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Analysis of aristolochlic acids and evaluation of antibacterial activity of *Aristolochia clematitis* L.

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Introduction: Several *Aristolochia* species were used as medicinal herb across Europe and in recent years, their antimicrobial activity has also been investigated. *Materials and methods:* In this study, *A. clematitis* was selected to evaluate the aristolochic acids I and II (AA I and AA II) concentrations and the antimicrobial activity of methanol, hexane, butanol, and ethyl acetate extracts of the root, stem, leaf, root, and fruit. AA I and AA II contents were measured by a validated high-performance liquid chromatography–ultraviolet method. *Results:* Each fraction of the plant contained AA I and AA II and the root was found to have the highest contents of AA I (1.09%) and AA II (0.7454%). The minimum inhibitory concentrations of all extracts were determined by standard microdilution method. The fruit's extracts showed the most efficient antimicrobial effect against both methicillin sensitive and resistant *Staphylococcus aureus* strains. *Conclusion:* Correlation between the AA I and AA II concentrations and the antimicrobial effect was not found.

INTRODUCTION

A number of recently discovered medications are based on herbs used as remedies for centuries. Some of them with antibacterial, antifungal and antiprotozoal activities are widely used both in human and veterinary medicine (Heinrich et al., 2004). Plants are possible sources of antimicrobial compounds for modern medicine with valuable therapeutic potential, which are not just effective in the treatment but may mitigate the side effects of synthetic antimicrobial agents (Iwu et al., 1999).

Aristolochia (birthwort) is a large genus belonging to *Aristolochiaceae* family. Many investigations analyzed the components of species of this genus. *Aristolochia trilobata* L. stem contains carboxylic acid ester (6-methyl-5-hepten-2-yl acetate), terpenes (limonene, linalool, and p-cymene), sesquiterpene (bicyclogermacrene), and sesquiterpenoid (spathulenol; Santos et al., 2014). Isoquinoline alkaloids (constrictosoines) were isolated from the aerial parts of *Aristolochia constricta* Grisebach. (Rastrelli et al., 1997). Futhermore isoquinolones, biphenyl ethers (aristogin F), and benzoyl benzyltetrahydroisoquinoline ether alkaloids were demonstrated in the root and stem extracts of *Aristolochia elegans* Mast. (Shi et al., 2004). Nitroaromatic compounds (9-methoxytariacuripyrone and 7,9-dimethoxytariacuripyrone) and aristolactams were detected in extracts of the rhizome of *Aristolochia brevipes* Benth. (Achenbach et al., 1992).

Aristolochic acids I and II (AA I and AA II; Fig. 1) as main compounds occurring in most of *Aristolochia* species have nephrotoxic and genotoxic effects (Heinrich et al., 2009). A case report of a patient, who had taken slimming pills contained *Aristolochia fangchi* Y. C. Wu ex L. D. Chow & S. M. Hwang (Chow & Hwang, 1975), described the nephropathy with renal fibrosis (Vanherweghem et al., 1993). Similar results were also observed in studies with larger sample size (Cosyns, 2003; Debelle et al., 2008).

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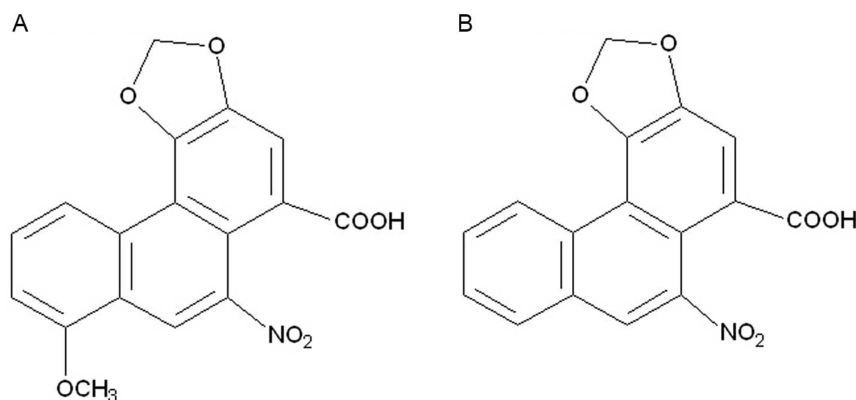

Fig. 1. Constitutional formulas of aristolochic acids I (A) and II (B)

Birthworts contain minerals (Na, K, Ca, Mn, Cu, Fe, Cr, and Zn), polyphenols (Butnariu et al., 2012; Crivineanu et al., 2009) such as flavonoids and tannins (Abbouyi et al., 2016), sesquiterpenic lactone aristolone, AA I and AA II, aristolactam *N*-β-D-glucoside, β-sitosterol and its β-D-glucoside, aporphine alkaloid magnoflorine, sitosterol β-D-glucoside, and methyl 4-coumarate (Košťálová et al., 1991).

In pharmacological reports, *A. clematitis* L. has shown antioxidant activity (Abbouyi et al., 2016). Wistar rats were treated with sodium salt of AA showed dose- and time-dependent development of tumors as papillomatosis of the forestomach; in low-dose treatment (0.1 mg/kg), no tumor was found in the first 6 months, and found only after 12 and 16 months (Mengs et al., 1982). After oral treatment of rats with different metabolites of AA I and AA II (aristolactam I, aristolactam Ia, AA 1a, and 3,4-methylenedioxy-8-hydroxy-1-phenanthrenecarboxylic acid as metabolites of AA I or aristolactam Ia, aristolactam II, and 3,4-methylenedioxy-l-phenanthrenecarboxylic acid as metabolites of AA II), these metabolites were detected in the urine (Krumbiegel et al., 1987). In spite of the known toxic effect of AA, these compounds could be used topically against wound infection based on several examples from ethnopharmacology.

In this study, *A. clematitis* (European birthwort) was selected for analyses, which has been cultivated all across Europe (Tutin et al., 2010). In the ethnomedicine, fresh leaves were used for infected wounds as a foment (Butură, 1979; Gub, 1993; Keszeg, 1981; Péntek & Szabó, 1985) or a decoction (Dénes et al., 2014; Gub, 2000), for abscess (Szabó, 2002; Tóth & Papp, 2014), ulcer (Bahmani & Eftekhari, 2013), eczema (Tóth & Papp, 2014), and rheumatic disease both in human and ethnoveterinary medicine (Bartha et al., 2015) in Romania. The aerial part was applied for wound infection in Kosovo (Mustafa et al., 2012) and Serbia (Jarič et al., 2007), similar to the use of the root's decoction in Bulgaria (Leporatti & Ivancheva, 2003), or that of the rhizome in Italy (Leporatti & Ivancheva, 2003).

The aims of this study were to determine the antimicrobial effect and the contents of AA I and AA II in extracts isolated from different parts of *A. clematitis*. Our hypothesis is that the studied plant parts contain different concentrations of AAs. AAs are slightly soluble in water but soluble in methanol, chloroform, ethyl acetate, and butanol. This supposes that the residual water extracts contain the smallest concentration of AAs and the presence of these compounds in the extract may explain the belief that the application of these plants helps in treating wound infection.

MATERIALS AND METHODS

Chemicals

AA I and AA II were purchased from Sigma-Aldrich (Budapest, Hungary). The materials and reagents applied in the preparation and analysis of *A. clematitis* were all of analytical reagent grade of the highest purity available, such as acetonitrile (ACN; VWR Chemicals, Belgium), methanol, hexane, chloroform, ethyl acetate, and butanol (Molar Chemicals, Hungary).

Sample collection

The aerial parts and the roots of *A. clematitis* were collected at weed community in Augustin (Romania) in 2017. Until the time of further processing, the samples were dried at room temperature. Voucher specimen was deposited at the Department of Pharmacognosy, University of Pécs.

Preparation of extracts

The preparation of the plant's extracts for high-performance liquid chromatography (HPLC) and microbiological analysis was performed according to Lee et al. (2014). Briefly, 3 g of dried leaf, fruit, root, and stem were ground separately. Each sample was suspended with methanol in a ratio 1:10 in Erlenmeyer flask individually and soaked by agitation at 150 rpm for 24 hr. The samples were filtered through No. 1 Whatman paper and the eluent was evaporated. Each residue was resuspended in 2 ml of methanol. An amount of 5 ml of distilled water and 5 ml of hexane were given to 1:1 ml suspension and mixed. The polar and non-polar solvent extracts were separated from each other and the hexane phase was collected and evaporated. Dried residues were measured and then chloroform, ethyl acetate, and butanol were used for further extraction. Dried residues were dissolved with dimethyl sulfoxide (DMSO; Sigma-Aldrich). Further dilutions were performed in Mueller–Hinton broth

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to reach the appropriate concentration of DMSO (1%–2%–2.5%) and six extracts (0.5–2 mg/ml) in each studied plant part for antimicrobial examination.

HPLC methods

The AA I and AA II contents of all 24 extracts were determined by HPLC–ultraviolet (HPLC-UV) method based on previously validated work (Sorenson & Sullivan, 2007) with minor modifications. HPLC analysis was performed by an Agilent 1260 Infinity LC system (G1312B binary gradient pump, G1367E autosampler, G1315C diode array detector, Agilent Technologies, Waldbronn, Germany). Chromatography was carried out using a Kinetex C18 column (100 mm × 4.6 mm, 2.6 μm; Phenomenex, Los Angeles, CA, USA), maintained at 20 °C. The following gradient elution program was applied at flow rate of 0.7 ml/min, where eluent A was 0.1% (v/v) formic acid, and eluent B was ACN: 0 min: 20% (v/v) B, 25 min: 70% (v/v) B, 30 min: 100% (v/v) B, 31 min: 20% (v/v) B, 40 min: 20% (v/v) B. UV detection was performed at 390 nm. Calibration curves were prepared using six concentrations between 1 and 500 μg/ml. Calibration curves were constructed by the least-square linear regression analysis with uniform weighting. Linear relationships were found for both isomers with the following equation $y = 41.164 \times -0.4646$ ($r^2 = .9998$) and $y = 40.3225 \times -0.4516$ ($r^2 = .9997$) for AA I and AA II, respectively (x = concentration of compounds in μg/ml and y = peak area of compounds). For checking the applicability of the method, intra- and interday relative standard deviations (low, mid, and high concentrations of the standards in three parallel runs on the same day and on three successive days, respectively) were determined that were less than 1.25% and 1.48%, respectively.

Microbial strains and culture media

All of 24 extracts were tested against *Staphylococcus aureus* ATCC 23923, methicillin-resistant *S. aureus* (MRSA) ATCC 700698, *Escherichia coli* ATCC 25922, clinical isolates of extensive spectrum β-lactamase (ESBL)-producing *E. coli*, and *Klebsiella pneumoniae* strains, *K. pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, clinical isolate of *P. aeruginosa* multidrug resistant (MDR), *Salmonella* Typhimurium [abbreviated scientific name of *Salmonella enterica* subsp. *enterica* (Le Minor and Popoff)] serovar. Typhimurium ATCC 14028, and clinical isolate of *Acinetobacter baumannii* MDR strain.

Mueller–Hinton broth and agar (Oxoid, Basingstoke, UK) were used as culture media for the microdilution methods and evaluation of minimum inhibitory and bactericidal concentration.

Broth microdilution method for determination of minimum inhibitory concentration (MIC) of the plant extracts

The procedure involved preparing twofold dilutions of the solved and diluted extracts (initial concentrations of extracts were 0.5–2 mg/ml depended on amounts of dried extracts, and initial DMSO concentrations were 1%–2%–2.5%) in 0.1 ml of Mueller–Hinton broth dispensed in the wells of sterile 96-wells tissue culture plate (Sarstedt, Nurnbrecht, Germany). Each extract fraction was diluted three times for each investigated bacterium strain. Bacterial inoculums were prepared in sterile physiological saline (0.9% NaCl) after dilution of standardized microbial suspension adjusted to 0.5 McFarland scale. The wells were inoculated with 0.01 ml of 5×10^6 CFU/ml bacterial suspension and then the microplates were incubated at 37 °C overnight. The wells were examined for turbidity by the unaided eye and the concentration of extract where the growth of bacteria was inhibited giving the MIC. Subcultures were performed from unturbid wells for the determination of the bactericidal concentration. Appropriate antibiotics (depending on the strain, e.g., vancomycin for MRSA strain) were used as a positive control in microdilution, and diluted DMSO solution was used as a negative control (Clinical and Laboratory Standards Institute, 2012).

RESULTS

AA I and AA II contents by HPLC

All extracts of the studied plant parts of *A. clematitis* contained AA I. The root showed the highest amount in each extract especially its ethyl acetate extract with the highest value of the compound (1,347.9 μg). In the stem, the chloroform phase contained the highest (160.4 μg), whereas in the leaf, the water phase contained the less amount of AA I (0.0004 mg; Table 1).

AA II could be also detected in each extract of the studied parts of the plant. The ethyl acetate phase of the root extract contained the highest concentration of AA II (0.9536 mg), whereas the aqueous phases showed the lowest value in each extract (Table 1). An exemplified HPLC-UV chromatogram was depicted in Fig. 2.

MIC of the studied extracts

Each root extract fraction, which was solved in DMSO solution, has shown antimicrobial effect against MRSA strain at MIC values 1–2 mg/ml. Butanol extract of the root inhibited the growth of *S. aureus* and *P. aeruginosa* strains, whereas water extract had effect for MRSA and MDR *P. aeruginosa* strains (MIC = 1–2 mg/ml; Table 2).

Butanol extract of stem inhibited the multiplication of the investigated strains by 1–2 mg/ml except for *E. coli*, *K. pneumoniae* ESBL, and *S.* Typhimurium.

In the studied leaf extracts, the growth of both selected *S. aureus* was inhibited with the methanol, hexane, and ethyl acetate extracts in 2 mg/ml concentration. The s ame concentration of the methanol extract did not allow the growth of *K. pneumoniae* and *P. aeruginosa* strains. *P. aeruginosa* was also inhibited by the ethyl acetate extract.

Multiplication of both *S. aureus* strains was inhibited with each extract of the fruit except for water. The most effective fraction of this part was made of ethyl acetate with 62.5–125 μg/ml against *S. aureus* strains, respectively, with 1 mg/ml for *P. aeruginosa* and 2 mg/ml for *K. pneumoniae* (Table 3).

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Table 1. Aristolochic acids I and II (AA I and AA II) contents in the studied extracts of *A. clematitis*

Studied part	Solvent	Weight of extract after evaporation (mg)	AA I (μg)	AA I (%)	AA II (μg)	AA II (%)
Root	Methanol	101.8	698.6	0.6862	480.8	0.4723
	Hexane	12.0	9.0	0.0750	5.4	0.0450
	Chloroform	7.2	572	**7.9444**	328.1	**4.5569**
	Ethyl acetate	32	**1,347.9**	4,2122	**953.6**	2.9800
	Butanol	57.4	194.4	0.3387	161.7	0.2817
	Water	48.6	1.3	0.0027	1.1	0.0023
	Total	259	**1,823.2**	1,0900	**1930.7**	0.7454
Stem	Methanol	50.4	8.5	0.0169	2.2	0.0044
	Hexane	0.8	0.7	0.0874	0.2	0.0250
	Chloroform	1.2	**160.4**	**13.3667**	**37.1**	**3.0917**
	Ethyl acetate	4.8	55.9	1.1646	16.1	0.3354
	Butanol	10.5	9.5	0.0900	2.8	0.0267
	Water	42.7	0.6	0.0014	0.4	0.0009
	Total	110.4	235.6	0.2134	58.8	0.0533
Leaf	Methanol	82.4	75.8	0.0920	12.0	0.0146
	Hexane	0.8	10.6	1.3250	0.16	0.0200
	Chloroform	1	**278.4**	**27.8400**	**44.2**	**4.4200**
	Ethyl acetate	5.3	200.5	3.7834	29.8	0.5623
	Butanol	34.8	131.9	0.3789	19.6	0.0563
	Water	46	0.4	0.0009	0.2	0.0004
	Total	170.3	697.6	0.4097	105.96	0.0622
Fruit	Methanol	250.9	203	0.0809	11.9	0.0047
	Hexane	4.2	13.1	0.3119	0.8	0.0190
	Chloroform	30.2	**821.0**	2.7185	56.9	0.1884
	Ethyl acetate	4.0	654.9	**16.3672**	**436.5**	**10.9125**
	Butanol	57.8	379	0.6557	23.7	0.0410
	Water	198.4	1.4	0.0007	0.9	0.0004
	Total	545.5	2072.4	0.3799	530.7	0.0973

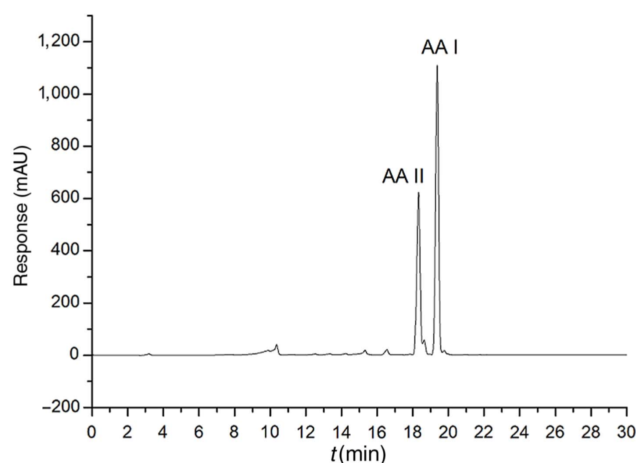

Fig. 2. HPLC-UV chromatogram of the chloroform phase of the root. AA I: aristolochic acid I; AA II: aristolochic acid II

DISCUSSION

In previous reports, more than 100 phytochemical compounds of different *Aristolochia* species have been analyzed with their biological activities (Kuo et al., 2012; Wu et al., 2004), but total phytochemical study of the root, stem, leaf, and fruit of *A. clematitis* and other species of the genus has not been carried out. *Aristolochia* species showed antibacterial and antifungal effects against various microorganisms, such as *Aristolochia indica* against *S. aureus*, *Staphylococcus epidermidis*, *Bacillus megaterium*, *E. coli*, *Salmonella* Typhi, and *Vibrio cholerae* (Farhana et al., 2016), *Aristolochia bracteolata* for *Aspergillus flavus* and *Botrytis cinerea* (Trayee et al., 2016), or *A. trilobata*, *A. brevipes*, *Aristolochia monticola* Brandegee and *Aristolochia kristsagathra* against *S. aureus* (Camporese et al., 2003; Moorthy et al., 2015; Murillo-Alvarez et al., 2001). Among the identified compounds, such as 7,9 dimethoxytariacuripyrone, licarin A and B have antimicrobial effect out of AAs (Kuo et al., 2012).

In this study, the examined extracts including methanol extracts of *A. clematitis* had no effect against *E. coli* similar to the report of *A. brevipes* and *A. monticola* (Murillo-Alvarez et al., 2001), in contrast with the methanol and acetone extract of *A. bracteolata* investigated previously (Vaghasiya & Chanda, 2007). Methanol, hexane, and ethyl acetate extracts of the leaf of *A. clematitis* showed the same effectivity against both *S. aureus* strains in higher concentration (2 mg/ml) similar to the same fractions of the leaf of *A. bracteolata* (Trayee et al., 2016). The most efficient antimicrobial activity against *S. aureus* strains was detected in the case of the fruit extracts. In contrast with earlier findings (Angalaparameswari et al., 2011), we could not

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Table 2. Minimum inhibitory concentrations (MICs) of the studied extracts of the root and stem of *A. clematitis*

Strains	Root						Stem					
	Methanol	Hexane	Chloroform	Ethyl acetate	Butanol	Water	Methanol	Hexane	Chloroform	Ethyl acetate	Butanol	Water
	MIC of the extracts (μg/ml)						MIC of the extracts (μg/ml)					
S. aureus ATCC 23923	N	N	N	N	2,000	2,000	N	N	N	N	1,000	N
MRSA ATCC 700698	2,000	2,000	2,000	1,000	1,000	2,000	N	N	N	N	1,000	N
E. coli ATCC 25922	N	N	N	N	N	N	N	N	N	N	N	N
E. coli ESBL	N	N	N	N	N	N	N	N	N	N	N	N
K. pneumoniae ATCC 13883	N	N	N	N	N	N	N	N	N	N	2,000	N
K. pneumoniae ESBL	N	N	N	N	N	N	N	N	N	N	N	N
P. aeruginosa ATCC 27853	N	N	N	N	1,000	2,000	N	N	N	N	2,000	N
P. aeruginosa MDR	N	N	N	N	N	1,000	N	N	N	N	2,000	N
S. Typhimurium ATCC 14028	N	N	N	N	N	N	N	N	N	N	N	N
A. baumannii MDR	N	N	N	N	N	N	N	N	N	N	1,000	N

Note. N: MIC value was not determined. ESBL: extensive spectrum β-lactamase; MDR: multidrug resistant; MRSA: methicillin-resistant *Staphylococcus aureus*.

Table 3. Minimum inhibitory concentrations (MICs) of the studied extracts of the leaf and fruit of *A. clematitis*

Tested strains	Leaf						Fruit					
	Methanol	Hexane	Chloroform	Ethyl acetate	Butanol	Water	Methanol	Hexane	Chloroform	Ethyl acetate	Butanol	Water
	MIC of the extracts (μg/ml)						MIC of the extracts (μg/ml)					
S. aureus ATCC 23923	2,000	2,000	N	2,000	N	N	2,000	500	500	125	2,000	N
MRSA ATCC 700698	2,000	2,000	N	2,000	N	N	2,000	500	500	62.5	2,000	N
E. coli ATCC 25922	N	N	N	N	N	N	N	N	N	N	N	N
E. coli ESBL	N	N	N	N	N	N	N	N	N	N	N	N
K. pneumoniae ATCC 13883	2,000	N	N	N	N	N	N	N	N	2,000	N	N
K. pneumoniae ESBL	N	N	N	N	N	N	N	N	N	N	N	N
P. aeruginosa ATCC 27853	2,000	N	N	2,000	N	N	N	2,000	N	1,000	N	N
P. aeruginosa MDR	N	N	N	N	N	N	N	N	N	N	N	N
S. Typhimurium ATCC 14028	N	N	N	N	N	N	N	N	N	N	N	N
A. baumannii MDR	N	N	N	N	N	N	N	N	N	N	N	N

Note. N: MIC value was not determined. ESBL: extensive spectrum β-lactamase; MDR: multidrug resistant; MRSA: methicillin-resistant *Staphylococcus aureus.*

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demonstrate more effective antimicrobial activity with the fractions containing the highest concentration of AA I and AA II, as in the case of the chloroform fraction of the leaf having the highest AA I content but no effect against any investigated strain. It could be explained that not AA is responsible for the antimicrobial effect or the negative interactions between the components of the extracts. However, correct explanation may require further investigations.

S. aureus strains more frequently cause skin and wound infections than pneumonia. Extracts of different *Aristolochia* species have highest activity against this strain. Probably, tannins, phenolic compounds (four coumaric acids and flavonoids), and saponins of *A. clematitis* may be responsible for the antimicrobial activity of the species (Abbouyi et al., 2016; Benmehdi et al., 2017; Košťálová et al., 1991). These compounds can be solved in different concentrations in polar and non-polar solvents, so they may be found in different concentrations in each extract resulting in various antimicrobial effects.

CONCLUSION FOR FUTURE BIOLOGY

However, AA I was previously described as a compound responsible for the antibacterial activity in some *Aristolochia* species but our results in *A. clematitis* do not confirm this fact. Further studies are required to determine whether other compounds may contribute to activity of AA I, and what kind of mechanism controls the action of the different fractions of *A. clematitis*, which may be responsible for the antibacterial activity. Our results could lay the scientific basic of future clinical perspectives of different parts of birthworts.

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Ethical Statement: The work does not require permission and ethical approval.

Data Accessibility: This work does not include Supplementary Material and digital research materials.

Competing Interests: The authors declare no competing interests.

Authors' Contributions SGB collected the plant samples, performed the preparation of plant's extracts, and participated in the microbiological studies. GT, PH, and EK participated in HPLC detection as well as analysis and interpretation of data. SGB and MK significantly contributed to planning of the study, acquisition and analysis of data, and interpretation of the results. MK helped in the microbiological study and evaluation of the results. SGB, GT, PH, EK, NP, and MK participated in the drafting and revising of the article. All authors agreed with the content of the manuscript and sent it to *Biologia Futura* for possible publication.

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