https://doi.org/10.31925/farmacia.2019.6.24 **ORIGINAL ARTICLE**

INSIGHTS OF THE ANTIMICROBIAL ACTIVITY OF PIPERINE EXTRACTED FROM *PIPER NIGRUM* L.

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Abstract

The aim of this study was to present new insights of the antimicrobial activity of piperine extracted from *Piper nigrum* as compared to commercial piperine and to the activity of other similar compounds (namely: protocatechuic acid, L-ascorbic acid, L-tyrosine and syringic acid) and in synergism with ampicillin and amphotericin B. For this, piperine was isolated, purified and its structure and high purity were determined by NMR, FTIR, UV-Vis and TLC analysis. A concentration of 100 mg/mL of piperine proved to have no cytotoxic effect on the opportunistic pathogens represented by *Staphylococcus aureus* and *Escherichia coli*, and was slightly efficient against *Candida albicans*. Nevertheless, piperine improved the ampicillin activity against *S. aureus* with 14% and in the same time decreased the activity of amphotericin B against *C. albicans* with 54.2%, their antimicrobial properties being quite different compared to the commercial piperine.

Rezumat

Scopul acestui studiu este de a prezenta noi perspective asupra activității antimicrobiene a piperinei extrase din *Piper nigrum* în comparație cu piperina comercială și cu activitatea altor compuși similari (și anume: acid protocatechic, acid L-ascorbic, L- tirozină și acid siringic) și în sinergism cu ampicilina și amfotericina B. Pentru aceasta, piperina a fost izolată, purificată și caracterizată prin analize RMN, FTIR, UV-Vis și TLC. O concentrație de piperină de 100 mg/mL nu a prezentat efect citotoxic asupra agenților conditionat patogeni reprezentați de *Staphylococcus aureus* și *Escherichia coli* și a fost eficient împotriva speciei *Candida albicans*. Cu toate acestea, piperina a îmbunătățit activitatea ampicilinei împotriva speciei *S. aureus* cu 14% și, în același timp, a scăzut activitatea amfotericinei B împotriva levurii *C. albicans* cu 54,2%, proprietățile ei antimicrobiene fiind destul de diferite față de piperina comercială.

Keywords: *Piper nigrum*, piperine, antimicrobial

Introduction

Piperine is the major alkaloid present in black pepper belonging to family *Piperaceae*, which is responsible for its hot and spicy taste and was first isolated from pepper extract by Hans Christian Ørsted in 1819 [1]. Pepper extracts contain alkaloids (e.g. piperine), terpenes, flavones and volatile oils (e.g. piperlyne) that exhibit sedating, detoxificativ, hypotensive, immuno-modulatory, anti-oxidant, anti-asthmatic, anti-carcinogenic, anti-inflammatory, anti-ulcer, anti-amoebic activities [2-4].

Piperine has increased the bioavailability of many drugs such as: vasicine [5], theophylline and phenytoin [6], pyrazinamide and isoniazid [7], rifampicin [8-9], propranolol [10], pro-vitamin beta-carotene [11], nimesulide [12], cefotaxime sodium and amoxicillin [13], ampicillin and norfloxacin [14], gatifloxacin [15], docetaxel [16], curcumin [17-18], and acyclovir [19]. Based on cell, animal and human studies, piperine has been found to have properties and it is considered to have the potential to reduce drug dosage, cost of the drugs, the incidence of drug resistance and the risk of adverse drug reaction/ side effects [20].

According to literature, piperine proved to be efficient against *Staphylococcus epidermidis* and *Salmonella enterica* [21], *Bacillus cereus* and *Bacillus subtilis* [22], *Staphylococcus aureus* [23]. On the other hand, piperine did not show any antibacterial activity, but in combination with ciprofloxacin was efficient against *Staphylococcus aureus* ATCC29213 and MRSA isolates [24].

Within this context, the aim of this study was to obtain pure piperine from *P. nigrum* and to perform a screening of their antimicrobial properties compared with the commercial version and with similar drugs. The piperine was extracted, purified and characterized. In order to select the potential medical applications of the extract, there were conducted a series of biological

assays: antibacterial and antifungal susceptibility test against three of the most known pathogens nowadays: *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25922 and *Candida albicans* ATCC10231. Moreover, it was investigated the interaction of the extract with an antibiotic and an antifungal drug used against the selected pathogens.

Materials and Methods

Materials

Black pepper (from BioWagner, country of origin: Sri Lanka), piperine (from Sigma, 97%, used as reference), extraction thimbles (from Macherey-Nagel GmbH, Germany), chloroform (from VWR Chemicals, 99.9% GPR RECTAPUR), diethyl ether (Et_2O) (from VWR Chemicals, 99.9%), ethylic alcohol (EtOH) (from VWR Chemicals, 99%), potassium hydroxide (KOH) (from Lach-Ner, 86.7%), dichloromethane (DCM) (from Honeywell, Riedel-de Haen, CHROMASOLV ≥ 99.8%), ethyl acetate (EtAc) (from VWR Chemicals, ACS, Reag. Ph. Eur.), double distilled water (obtained with FiSTREEM Cyclon WSC044.MH3.4 system), ultrapure water (obtained with TKA-GenPure 08.2204 system).

Isolation and purification

The extraction procedure was accomplished using slightly modified literature protocols [25-27], as follows: black pepper (160 g) was very fine milled using a Philips HR2860 grinder equipped with dry samples blades. The obtained black pepper fine powder was filled into the extraction thimble and sealed with a cotton stopper. The extraction cartridge loaded with the sample of interest was inserted into the Soxhlet extractor. The round bottom flask was filled with 320 mL chloroform, connected to Soxhlet extractor and inserted into silicone oil bath heated to 86°C. To limit the extraction of by-products, the extraction process was stopped after 3.5 - 4 hours (two extraction cycles).

The resulting mixture solution was concentrated to dryness under vacuum using a rotary evaporator. The remaining residue as a viscous green-brownish oil was cooled to the freezer overnight. The next day, 50 - 100 mL of diethyl ether were added over the cold residue and the mixture was triturated until a yellow precipitate appeared. The precipitate was filtered, washed three times with cold diethyl ether and dried under vacuum, obtaining 5.5 g (3.44% yield) pale yellow precipitate of piperine.

The precipitate was solubilized in a minimum volume of hot ethanol and the resulting solution was treated with 100 mL solution of 10% KOH in warm ethanol and magnetically stirred for 30 minutes. Double distilled water was added to the solution in small portions, using the glass walls as support and the apparition of a fine yellow precipitate was observed. The mixture was diluted with double distilled water to a final volume of 800 - 1000 mL and kept in the refrigerator overnight to fully precipitate the piperine. The next day, the solution was filtered and the obtained yellow precipitate was washed twice with cold double distilled water and once with cold diethyl ether. The filtered precipitate was dried under vacuum using a drying oven for a couple of hours. After complete drying, the precipitate was solubilized in a minimum volume of hot ethanol, filtered and allowed to crystalize. After two hours was observed the apparition of small needle shaped crystals, but the solution was left overnight to fully crystallize. Further, yellow needle crystals were filtered and washed twice with cold ethanol. For the next crystallization, the crystals were solubilized in a minimal amount of hot ethanol, filtered, and the obtained solution was again allowed to crystallize overnight. The crystals were filtered, washed once with cold ethanol and twice with cold diethyl ether and dried under vacuum, obtaining 5.305 g (96% yield) of pure yellow crystals of piperine.

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 400 instrument (Bruker, Bremen, Germany), operated at 400 and 100 MHz for ^{1}H and ^{13}C nuclei, respectively, at room temperature (23ºC). All chemical shifts are quoted on the δ-scale in ppm, referred to tetramethylsilane (TMS) as internal standard and the coupling constants are given in Hz.

Fourier-Transform Infrared Spectroscopy (FTIR). IR spectra were recorded on a FTIR Bruker Vertex 70 Spectrophotometer (Bruker, Bremen, Germany) equipped with a ZnSe single reflection ATR accessory. UV-Vis spectra were recorded with a LAMBDA 35 spectrophotometer (from Perkin Elmer Inc. USA).

Thin layer chromatography (TLC) was carried out on ALUGRAM Xtra SIL G/UV_{254} plates (Macherey-Nagel GmbH, Germany).

Melting points were recorded on an A. Krüss Optronic Melting Point Meter KSPI (Germany).

The antimicrobial activity screening of the samples (piperine, standard piperine, protocatechuic acid, L-ascorbic acid, L-tyrosine and syringic acid) was determined by the disk diffusion assay against six different reference strains: *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25922 and *Candida albicans* ATCC10231. The tested concentration was 100mg/mL dissolved in DMSO. Afterwards, this concentration of the samples was mixed with ampicillin (100 mg/mL, Epigenetics, USA) and tested again against the bacterial reference strains and also with amphotericin B (µg/mL, Lonza, USA) and tested against the yeast strain.

All microorganisms were stored at -80°C in 20 - 40% glycerol. The bacterial strains were refreshed in tryptic soy agar (TSA), and the yeast strain was refreshed with Sabouraud dextrose agar (SDA) at 36 ± 1°C. The microbial suspensions were prepared with these cultures

in sterile solution to obtain turbidity optically comparable to that of 0.5 McFarland standards.

Volumes of 0.5 mL from each inoculum were spread on the Petri dishes. The sterilized paper disks were placed on the plates and an aliquot (50 µL) of the samples were added. To evaluate the antimicrobial properties, the growth inhibition was measured under standard conditions after 24 hours of incubation at 36°C. All tests were carried out in triplicate to verify the results [28]. After incubation, the diameters of the inhibition zones were measured by using Image J software.

Results and Discussion

Melting point studies

For the isolated and reference piperine were determined the melting points which must have an appropriate value of 131.5°C [29]. In the present study, isolated piperine undergoes melting between 130 - 131.5°C, meanwhile, the reference piperine melts in the interval of 131 - 132.7°C. Both melting point intervals are comparable with the literature value.

TLC analysis

Purity analysis of the isolated and reference piperine, appeared as single circular spot on the TLC plate (Rf = 0.69, DCM/40% ethyl acetate) under UV light at 254 nm and blue fluorescent spot at 365 nm for both studied compounds.

NMR spectra studies:

The chemical structures of both types of piperine were assigned by ^{1}H-NMR and ^{13}C-NMR spectroscopic methods. ^{1}H-NMR (400 MHz, $CDCl_3$) spectra of isolated piperine (Figure 1A) confirms the chemical structure as follow: δ (ppm) 7.39 (ddd, *J* = 14.7, 8.1, 2.0 Hz, 1H, H_9), 6.98 (d, *J* = 1.5 Hz, 1H, H_{13}), 6.88 (dd, *J* = 8.0, 1.5 Hz, 1H, H_{16}), 6.79 – 6.73 (overlapped signals, 3H, H_{10}, H_{11}, H_{15}), 6.43 (d, *J* = 14.7 Hz, 1H, H_8), 5.96 (s, 2H, H_{14}), 3.57 (as, 4H, H_2, H_6), 1.66 (m, 2H, H_4), 1.57 (m, 4H, H_3, H_5). As expected, the ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of reference piperine (Figure 1B) is superposing completely over the isolated piperine spectrum: δ (ppm) 7.40 (ddd, *J* = 14.7, 8.3, 1.9 Hz, 1H, H_9), 6.97 (d, *J* = 1.5 Hz, 1H, H_{13}), 6.88 (dd, *J* = 8.0, 1.6 Hz, 1H, H_{16}), 6.78 – 6.72 (overlapped signals, 3H, H_{10}, H_{11}, H_{15}), 6.43 (d, *J* = 14.6 Hz, 1H, H_8), 5.96 (s, 2H, H_{14}), 3.57 (as, 4H, H_2, H_6), 1.66 (m, 2H, H_4), 1.58 (m, 4H, H_3, H_5).

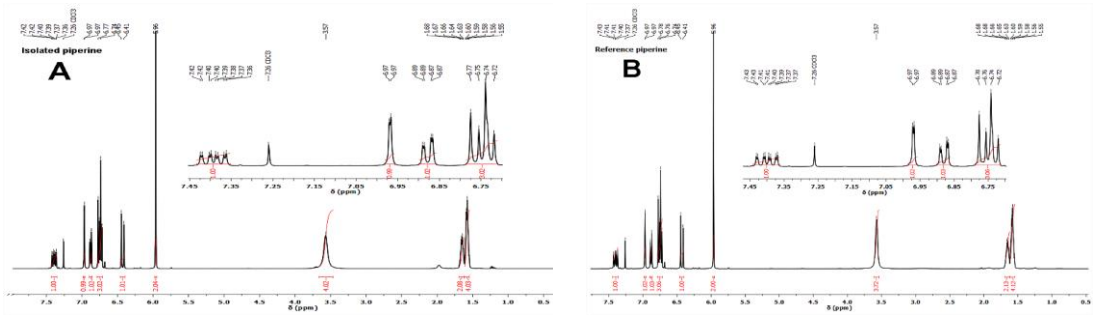

Figure 1.

^{1}H-NMR (400 MHz, $CDCl_3$) spectra of isolated piperine (A) and reference piperine (B)

The perfect superposing of both spectra was also observed in ^{13}C-NMR spectra with some few exceptions. The signal appeared at δ = 26.13 ppm from the isolated piperine spectra (Figure 2A) assigned for the C2 and C6 atoms showed a considerable decrease in intensity into ^{13}C-NMR spectrum of reference piperine (Figure 2B) and also, the signal expected at δ = 45.2 ppm assigned for C4 atom is completely absent in the ^{13}C-NMR spectrum of reference piperine, probably caused by the chirality of the molecule. All other signals are associated with the existing carbon atoms from the molecule. ^{13}C-NMR (100 MHz, CDCl3) of isolated piperine: δ (ppm) 165.5 (C7), 148.1 (C17, C18), 142.9 (C9), 138.5 (C11), 130.9 (C12), 125.2 (C9), 122.6 (C16), 119.6 (C11), 108.4 (C15), 105.6 (C13), 101.2 (C14), 45.2 (C4), 26.1 (C2, C6), 24.6 (C3, C5) and ^{13}C-NMR (100 MHz, CDCl3) of reference piperine: 165.4 (C7), 148.1 (C17, C18), 142.5 (C9), 138.2 (C11), 131.0 (C12), 125.3 (C9), 122.5 (C16), 120.0 (C11), 108.5 (C15), 105.6 (C13), 101.3 (C14), 26.1 (C2, C6), 24.6 (C3, C5).

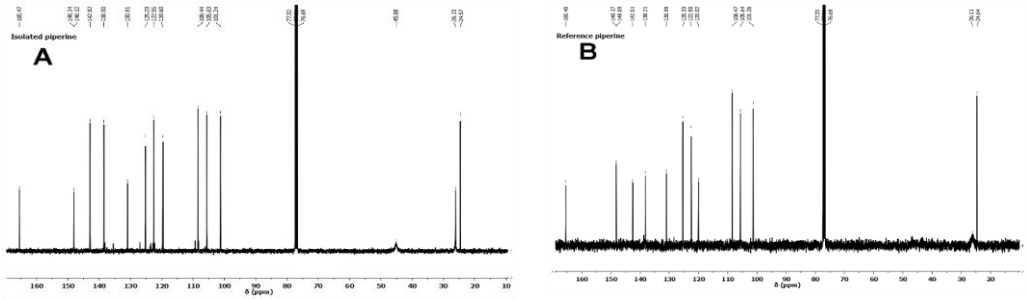

Figure 2.
^{13}C-NMR (100 MHz, $CDCl_3$) spectra of isolated piperine (A) and reference piperine (B)

IR spectra studies

The structures of both types of piperine were confirmed by FTIR spectroscopy. As in the case of NMR spectra, both IR spectra of isolated and reference piperine are perfectly superposing (Figure 3). Obtained wavenumbers (ATR, cm^{-1}) are assigned as follows: for isolated piperine: 3011 (arom. C-H st.), 2939, 2854 (aliph. C-H st.), 1632 (-CO-N-), 1610, 1582, 1489 (arom. C=C st.), 1441 (methylenedioxy CH_2 bending), 1250, 1194 (=C-O-C asym. st.), 1028 (=C-O-C sym. st.), 995 (C-H bending of *trans* –CH=CH-), 928 (C-O st.) and for reference piperine 3011 (arom. C-H st.), 2941, 2854 (aliph. C-H st.), 1631 (-CO-N-), 1610, 1581, 1489 (arom. C=C st.), 1440 (methylenedioxy CH_2bending), 1250, 1194 (=C-O-C asym. st.), 1028 (=C-O-C sym. st.), 996 (C-H bending of *trans* –CH=CH-), 928 (C-O st.).

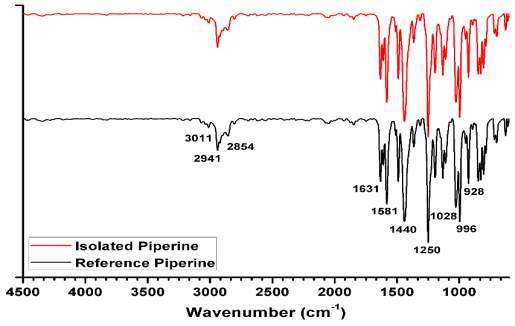

Figure 3.
ATR-FTIR overlaid spectra of isolated and reference piperine

UV-Vis analysis

The UV-Vis spectra of isolated and reference piperine (Figure 4) were recorded at the same concentration in water (0.545 x 10^{-5} M) and were perfectly over-lapping, presenting the UV absorption at λ_{max} value = 342 nm which is in good correlation with the value reported in literature [30-32]. In the inserted graph it could be observed that the increasing or decreasing of the piperine concentration have no influence in the characteristic slope.

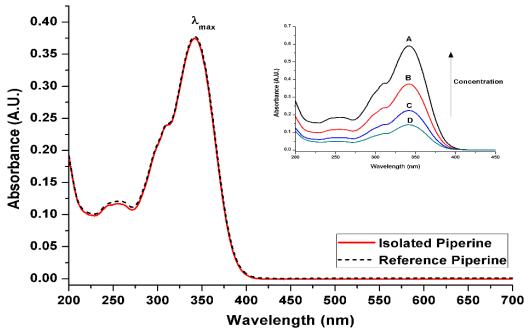

Figure 4.
Overlaid UV-Vis spectra of isolated and reference piperine (λ_{max}= 342 nm, 0.545 x 10^{-5} M in water). Inset graphic represents UV-Vis spectra of isolated piperine at different concentrations (A - 1.09 x 10^{-5} M, B - 0.545 x 10^{-5} M, C - 0.2725 x 10^{-5} M and D - 0.1363 x 10^{-5} M)

Antimicrobial activity

According to series of studies regarding different black pepper extracts showed significant inhibitory effect against varied Gram-negative and Gram-positive bacteria, although some studies revealed that the black pepper have no antibacterial activity, which could be generally attributed to differences in plant varieties, microbiological methods, solvents used for compound extraction and tested microorganisms.

In this study, the antimicrobial activity was measured by the agar disk diffusion method [28] which supposes the addition of the compounds on the culture medium pre-inoculated with the microbial suspension, and measuring of the clear zone caused by the growth inhibition around the film disks after 24 h of incubation for the bacterial strains. The antimicrobial activity of the extracted piperine was compared to that of the commercial piperine, protocatehuic acid, syringic acid, L-ascorbic acid, L-tyrosine and syringic acid, and also with the combination of these compounds with Ampicillin and Amphotericin B.

Ampicillin is a penicillin beta-lactam antibiotic used in the treatment of bacterial infections caused by susceptible, Gram-positive and Gram-negative organisms. By binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall, ampicillin

inhibits the third and last stage of bacterial cell wall synthesis [33-34].

On the other hand, Amphotericin B showed a high order of *in vitro* activity against many species of fungi, especially against *Candida albicans*; it binds irreversibly to ergosterol, resulting in the disruption of membrane integrity and ultimately cell death [35].

Within this context, our results proved that the protocatechuic acid and the syringic acid were slightly efficient against *S. aureus* ATCC25923, *E. coli* ATCC25922 and *C. albicans* ATCC10231 (Table I). Syringic acid is a phenolic compound that proved to have antibacterial activity against *Escherichia coli*, *Oenococcus oeni* and *Cronobacter sakazakii* [36-38]. *In vitro* studies demonstrated that protocatechuic acid has the ability to inhibit bacterial growth and increase the synergistic effects of antibiotics hence reducing the possibility of resistance to drugs [39].

L-ascorbic acid has been shown to have an inhibitory effect on the growth of *Staphylococcus aureus*, *Enterococcus fecalis*, *Helicobacter pylory*, *Campylobacter jejuni*, *Mycobacterium tuberculosis*, *Escherichia coli* and *Klebsiella pneumoniae* [40-43] and even on fungi [44]. Furthermore, *in vitro* studies have shown that vitamin C can enhance the inhibitory effect of antibiotics such as levofloxacin [45] and azithromycin [46]. In our study L-ascorbic acid did not present antimicrobial activity and in combination with Ampicillin slightly increased the activity against *S. aureus* (+8%) and decreased the activity against *E. coli* (-19.9%) (Table I).

Table I

Antimicrobial activity of the tested compounds against the references strains (mm)

Tested compounds	*S. aureus* ATCC25923		*E. coli* ATCC25623		*C. albicans* ATCC10231	
	Inhibition zone	*%**	*Inhibition zone*	*%**	*Inhibition zone*	*%***
Ampicillin	22.20 ± 0.031		27.62 ± 0.051		Not tested	
Amphotericin B	Not tested		Not tested		17.25 ± 0.123	
Piperine	0		0		01.90 ± 0.012	
Piperine + Ampicillin	26.13 ± 0.023	+14.00	27.46 ± 0.015	-0.57	Not tested	
Piperine + Amphotericin B	Not tested		Not tested		07.90 ± 0.024	-54.2
Piperine Standard	0		0		01.5	
Piperine Standard + Ampicillin	22.83 ± 0.05	+2.83	26.40 ± 0.01	-4.41	Not tested	
Piperine Standard + Amphotericin B	Not tested		Not tested		11.84 ± 0.015	-31.36
Protocatehuic acid	03.88 ± 0.210		02.52 ± 0.012		01.75 ± 0.132	
Protocatehuic acid + Ampicillin	24.49 ± 0.012	+10.31	25.00 ± 0.023	-9.48	Not tested	
Protocatehuic acid + Amphotericin B	Not tested		Not tested		13.64 ± 0.010	-20.92
L-ascorbic acid	00.75 ± 0.011		0		0	
L-ascorbic acid + Ampicillin	23.98 ± 0.012	+8.01	22.10 ± 0.015	-19.9	Not tested	
L-ascorbic acid + Amphotericin B	Not tested		Not tested		15.24 ± 0.018	-11.65
L-tyrosine	0		0		00.30 ± 0.012	
L-tyrosine + Ampicillin	26.74 ± 0.011	+20.45	23.78 ± 0.013	-13.9	Not tested	
L-tyrosine + Amphotericin B	Not tested		Not tested		12.83 ± 0.015	-25.62
Syringic acid	03.88 ± 0.014		02.28 ± 0.015		01.80 ± 0.018	
Syringic acid + Ampicillin	21.75 ± 0.013	-2%	23.02 ± 0.012	-16.6	Not tested	
Syringic acid + Amphotericin B	Not tested		Not tested		15.95 ± 0.017	-4.53

*percentage from the ampicillin activity; ** percentage from amphotericin B activity

The combination of the tested compounds with the ampicillin slightly improved the antibiotic activity against *S. aureus* ATCC25923, the most notable activity proved to have the combination between ampicillin and L-tyrosine (20.45%) and between ampicillin and piperine (14%). The combination between syringic acid and ampicillin decrease the antibiotic activity with 2%. The co-administration of piperine with ampicillin can cut down the frequency of administration and the related side effects. Piperine is a natural product used in food preparation with less toxicological consequences. This may also bring down the cost of the treatment of many infections which requires the use of antibiotics.

The same combinations determine a decrease of the antibiotic activity against *E. coli* ATCC25922 of 0.57% for piperine combined with ampicillin up to 19.98% for L-ascorbic acid combined with ampicillin (Table I).

Piperine presented a very low activity against *C. albicans* ATCC10231. However, the most dramatic decrease of activity was for the combination between the tested compounds and amphotericin B against *C. albicans* ATCC10231. In this case piperine managed to decrease the antifungal activity of amphotericin B up to 54.20% (Table I). Similar results were obtained for the other tested compounds, but in a lower percentage.

Conclusions

In conclusion, we isolated and purified piperine from *Piper nigrum*, which had the same characteristics with the standard one, but a different *in vitro* activity. The isolated compound did not present antimicrobial activity by itself but improved the ampicillin activity against *S. aureus* ATCC25923 with 14% and in the same time decreased the activity of amphotericin B against *C. albicans* ATCC10231 with 54.2%.

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