The aim of this investigation was to evaluate the phytochemical, antibacterial and cytotoxic activities of ethanol extract and their fractions of Aristolochia galeata's rhizomes. The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) were evaluated by the broth microdilution assay to investigate the antibacterial activity of various extracts and fractions of Aristolochia galeata against Gram-positive and Gram-negative bacteria. The cytotoxicity of plant samples was evaluated in human cervix carcinoma cell line (HeLa) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The phytochemical study showed the presence of main secondary metabolites, steroids, flavonoids, coumarins and alkaloids. The ethanol extract and their fractions presented antimicrobial activity against Gram-positive bacteria, and Staphylococcus aureus was regarded the most sensitive strain with MIC of 250 µg/ml for the ethanol extract. The dichlorometane fraction showed bactericidal activity with the value of 1250 µg/ml and moderate cytotoxicity in front of the HeLa cell line tested (CC_{50} = 90 µg/ml). The results showed that A. galeata had effective antibacterial activity against Gram-negative bacteria and compounds extracted from Aristolochia galeata Mart. ex Zucc could be used as possible antimicrobials. The good antimicrobial activity and the low cytotoxicity presented by the hexane fraction can be promised for the new molecules with antibiotic activity.

**Key words:** Aristolochia galeata, antibacterial activity, minimal inhibitory concentration, cytotoxic activity, phytochemistry.

**INTRODUCTION**

The Aristolochia genus presents approximately 400 species distributed in areas from tropics to the temperate...
zones (Wu et al., 2001). *Aristolochia galeata* Mart. ex Zucc (Aristolochiaceae Family), is a native climber with a wide distribution in the Brazilian Cerrado biome, associated with road and gallery forest edges (Alves-Da-Silva et al., 2011). Some studies have indicated that many plants of *Aristolochia* genus have therapeutic properties such as analgesic, anti-diuretic, anti-inflammatory, antimicrobial, antioxidant and antiparasitic (Shafi et al., 2002; Yu et al., 2007; Pacheco et al., 2010; Papuc et al., 2010; Ahmed et al., 2010). However, many species of *Aristolochia* genus contain aristolochic acids, which can cause nephrotoxicity and mutagenicity (Kohara et al., 2002; Chen et al., 2007). Therefore, many countries have prohibited the use of phytotherapeutic drugs containing aristolochic acid and thus, numerous plant species with bioactive properties are no longer used, despite the possibility of separating the potentially toxic compounds (Yu et al., 2007).

Although there are no registers of antimicrobial activity of *A. galeata*, some compounds have already been isolated, and among them, stand out clerodane and labdane diterpenoids (Lopes and Bolzani, 1988). Compounds of these classes isolated from other species, present diverse biological properties, such as antiviral, antifungal and antibacterial (Salah et al., 2003; Vidal et al., 2011; Porto et al., 2012).

Studies on new therapeutic options from herbal products as antimicrobials are necessary, since bacterial infections have grown significantly, contributing to increased morbidity and mortality, especially in hospitalized and immunocompromised patients (Zhong et al., 2012; Schmitt et al., 2012; Pandey et al., 2012). Allied to the increased infections, treatment is becoming increasingly difficult in view of the notable ability of these pathogens to acquire new mechanisms of selective resistance to antibiotics (Tenover, 2006).

Therefore, in front of the lack of studies on phytochemistry, antimicrobial and cytotoxicity activity of *A. galeata* with the potential use of medicinal plants in the treatment of diseases caused by Gram-negative and -positive bacteria. The current investigation carried out a screening of ethanol extract and hexane, dichloromethane, ethyl acetate and hydroethanolic fractions of *A. galeata* against important pathogenic bacteria and evaluates its cytotoxicity potential to develop new antibacterial therapy.

**MATERIALS AND METHODS**

**Plant and extract preparation**

*A. galeata* rhizomes were collected in São Sebastião do Oeste, Minas Gerais, Brazil, located in coordinates -20° 14' 38.96"S and -45° 2' 14.38"W, altitude of 712 m, in August 2011. A voucher specimen (BHC 159396) was deposited at the Instituto de Ciências Biológicas Herbarium, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. The plant material (486.95 g) was dried at 40°C, triturated and extracted by cold maceration in ethanol P.A (Vetec, Brazil) for a period of 10 days at room temperature. The extract was filtrate and concentrated in a rotary evaporator (IKA equipment, model RV10, Germany) at 40°C under reduced pressure to yield ethanol extract. The dried extract (14.28 g) was obtained after lyophilization (Liobras equipment, model K 105, Brazil). Part of this extract (4 g) was dissolved in ethanol/water (7:3) (Vetec, Brazil) and then partitioned successively with hexane, dichloromethane and ethyl acetate (Vetec, Brazil) 15 ml, three times with each solvent, resulting in 0.471, 0.783, 0.823 and 1.741 g of hexane (F1), dichloromethane (F2), ethyl acetate (F3), and hydroalcoholic (F4) fractions, respectively. The extract and fractions were screened for the presence of different phytoconstituents like saponins, tannins, alkaloids, steroids, triterpenes, coumarins and flavonoids (Wagner et al., 2001).

**Bacterial strains and antimicrobial tests**

The minimum inhibitory concentration (MIC) of *A. galeata* ethanol extract and their fractions were determined using a broth microdilution method as described by Clinical and Laboratory Standards Institute (CLSI, 2003) with modifications. Nine reference bacterial strains of American Type Culture Collection (ATCC) were chosen due to their ability of present multi resistant to the drugs as follows: Gram-negative Escherichia coli ATCC 43895, Pseudomonas aeruginosa (ATCC 27853), Klebsiella pneumoniae (ATCC 27736), Salmonella typhi (ATCC 19430) and Gram-positive Staphylococcus aureus (ATCC 29213), Streptococcus mutans (ATCC 25175), Staphylococcus saprophyticus (ATCC 15305), Staphylococcus epidermidis (ATCC 12228), Enterococcus faecalis (ATCC 19433), donated by the Laboratory for Reference Microorganisms of the Oswaldo Cruz Foundation, FIOCRUZ, Brazil.

The ethanol extract and fractions were dissolved in sterile dimethylsulfoxide 2% (DMSO) (Synth, Brazil) and were used in serial dilution from of 1250 until 125 µg/ml. An inoculum of 125 µl of cell culture was added to 25 µl of each concentration of samples in Mueller-Hinton broth (MH) (Himedia, India) in 96-well plates. For negative controls, wells containing MH medium or sterile DMSO 2% were used and for positive control, MH plus bacteria and the antimicrobial agent streptomycin 100 µg/ml (Sigma-Aldrich, USA) were used (growth inhibition). Plates were incubated at 35 ± 1°C for 24 h.

The MIC was assessed based on the lowest concentration of sample required to inhibit the microbial growth and was determined by measuring the absorbance at 490 nm (Powder Wave XS2, Biotec, USA). The experiments were performed in triplicate.

For assays to determine the minimum lethal concentration (MLC), aliquots of 25 µl were removed from wells without visible turbidity and placed on Agar Plate-count by a Pour-Plate Method (Costa et al., 2010). After incubation at 37°C for 24 h, colonies were counted. The concentration of sample that resulted in a growth 0.1% of initial inoculum (1.5 × 10³ UFC/ml) was determined as the MLC.

**Cytotoxicity analysis by the MTT assay**

Human cervix carcinoma cell line (HeLa) was grown in Dulbecco's Modified Eagle Medium (DMEM) with 2% of Fetal Bovine Serum (FBS), at 37°C, 5% of CO₂ atmosphere and in 96-well microplate, until it reaches 95% of confluence. After 72 h exposure at dosages from 1000 to 0.025 µg/ml, 20 µl (2 mg/ml) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Bio Basic INC, Canada) in phosphate buffered saline (PBS) were added on each well and the plate was incubated at 37°C for 3 h. The medium was removed and 130 µl of DMSO were added and after incubation at 37°C for 10 min, the absorbance was read at 540 nm in ELISA spectrophotometer (Powder Wave XS2, Biotec, USA) to determine the concentration that killed 50% of cells (IC₅₀) (Twentyman and...
Dependent experiments. The results showed no bactericidal or bacteriostatic activity. This extract showed bacteriostatic activity against Gram-negative bacteria, and dichloromethane had MIC ranging from 500 to 1000 μg/ml. However, no Gram-negative bacteria showed sensitivity to components of plant extracts, but this may be related to the chemically more complex cell wall with the presence of additional membrane that can act as a selective barrier compared to the Gram-positive bacteria (Deans and Ritchie, 1987; Srinivasan et al., 2013a). Rios and Recio (2005) suggested that MIC greater than 1 mg/ml for crude extracts or 0.1 mg/ml for isolated compounds should be avoided and proposed that activity would be very interesting in MICs of 0.1 and 0.01 mg/ml for extracts and isolated compounds, respectively. On the other hand, Fabry et al. (1998) defined active crude extracts as those having MIC values <8 mg/ml. In this study, however, MIC and MFC values of less than 1 mg/ml were considered to be of good activity.

The DMSO used as a negative control, showed no bacteriostatic or bactericidal activity, as expected (data not show) (Table 3).

### Cytotoxicity activity

Analyzing the A. galeata cytotoxicity in vitro, the results show that the dichloromethane fraction presented moderate cytotoxic effect with CC$_{50}$ = 90 μg/ml (Table 3). However, the crude extract and other fractions showed little cytotoxicity activity with the CC$_{50}$ ranging between 1620 and 369 μg/ml.

### DISCUSSION

Many studies have demonstrated the antibacterial activity of plants commonly used in traditional medicine (Rakholiya and Chanda, 2012; Tekwu et al., 2012, Mishra et al., 2013a). Rios and Recio (2005) suggested that MIC greater than 1 mg/ml for crude extracts or 0.1 mg/ml for isolated compounds should be avoided and proposed that activity would be very interesting in MICs of 0.1 and 0.01 mg/ml for extracts and isolated compounds, respectively. On the other hand, Fabry et al. (1998) defined active crude extracts as those having MIC values <8 mg/ml. In this study, however, MIC and MFC values of less than 1 mg/ml were considered to be of good activity.

Our results showed that the crude extracts of A. galeata, and their fractions presented selective antibacterial activity against Gram-positive bacteria. It is not known exactly why Gram-negative bacteria typically have lower sensitivity to components of plant extracts, but this may be related to the chemically more complex cell wall with the presence of additional membrane that can act as a selective barrier compared to the Gram-positive bacteria (Deans and Ritchie, 1987; Srinivasan et al., 2001; Nikaido, 2003).

Gram-positive bacteria belonging to the genera Staphylococcus and Enterococcus, with clinical relevance, showed sensitivity to A. galeata. These genera are the most frequent cause of nosocomial infections by Gram-positive strains, and the species S. aureus,

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**Table 1.** Phytochemical study of ethanol extract (CE) and hexane (F1), dichlorometane (F2), ethyl acetate (F3) and hydroethanolic (F4) fractions from of Aristolochia galeata.

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Steroids/Triterpenoids</th>
<th>Flavonoids</th>
<th>Coumarins</th>
<th>Saponins</th>
<th>Alkaloids</th>
<th>Tanins</th>
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<tbody>
<tr>
<td>CE</td>
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<td>+++</td>
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(-) absence; (+/-) minimal presence; (+), (++) and (+++) grading presence.
S. epidermidis, S. saprophyticus and E. faecalis are the most common in this scenario (Boneca and Chiosis, 2003; Kuroda et al., 2005). These microorganisms may acquire resistance against antibacterial drugs by a variety of mechanisms that includes drug modification or destruction, alteration of binding sites, altered metabolism, and prevention of drug entry into the cell (Tenover, 2006; Barie, 2012).

The bacterial samples analyzed presented great sensitivity to aminoglycoside streptomycin used in this study. However, many studies have reported the resistance of both Gram-positive and Gram-negative bacteria to this class of antibiotics. This fact justifies the search for new molecules with antibacterial properties (Josephson, 2006; Coutinho et al., 2010; Zhong et al., 2012).

The Aristolochiaceae genus has revealed its potential as antimicrobial agent. Aristolochia species have also been described as getting antibacterial properties against strains of medical importance (Shafi et al., 2002). The A. esperanzae species showed antibacterial activity against samples of S. aureus, E. coli, S. typhimurium, B. cereus, C. freundii and L. monocytogenes (Pacheco et al., 2010). Some species of this genus stood out due to the potential cytotoxicity of cancer cell lines (Yu et al., 2007). In our study, A. galeata presented low cytotoxicity in HeLa cells, except for dichloromethane fraction. The presence of large amounts of steroids/triterpenoids on the dichloromethane fraction may be related with the antimicrobial activity. These results corroborate with literature data that showed antistaphylococcal activity of terpenes (Gibbons, 2004) and antifungal of steroids (Johann et al., 2010). However, the moderate cytotoxicity shown for this fraction (CC₅₀ = 90 µg/ml) may be related to compounds belonging to this class, such as sesquiterpenes, very common in species of this genus (Yu et al., 2007).

Coumarins and alkaloids were the main secondary metabolites found in the hexane fraction, which also demonstrated antibacterial activity. These classes of compounds are also described in the literature as natural antimicrobial agents (Cottiglia et al., 2001; Gibbons, 2004; Mishra et al., 2009; Mishra et al., 2013b). The hexane fraction has been promising, because besides having good antibacterial activity it also showed low cytotoxicity (CC₅₀ = 0.726 mg/ml) against the cell line studied. Moreover, the hydrophobicity of this fraction determines the minimal presence of triterpenoids compounds and aristolochic acid, which is expected, because these substances have cytotoxic and nephrotoxic effects.

### Conclusion

The results of the present study revealed for the first time evidence for prospect in A. galeata to new molecules with antibacterial activity, especially in the hexane fraction. It is relevant in front of the growing resistance that bacterial strains have shown to actual drugs.

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