

Artigo original

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KEYWORDS

Candida; *Leishmania*; Macrophage;
Staphylococcus; Tannins.

PALAVRAS-CHAVE

Candida; *Leishmania*; Macrófago,
Staphylococcus; Taninos.

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Submetido em: 22/12/2017

Aceito em: 14/04/2018

Antimicrobial Activity and Cytotoxic Assessment of Gallic and Ellagic Acids

Atividade microbiana e avaliação citotóxica dos Ácidos Gálico
 e Elágico

ABSTRACT

The aims of this study were to investigate the antimicrobial activity of the gallic acid (GA) and ellagic acid (EA) against bacteria strains, *Candida albicans* and *Leishmania amazonensis*, as well as to evaluate their cytotoxicity on murine peritoneal macrophages. Minimal inhibitory concentrations (MICs) of gallic and ellagic acids were determined by microdilution assay. MICs of the norfloxacin against a *Staphylococcus aureus* strain with NorA overexpression were determined in the absence or presence of each compound at subinhibitory concentrations, in order to verify the ability of this compounds as potential efflux pump inhibitors. Gallic acid was inactive against all strains tested, meanwhile ellagic acid showed activity against *S. aureus* and *C. albicans*. On the other hand, both compounds did not able to modulate the fluoroquinolone resistance, indicating that they are not NorA inhibitors. Besides, they were actives against *L. amazonensis*, with IC₅₀ values of 10.94 and 3.64 µg·mL⁻¹ for GA and EA, respectively. They also showed cytotoxicity on murine peritoneal macrophages with CC₅₀ values of 126.5 and 23.811 µg·mL⁻¹ for GA and EA, respectively. Interestingly, both compounds have shown to be more selective to parasite rather than macrophages. These results demonstrated that gallic and ellagic acids represent a potential alternative for therapy of staphylococci infections, mycoses and leishmaniasis.

RESUMO

Os objetivos deste estudo foram investigar a atividade antimicrobiana do ácido gálico (AG) e ácido elágico (AE) contra estirpes de bactérias, *Candida albicans* e *Leishmania amazonensis*, bem como avaliar sua citotoxicidade em macrófagos peritoneais murinos. As concentrações mínimas de inibição (CMI) de ácidos gálico e elágico foram determinadas pelo ensaio de microdiluição. As CMIs da norfloxacina contra uma estirpe de *Staphylococcus aureus* com sobre-expressão de NorA foram determinadas na ausência ou presença de cada composto em concentrações sub-inibitórias, a fim de verificar a capacidade destes compostos como potenciais inibidores da bomba de efluxo. O ácido gálico foi inativo contra todas as cepas testadas, enquanto isso o ácido elágico mostrou atividade contra *S. aureus* e *C. albicans*. Por outro lado, ambos os compostos não conseguiram modular a resistência à fluoroquinolona, indicando que não são inibidores de NorA. Além disso, eles foram ativos contra *L. amazonensis*, com valores de IC₅₀ de 10,94 e 3,64 µg · mL⁻¹ para AG e AE, respectivamente. Eles também mostraram citotoxicidade em macrófagos peritoneais murinos com valores de CC₅₀ de 126,5 e 23,811 µg · mL⁻¹ para AG e AE, respectivamente. Curiosamente, ambos os compostos mostraram ser mais seletivos para parasitas que para macrófagos. Estes resultados demonstraram que os ácidos gálico e elágico representam uma alternativa potencial para a terapia de infecções por estafilococos, micoses e leishmaniose.

INTRODUCTION

Infectious diseases possess high prevalence rates throughout the world. Despite the advances in the therapy of bacterial infections, diseases caused by multiresistant bacteria continue to occur with high frequency (LAXMINARAYAN, 2014). A similar problem can be observed in the therapy of mycoses, since the development of resistance to different antifungal agents has been evidenced in strains of clinical origin (BONDARYK; KURZATKOWSKI; STANISZEWSKA, 2013; FEKKAR et al., 2013). Such drawbacks could be minimized with the discovery of novel compounds with antimicrobial potential.

Leishmaniasis caused by protozoa from *Leishmania* genus has affected more than 12 million people worldwide, with about 1,3 million of new cases reported each year (WHO, 2015). Due to the limited viability of chemotherapeutics with antileishmania activity, as well as the increasing resistance and their range of side effects (ALIZADEH et al., 2008; ASHFORD, 2000), the discovery of new drugs with high therapeutic potential against the parasite that minimizes the drawbacks from conventional treatments is markedly urgent.

In this sense, tannins are phenolic compounds derived from the secondary metabolism of plants, most of which are derived from glucose metabolism by different biochemical reactions, such as from either shikimic acid or acetate pathways (DE JESUS et al., 2012). Gallic acid and its dimers, such as digallic acid and ellagic acid, are phenolic lactones found in Brazilian flora, such as *Anacardium occidentale* L., *Myracrodruon urundeuva* Allemão, *Anogeissus leiocarpus* (DC.) Guill. & Perr., *Quercus infectoria* Olivier, *Stryphnodendron obovatum* Benth., in the form of hydrolysable tannins called ellagitannins (MURAKAMI et al., 1991; VATTEM; GHAEDIAN; SHETTY, 2005; RIBEIRO et al., 2015).

Some important pharmacological activities have already been described for gallic acid, such as antioxidant and anti-inflammatory properties (YANG et al., 2015), antimutagenic and anticarcinogenic properties (LEE; HARRISON; GRINSTEIN, 2003; LU et al., 2010; PAOLINI et al., 2015). For ellagic acid, the following activities have been described: anticarcinogenic, hepatoprotective, DNA inhibitor topoisomerase (VATTEM et al., 2005; AGGARWAL; SHISHODIA, 2006; CORTAZAR; COOMBS; WALKER, 2007), antioxidant (DEVIPRIYA et al., 2007), anti-inflammatory (YUCE et al., 2007; PAPOUTSI et al., 2008) and gastroprotective activities (IINO et al., 2001).

The present study aimed to investigate the antimicrobial and antileishmanial potential of gallic acid and ellagic acid (Fig 1), as well as to evaluate its possible cytotoxic action in

murine peritoneal macrophages, based on the determination of their selectivity index.

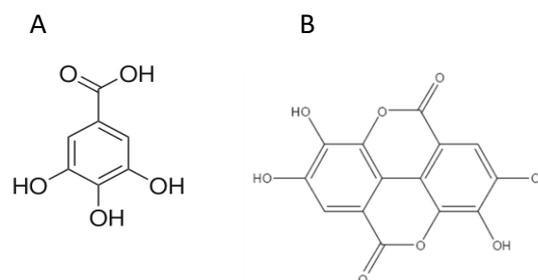


Figure 01. Molecular structures of the phenolic compounds gallic acid (A) and ellagic acid (B). Gallic acid (GA) has molecular weight of 170.12 g/mol; Ellagic acid (EA) is classified as a GA dimer, and has molecular weight of 302.197 g/mol.

MATERIAIS E MÉTODOS

CHEMICALS

Dimethyl sulfoxide (DMSO: 99%; PubChem CID: 679), GIEMSA (PubChem CID: 13735) was purchased from Merck Chemical Company (Germany). The Schneider's medium (PubChem CID: 2723893, RPMI medium (PubChem CID: 1640), fetal bovine serum (FBS; PubChem CID: 86289556), MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; PubChem CID: 64965), Alamar blue (PubChem CID: 11077), Gallic Acid (GA) (PubChem CID: 370), Ellagic Acid (EA) (PubChem CID: 5281855) and the antibiotics penicillin and streptomycin (PubChem CID: 71311919) were purchased from Sigma Chemical (St. Louis, MO, USA). The antibiotic amphotericin B (90%) was purchased from Cristália (São Paulo, SP, Brazil). Norfloxacin, Ciprofloxacin and Chlorpromazine were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

BACTERIAL STRAINS AND GROWTH CONDITIONS

The intrinsic antimicrobial activity was tested against Gram-positive (*Staphylococcus aureus* ATCC 25923 and SA1199B) and Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* NEWP53), as well as against a yeast strain (*Candida albicans* NEWP31). The inhibitory effect on NorA activity was performed with *S. aureus* SA1199-B strain which over-express the *norA* gene encoding NorA. NorA can efflux hydrophilic fluoroquinolones and other drugs such as DNA-intercalating dyes (KAATZ; SEO, 1995). Bacterial strains were maintained on Brain Heart Infusion Agar (BHIA, Himedia, India) slant at 4 °C, and prior to assay the cells were grown overnight at 37 °C in Brain Heart Infusion (BHI, Himedia, India). The yeast strain was maintained on Sabouraud Dextrose Agar (SDA, Himedia, India) slant at 4 °C and prior

to assay the cells were grown for 24 h at 37°C in Sabouraud Dextrose Broth (SDB, Himedia, India).

EVALUATION OF THE INTRINSIC ANTIMICROBIAL ACTIVITY

Stock solutions of GA and EA were prepared by dissolving 10000 µg of each monoterpene in 1 mL of dimethyl sulfoxide. This stock solution was diluted in sterile distilled water to obtain the test solution (1024 µg·mL⁻¹). Minimal inhibitory concentrations (MICs) were determined by micro-dilution assay in BHI broth with bacterial suspensions of 10⁵ CFU·mL⁻¹ and compound solutions ranging from 8 to 512 µg·mL⁻¹. Microtiter plates were incubated at 37 °C for 24 h, then 20 µL of resazurin (0.01% w/v in sterile distilled water) was added to each well to detect bacterial growth by color change from blue to pink. MICs were defined as the lowest concentration at which no bacterial growth was observed. Inhibition of the bacterial growth was confirmed transferring an aliquot from each well of the MIC test microtiter plate to a Petri dish containing BHIA and checking cell viability after incubation at 37 °C for 24 h.

Antifungal assays were performed by micro-dilution method in SDB double concentrated with yeast suspension of 10⁵ CFU·mL⁻¹ and compound ranging from 8 to 512 µg·mL⁻¹. Microtiter plates were incubated at 37 °C for 24 h. Inhibition of the fungal growth was confirmed transferring an aliquot from each well of the MIC test microtiter plate to a Petri dish containing SDA and checking cell viability after incubation at 37 °C for 24 h (COELHO et al., 2016).

MODULATION OF THE FLUOROQUINOLONE-RESISTANCE ASSAY

For evaluation of the monoterpenes as modulators of fluoroquinolone resistance, MICs of the norfloxacin for SA1199-B strain were determined in the presence or absence of GA, EA or Chlorpromazine (a known NorA inhibitor) at sub-inhibitory concentrations (1/8 MIC). Antibiotic concentrations ranged from 0.125 to 128 µg·mL⁻¹. Microtiter plates were incubated at 37 °C for 24 h and readings were performed with resazurin as previously described (COELHO et al., 2016).

PARASITES AND MICE

Leishmania (*Leishmania*) *amazonensis* (IFLA/BR/67/PH8) was used for the determination of the antileishmania activity. Parasites were grown in supplemented Schneider's medium (10% heat-inactivated fetal bovine serum (FBS), 100 U·mL⁻¹ penicillin, and 100 µg·mL⁻¹ streptomycin at 26 °C). Murine macrophages were collected from the peritoneal cavities of male and female BALB/c mice (4-5 weeks old), obtained from Medicinal Plants Research Center (UFPI, Teresina, PI, Brazil). Mice were maintained at a controlled temperature (24 ± 1 °C) and

light conditions (12 h light/dark cycle), with water and food *ad libidum*. All protocols were approved by the Animal Research Ethics Committee (CEEA-PI No. 053/2015).

ANTILEISHMANIA ACTIVITY ASSAY

Promastigotes in the logarithmic growth phase were seeded in 96-well cell culture plates at 1×10⁶ promastigotes per well. Then, GA or EA was added to the wells in serial dilutions of 400, 50, and 6.25 µg·mL⁻¹. The plate was kept at 26 °C in a biological oxygen demand (BOD) *Leishmania* was observed and counted by using a Neubauer hemocytometer after 24, 48, and 72 h to monitor growth and viability (VALADARES et al., 2011).

Amphotericin B (Amph B) was used as positive control in serial dilutions of 8, 4, 2, 1 and 0.5 µg·mL⁻¹. The negative control was the Schneider's medium with promastigotes (1×10⁶ cells/well). The cell viability was considered as 100% for the parasite. The blank was read for each concentration and controls in order to avoid interference of absorbance of medium other compounds. Assays were performed in triplicate and were repeated 3 times on different days.

CYTOTOXICITY DETERMINATION

Cytotoxicity of GA and EA was assessed using the MTT test (GONÇALVES et al., 2016). In a 96-well plate, 100 µL of supplemented RPMI 1640 medium and about 1×10⁵ macrophages were added per well. They were then incubated at 37 °C in 5% CO₂ for 4 h in order to allow cell adhesion. After this time, two washes with supplemented RPMI 1640 medium were performed in order to remove non-adhered cells. Then, GA and EA was added in triplicate, after being previously diluted in supplemented RPMI 1640 medium to a final volume of 100 µL for each well at the tested concentrations (400, 50, and 6.25 µg·mL⁻¹). Cells were then incubated for 48 h. At the end of the incubation, 10 µL of MTT [5 mg·mL⁻¹] diluted in RPMI 1640 medium was added at a final concentration of 5 mg·mL⁻¹ (10% of volume, i.e., 10 µL for each 100 µL well), and was then incubated for 4 h at 37 °C in 5% CO₂. Afterwards, the supernatant was discarded, and 100 µL of DMSO was added to all wells. The plate was then stirred for about 30 min at room temperature in order to complete formazan dissolution. Finally, spectrophotometric reading was conducted at 550 nm in an ELISA plate reader.

STATISTICAL ANALYSIS

All experiments were performed in triplicate, and results were normalized by calculation of geometric average values. Error deviation and standard deviation of the geometric average were revealed. Differences between treatment with antibiotics alone or associated with GA, EA or Chlorpromazine were examined using one-way analysis of

Compound	Strains	(µg·mL ⁻¹)			Effect
		MIC	MMC	MMC/MIC	
GA	<i>S. aureus</i> ATCC 25923	≥1024	-	-	No activity
	<i>S. aureus</i> SA1199B	≥1024	-	-	No activity
	<i>P. aeruginosa</i> NEWP53	≥1024	-	-	No activity
	<i>C. albicans</i> NEWP31	≥1024	-	-	No activity
EA	<i>S. aureus</i> ATCC 25923	128	≥1024	≥8	Bacteriostatic
	<i>S. aureus</i> SA1199B	256	≥1024	≥4	Bacteriostatic
	<i>P. aeruginosa</i> NEWP53	≥1024	-	-	No activity
	<i>C. albicans</i> NEWP31	128	≥1024	≥8	Fungistatic
DMSO	<i>S. aureus</i> ATCC 25923	2048	-	-	-
	<i>S. aureus</i> SA1199B	2048	-	-	-
	<i>P. aeruginosa</i> NEWP53	4096	-	-	-
	<i>C. albicans</i> NEWP31	1024	-	-	-

Table 01. Minimal inhibitory concentrations (MIC) and minimal microbicide concentration (MMC) showed by GA or EA against microbial strains (geometrical means of three simultaneous tests).

variance (ANOVA). The mean inhibitory concentrations (CI₅₀) and mean cytotoxic concentrations (CC₅₀) with confidence limits of 95% were determined by regression of probits using the software SPSS 13.0. The selectivity index was calculated as the ratio of CC₅₀ to CI₅₀. Differences were analyzed by Bonferroni post-hoc test using GraphPad Prism version 5.0 program and were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

EVALUATION OF THE INTRINSIC ANTIMICROBIAL ACTIVITY

Minimal inhibitory (MIC) and minimal microbicide (MMC) concentrations found to GA and EA against the microbial strains are presented in the Table 1. GA did not present intrinsic antimicrobial activity at clinically relevant concentrations. On the other hand, EA showed antibacterial activity against *S. aureus* strains, as well as antifungal activity against *C. albicans*.

MODULATION OF THE FLUOROQUINOLONE-RESISTANCE

Modulatory activity of compounds on resistance to Norfloxacin or Ciprofloxacin was evaluated using SA-1199B strain overexpressing the *norA* gene encoding the multidrug-transporter NorA, which confer fluoroquinolone-resistance by active extrusion of these antibiotics. In this assay, the MIC for each antibiotic was determined in the absence or presence of the compounds at subinhibitory concentrations. Chlorpromazine, a well-known NorA inhibitor was tested as a positive control. The results showed that GA and EA were not able to reduce the MIC of the fluoroquinolones tested, indicating that they are not NorA inhibitors (Figures 2 and 3).

ANTILEISHMANIAL ACTIVITY ASSAY

The inhibitory effect of GA and EA against *L. amazonensis* promastigotes showed a significant concentration-dependent decrease ($p < 0.05$) of parasite viability, with

approximately 100% of promastigotes killing at concentrations of 400 µg·mL⁻¹ for both GA and EA (Figure 4) At 48 h of exposure, the IC₅₀ values for GA and EA were 10.94 µg·mL⁻¹ and 3.64 µg·mL⁻¹, respectively. In cultures treated with GA and EA, the Alamar blue colorimetric was used, and the optical density was determined at 550 nm. Furthermore, morphological changes in the promastigotes were observed by optical microscopy after treatment with GA or EA, such as cells with rounded or completely spherical shapes, as well as cellular debris, instead of spindle forms present in the negative control (data not shown). Amphotericin B (Amph B) was used as a positive control and was tested at a concentration of 2 µg·mL⁻¹. The highest inhibitory effect of Amph B was observed after 48 h of incubation.

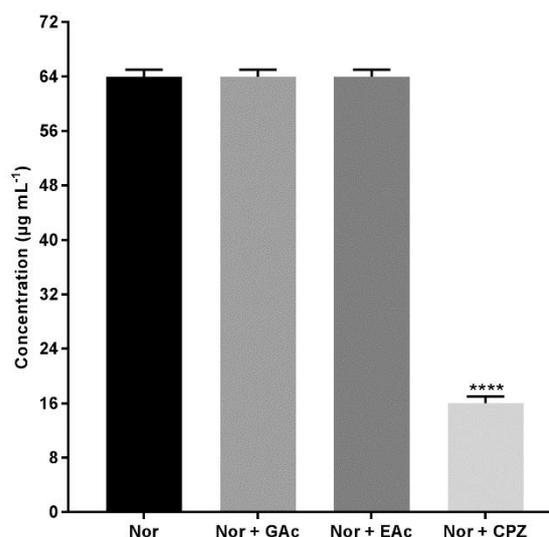


Figure 02. MIC of the Norfloxacin in absence or presence of GA, EA and Chlorpromazine (CPZ) at subinhibitory concentration against *S. aureus* SA-1199B. Each result is the geometric mean of three simultaneous experiments. Statistically significant value (**** $p < 0.0001$).

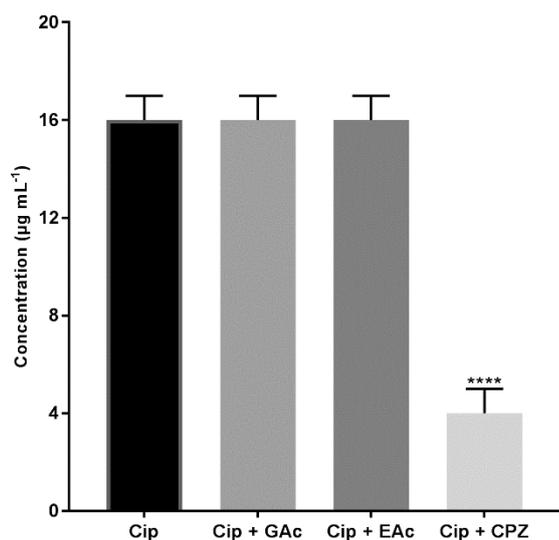


Figure 03. MIC of the Ciprofloxacin (Cip) in absence or presence of GA, EA and Chlorpromazine (CPZ) at subinhibitory concentration against *S. aureus* SA-1199B. Each result is the geometric mean of three simultaneous experiments. Statistically significant value (**** $p < 0.0001$).

CYTOTOXICITY ON MURINE PERITONEAL MACROPHAGES

The cytotoxicity assessment of GA and EA is shown in Figure 5. The GA and EA significantly decreased the macrophages viability starting at concentrations of 50 $\mu\text{g}\cdot\text{mL}^{-1}$ and 6.25 $\mu\text{g}\cdot\text{mL}^{-1}$ ($p < 0.05$), respectively. The mean cytotoxic concentrations (CC_{50}) of GA, EA and Amph B, as well as their selectivity index, are stated in Table 2.

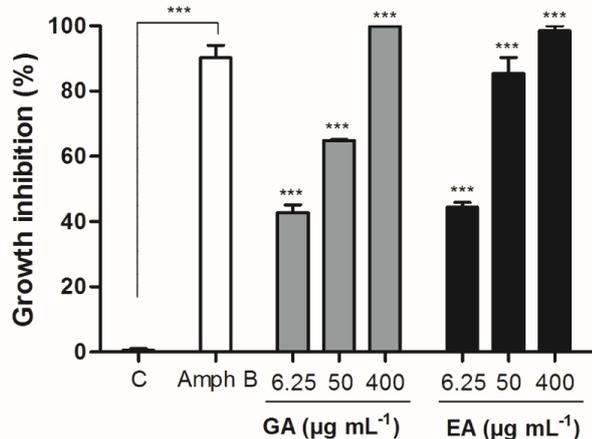


Figure 04. Effect of GA and EA (400, 50 and 6.25 $\mu\text{g}\cdot\text{mL}^{-1}$) or amphotericin B (Amph) (2 $\mu\text{g}\cdot\text{mL}^{-1}$) on *Leishmania amazonensis* promastigotes. Cultures of log-phase promastigotes (1×10^6 per well) were incubated at 26 °C for 48h in different GA and EA concentrations. Data represent the mean percentage of growth inhibition \pm standard error of 3 experiments carried out in triplicate. The same letter do not differ in Bonferroni's post-test ($p < 0.05$).

Infectious diseases caused by multidrug-resistant microorganisms had become a global problem leading to high morbidity and mortality rates (LOPEZ-CAMACHO et

al., 2014; STROMMENDER et al., 2014). In this context, some studies have proposed phytochemicals as promising alternatives to current antimicrobial agents (MARCHESE et al., 2016). Besides, they could be able to inhibit bacterial resistance mechanisms, recovering the effectiveness of current antibiotics against multidrug-resistant bacteria (LIMA et al., 2016; TINTINO et al., 2016).

Intrinsic antimicrobial activity was not observed for GA against bacterial or yeast strains tested in the present work. These results agree with a previous study which verified that GA did not present activity against isolate clinical strains of *S. aureus*, *P. aeruginosa* and *E. coli* (LIMA et al., 2016). On the other hand, EA showed antibacterial activity against *S. aureus* strains (MIC ranging from 128 to 256 $\mu\text{g}\cdot\text{mL}^{-1}$), as well as, antifungal activity against *C. albicans* (MIC 128 $\mu\text{g}/\text{mL}$). The inhibitory effects induced by EA against *S. aureus* and *C. albicans* were microbe static. The Gram-negative strain *P. aeruginosa* was not inhibited by EA.

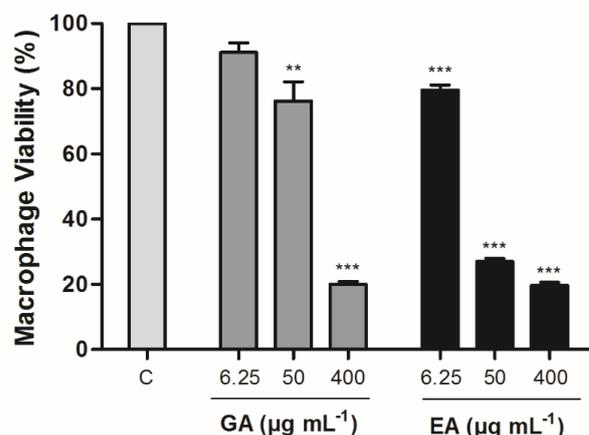


Figure 05: Cytotoxicity of GA and EA on the viability of murine peritoneal macrophages. Peritoneal macrophages were seeded at 1×10^5 per well in 96-well microplates and incubated for 48h in the presence of GA and EA at concentrations 400, 50 and 6.25 $\mu\text{g}\cdot\text{mL}^{-1}$. Viability was determined using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay. The optical density \pm standard error of 3 experiments carried out in triplicate. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

Modulation assays are important to verify if a substance is able to potentiate the activity of traditionally used antibiotics by inhibition of antibiotic-resistance mechanisms. Gallic acid has been related as modulator of drug resistance to Norfloxacin and Gentamicin in *S. aureus* strain isolated from clinical specimen (LIMA et al., 2016). In the present study, modulatory activity on resistance to Norfloxacin or Ciprofloxacin was evaluated using SA1199-B strain overexpressing the *norA* gene encoding the multidrug-transporter NorA, which confer fluoroquinolone-resistance by active efflux. (KAATZ; SEO, 1995). In this assay, the MIC for each antibiotic was determined in the

absence or presence of the compounds at subinhibitory concentrations. The results showed that GA and EA were not able to reduce the MIC of the fluoroquinolones tested, indicating that they are not NorA inhibitors.

	Macrophages CC ₅₀ (µg·mL ⁻¹)	Promastigotes IC ₅₀ (µg·mL ⁻¹)	SI
GA	126.5	10.94	11.56
EA	23.8	3.64	6.5
Amph B	8.750	1.742	5.02

Table 02. Antileishmanial and cytotoxic effects of GA and EA. SI (Selectivity index) = CC₅₀ on macrophages/IC₅₀ on promastigotes (48 h).

The need of discovery of novel antileishmanial compounds which are more effective and less toxic than conventional drugs has motivated research on natural products obtained from plant species. These products predominantly consist in alkaloids, terpenes, flavonoids, benzopyrans, phenolics, and sesquiterpene lactones, which have been identified in plant species with documented antileishmania activity (DE JESUS et al., 2012; RODRIGUES et al., 2013; RIBEIRO et al., 2015; RODRIGUES et al., 2015). In this sense, plant-derived polyphenols, such as stilbenoids, phenylpropanoids, flavonoids and quinones, have demonstrated high antileishmania activity against different species and forms of parasite in both *in vitro* and *in vivo* studies (DE JESUS et al., 2012).

The present study was conducted in order to evaluate the antileishmania activity of GA and EA against promastigote forms of *L. amazonensis*, as well as their cytotoxicity on mammalian cells. GA and EA showed significant concentration-dependent activity against promastigotes of *L. amazonensis*. Ogungbe; Erwin; Setzer., (2014) (OGUNGBE; ERWIN; SETZER, 2014) described around 352 phenolic compounds with *in silico* antileishmanial activity towards protein targets. Similar IC₅₀ values for GA and EA as major compounds of plant species were found for *Stryphnodendron obovatum* Benth. and *Anogeissus leiocarpus* (DC.) Guill. & Perr, with IC₅₀ values less than 30 µg·mL⁻¹ (SHUAIBU et al., 2008; RIBEIRO et al., 2015).

In this study, the EA has potential growth inhibitory action against the microorganisms used in our investigation, both in antimicrobial and antileishmania activities. This effect might be related to the previously reported EA-induced potent inhibitory effect of DNA topoisomerase II enzyme, hypothesizing the possible action on the process of replication or gene expression, and then opening perspectives for further approaches (VATTEM et al., 2005; AGGARWAL; SHISHODIA, 2006; CORTAZAR et al., 2007).

Considering the need of antileishmania substances which are more selective for the parasite and less toxic to host cells, as well as GA and EA showed low IC₅₀ values for parasite, the investigation of cytotoxic activity of GA and EA against macrophages was markedly important, since they are the main cells of the vertebrate host parasitized by *Leishmania* spp. (KAMHAWI, 2006; DE MEDEIROS et al., 2011; CARNEIRO et al., 2012). Although they demonstrated significant toxicity to these cells, both GA and EA demonstrated to be more toxic to the parasites than to the host cells, being even more selective to parasite than Amph B, which is the conventional drug widely used in the treatment of leishmaniasis.

CONCLUSION

The ellagic acid (EA) showed intrinsic antimicrobial activity against *S. aureus* and *C. albicans*, suggesting a potential use in the prevention or treatment of staphylococci-related diseases and candidiasis. Besides, GA and EA showed activity against promastigotes forms of *L. amazonensis* and cytotoxicity on BALB/c murine macrophages, indicating a promising application in further *in vivo* studies focused on the treatment of leishmaniasis.

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