry fractions. All the fractions had different total polyphenol contents ($p < 0.05$). The butanolic fraction from the fruits showed the lowest value ($54.25 \pm 1 \times 10^{-3}$ mg equiv pyrogallol/g DF) and the ethyl acetate fraction from the leaves showed the highest ($199.91 \pm 3 \times 10^{-3}$ mg equiv pyrogallol/g DF). These results are higher than those obtained by Turkmen et al. (2006) in "mate" tea. Comparing the sum of ethyl acetate and butanolic fractions from the leaves (mg/g DF) and the sum of ethyl acetate and butanolic fractions from the fruits, the polyphenol content in the leaves is nearly 2.3 times higher than in the fruits. Within the fruits, the polyphenols content of the ethyl acetate fraction was almost twice that of the butanolic fraction. However, this fraction showed very weak antioxidant activity ($IC_{50} = 285.78$ µg/mL) when compared with the leaves. Regarding ethyl acetate fractions, although the phenolic contents from leaves was near twice that of the fruits, the IC_{50} of both fractions showed a difference higher than 20 times. All these differences can be attributed to the difference in the composition of the fractions, including the differences between the composition of leaves and fruits. The presence of rutine, quercetin, kaempherol and caffeoyl-derivatives in *Ilex paraguariensis* leaves (Filip et al. 2001) could be responsible for the observed antioxidant activity. However, the polyphenol patterns in fruits remain unclear. From these results it seems that the polyphenol pattern in the fruit fractions may have a direct effect in their performance against the DPPH radical. Here we also observed that the radical scavenging activity increased with the increase of phenolic compound content, mainly in the leaf fractions. These results are in agreement to others reports in the literature (Chandra and Mejia 2004; Turkmen et al. 2006), showing a positive correlation between free radical scavenging activity and total phenolic compounds in *Ilex paraguariensis*.

Considering the practice of mixing the fruits with the leaves in summer, we measured the total methylxanthine contents in the leaves and in the fruits separately in order to verify the possible contribution of the methylxanthine contents from the fruits in the commercial "mate". Total methylxanthine contents, expressed as caffeine were 1.16 ± 0.06 mg/g dry weight in the fruits and 8.78 ± 0.01 mg/g in the leaves ($y = 0.0392x + 0.0161$, $r^2 = 0.99$). Comparing the values, methylxanthine content in the leaves is nearly 8-fold higher than in the fruits. Therefore, the addition of the fruits in the commercial product could lead to a decrease in the levels of methylxanthine contents in "mate". Since the fruits may be present in the summer harvest, our results suggest that the profile of methylxanthine contents of "mate" along the year could be different compared to our previous report in absence of them (Schubert et al. 2006). Currently, there is a growing interest in using natural antimicrobial compounds, such as plant extracts, herbs and spices. In the present study, we tested two saponin enriched fractions (A and B) obtained from the *n*-butanolic fraction of the fruits. Fractions A and B did not show activity against the tested bacteria and fungi. The MIC for fractions A and B for all microorganisms tested were >2.500 µg/mL. This finding does not support the hypothesis that fruit saponins take action as a chemical defence against bacteria and fungi to protect the unripe fruit. "Mate" is a widely accepted beverage in many parts of South America and it is an important source of protective substances (mainly polyphenols) that may have relevance in health care, decreasing the risk of degenerative diseases by reducing oxidative stress. Also, it is a good source of methylxanthines, appreciated for their stimulant effect. Considering the fruits, the results here showed weak antioxidant activity, low phenolic and methylxanthine contents and no antimicrobial activity. Therefore, for commercial purposes, a possible way to build up the value of *Ilex* fruits, which are byproducts of the "mate" production, is to utilise them as a natural, low cost and yearly available source of saponins for future use in the pharmaceutical or cosmetic industry. Further studies with *Ilex paraguariensis* fruits are underway.

3. Experimental

3.1. Chemicals

All the chemicals were of analytical grade. Solvents for the extractions, ascorbic acid, caffeine and pyrogallol were purchased from Merck (Darmstadt, Germany). Folin-Ciocalteu's phenol reagent 2 N and DPPH radical were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Analytical TLC was carried out on silica gel Merck GF254nm plates, using chloroform : ethanol : H_2O (80 : 40 : 5 v/v, lower phase) and chloroform : ethanol : H_2O (60 : 40 : 5 v/v and 40 : 40 : 5 v/v). Detection was performed with anisaldehyde-H_2SO_4/100 °C. For TLC, authentic samples of matessaponin 1 and matessaponin 2 (isolated from the leaves of *I. paraguariensis* available in the laboratory) were used.

3.2. Sample preparation

Leaves and unripe fruits of *Ilex paraguariensis* were harvested in Barão do Cotegipe (State of Rio Grande do Sul, Brazil) in February 2001. Samples of the collected material were identified by Botanist Gilberto Dolejal Zanetti and archived as voucher specimens in the Herbarium of the Industrial Pharmacy Department of the University of Santa Maria (HDFI n° 243).

3.3. Extraction and partition of the leaves

Dried and powdered leaves of *Ilex paraguariensis* (3×70 g) were extracted three times with ethanol-water (6 : 4, v/v, 700 mL for each extraction) in a water bath during 30 min at 100 °C. After filtration, the

combined extracts were evaporated under reduced pressure to remove the ethanol in order to obtain an aqueous suspension. The aqueous suspension was partitioned successively with chloroform, ethyl acetate and *n*-butanol (3×100 mL for each solvent), yielding chloroform, ethyl acetate, and *n*-butanol extracts, respectively. The extracts were evaporated to dryness under vacuum, to yield the chloroform (6.93 g), ethyl acetate- (8.61g) and *n*-butanol-soluble (7.98 g) fractions respectively.

3.4. Extraction and partition of the unripe fruits

Dried and powdered fruits (20 g) were extracted with ethanol/water (6 : 4 v/v, 200 mL) in a water bath during 30 min. After filtration, the extract was evaporated under reduced pressure to yield the water suspension. Then, it was successively partitioned with ethyl acetate and *n*-butanol (3×60 mL for each solvent). Partition with chloroform was not carried out because their lower amount of chlorophylls and fats. The extracts were concentrated in vacuum, yielding the ethyl acetate (1.34 g) and *n*-butanol-soluble (3.59 g) fractions, respectively.

3.5. Methylxanthine extraction from leaves and fruits

Extracts of 3.0 g of crushed leaves and powdered fruits were prepared separately. The materials were boiled with H_2SO_4 (20%, aq.) for 10 min and filtered before cooling. Once cool, the filtrates were neutralized with NH_4OH (50%, aq.) and extracted with chloroform:isopropanol (3 : 1 v/v) in triplicate. The resulting solutions were dried over Na_2SO_4 and evaporated to dryness under reduced pressure (Reginatto et al. 1999).

3.6. Determination of total methylxanthines

Total methylxanthine contents from leaves and fruits were determined spectrophotometrically as described by Schubert et al. (2006). The methylxanthine extracts were diluted in ethanol 40% and the absorbance was measured at 273 nm in a UV–VIS spectrophotometer (SHIMADZU-UV-1201, Kyoto, Japan) against a blank (ethanol 40%). For the calibration curve of caffeine, standard solutions of caffeine (2.0, 4.0, 8.0, 10.0, 16.0 and 20.0 μg/mL) were prepared using ethanol 40% as solvent. The standard solutions were measured in triplicate. The linearity was evaluated by linear regression and the precision and accuracy were determined by coefficient of variation (CV%). The estimation of total methylxanthines contents in the fractions was carried out in triplicate and the results were expressed as milligram equivalents of caffeine per gram of dry fraction.

3.7. Preparation of saponin enriched fractions A and B from the n-butanol fraction of the fruits

The n-BuOH fraction was repeatedly subjected to column chromatography on silicagel (on elution with chloroform:ethanol:H_2O 80 : 40 : 5 v/v, lower phase and chloroform:ethanol:H_2O 60 : 40 : 5 v/v). Two saponin mixtures were obtained and named A (242.6 mg) and B (210.1 mg). On elution with chloroform:ethanol:H_2O (60 : 40 : 5 v/v), the saponin mixtures A and B migrated to the same Rf-values as the authentic samples of matessaponin 1 and matessaponin 2, respectively. They also presented characteristic purple spots with anisaldehyde-sulphuric acid reagent. The precise chemical structures of all saponins from fractions A and B have not been elucidated yet; the chemical structures of matessaponin 1 and 2 isolated from the leaves have already been reported (Schenkel et al. 1996).

3.8. Radical-scavenging activity – DPPH assay

Six different ethanol dilutions of each extract (2.5 mL), at 250; 125; 62.5; 31.25; 15.625; and 7.8125 μg/mL were mixed with 1.0 mL of a 0.3 mM DPPH ethanol solution. The absorbance was measured at 518 nm with an UV–VIS spectrophotometer (SHIMADZU-UV-1201, Kyoto, Japan) against a blank after 30 min of reaction at room temperature. DPPH solution (1.0 mL, 0.3 mM) plus ethanol (2.5 mL) was used as a control. Relative activities were calculated from the calibration curve of L-ascorbic acid standard solutions under the same experimental conditions. Inhibition of free radicals by DPPH in percent (IP%) was calculated according to the equation $IP\% = 100 - [(Ab_{sample} \times 100)/Ab_{control}]$ where Ab_{sample} is the absorbance of the test compound and $Ab_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound). IP% was plotted against the sample extract concentration, and a linear regression curve was established in order to calculate the IC_{50}, which means the amount of each sample necessary to decrease the absorbance of DPPH by 50%. Tests were carried out in triplicate. Correlation coefficients were optimized.

3.9. Determination of total phenolics

Each dried fraction (0.5 g) was dissolved in 10 mL of ethanol and the volume was adjusted to 100mL with water. An aliquot of 3 mL was dissolved in 100 mL of water. The final concentration of each fraction was 0.15 mg/mL. The total polyphenol concentrations in chloroformic, ethyl acetate and butanolic fractions from the leaves and ethyl acetate and butanolic fractions from the fruits were measured spectrophotometrically as described by a modified Folin-Ciocalteau method (Chandra and Mejia 2004). Briefly, 0.5 mL of 2 N Folin-Ciocalteau reagent was added to 1 mL of each sample (0.15 mg/mL), and this mixture was allowed to stand for 5 min before the addition of 2 mL of 20% Na_2CO_3. The solution was then allowed to stand for 10 min before reading at 730 nm by an UV–VIS spectrophotometer (SHIMADZU-UV-1201, Kyoto, Japan). The estimation of phenolic compounds in the fractions was carried out in triplicate. The total polyphenol content was expressed as milligram equivalents of pyrogallol per milliliter of the extracts or as milligram equivalents of pyrogallol per gram of dry fraction (DF). The equation obtained for the standard curve of pyrogallol in the range of 0.005–0.030 mg/mL was $y = 41.071x - 0.2259$ ($r = 0.99$).

3.10. Antimicrobial screening

Saponin enriched fractions A and B were individually tested against a panel of microorganisms including yeast like fungi, such as *Candida albicans* ATCC 44373, *Candida dubliniensis* CBS 7987, *Candida glabrata* (clinical isolate), *Candida parapsilosis* HUSM 523, *Candida krusei* FM 1023, *Cryptococcus neoformans* var. gatti sorotype C ATCC 28957, *Cryptococcus neoformans* var. gatti sorotype B ATCC 56990, *Saccharomyces cerevisiae* ATCC 2606, bacteria, such as *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and fungi, such as *Microsporum gypseum* (clinical isolate), and *M. canis* (clinical isolate). Bacterial strains were cultured overnight at 37 °C in Mueller-Hinton Agar (MHA). Yeasts were cultured overnight at 30 °C in Sabouraud dextrose agar.

3.11. Microdilution method for MIC determination

The MICs of the saponin fractions A and B against the test microorganisms were determined by the broth microdilution method (NCCLS, 2002). All tests were performed in duplicate. Seven different dilutions of each Fraction A and B (2.500, 1.250, 625, 312.5, 156.25, 78.125, and 39.06 μg/mL) in DMSO : TWEEN 80 (1 : 2 v/v) were prepared. Bacteria were inoculated into Mueller-Hinton agar and, after overnight growth, four or five colonies were directly suspended in saline solution so that the turbidity matched the turbidity of the McFarland standard ($\approx 10^8$ cfu/mL). The minimal inhibitory concentration (MIC) was determined by broth microdilution methods according to documents M7-A5 (NCCLS, 2002). By further progressive dilutions with the test medium, the required concentrations were obtained. The suspension was diluted by 1 : 100 in saline followed by a new dilution of 1 : 20 in Mueller-Hinton broth, resulting in a final inoculum concentration of 5×10^4 cfu per well. Yeasts like fungi were inoculated into potato dextrose agar and the MIC was determinated by broth microdilution technique according to M27-A2 (NCCLS, 2002). The plates were incubated at 35 °C for 24 h for bacteria and *Candida*; 72 h for *S. cerevisae* and *C. neoformans*. Growth or a lack thereof in the antimicrobial agent containing wells was determined by comparing with the growth control, indicated by turbidity. All tests were carried out in duplicate and the lowest concentration that completely inhibited visible growth of the organism was recorded as the MIC.

3.12. Statistical analysis

Student's test was used to determine the levels of significance. Statistical analysis was performed by one-way analysis of variance ($p < 0.05$). IC_{50} was calculated from the concentration/effect linear regression lines.

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Manuscript retraction

The editors apologize for publishing the article

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