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Original Article

Polycarpol in *Unonopsis*, *Bocageopsis* and *Onychopetalum* Amazonian species: chemosystematical implications and antimicrobial evaluation

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ABSTRACT

Polycarpol, a recurrent lanostane-type triterpene in Annonaceae family, was confirmed by thin layer chromatography and mass spectrometry analysis in the aerial parts (twigs and trunk barks) of *Unonopsis duckei* R.E. Fr., *U. floribunda* Diels, *U. rufescens* (Baill.) R.E. Fr., *U. stipitata* Diels, *Onychopetalum amazonicum* R.E. Fr. and *Bocageopsis pleiosperma* Maas. Its chemotaxonomic significance was discussed for these three genera, as well for the Annonaceae family. In addition, the antimicrobial activity against several strains of microorganisms was evaluated for the first time for this compound, being observed significant antibacterial activity against *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 1228) and *Escherichia coli* (ATCC 10538 and ATCC 10799) with minimal inhibitory concentration values between 25 and 50 $\mu g\,ml^{-1}$.

Introduction

The Annonaceae family comprises about 2500 species distributed in 130 genera. This family consists of trees, shrubs and climbers, with a predominant distribution in lowlands of tropical and subtropical regions, being considered a pantropical family (Richardson et al., 2004). This family is chemically characterized by the presence of alkaloids, particularly isoquinoline-derived and terpenoids, where monoterpenes and sesquiterpenes are predominant in the composition of essential oils (Leboeuf et al., 1982; Fournier et al., 1999). Another remarkable feature is the presence of annonaceous acetogenins, a class exclusive from this family, that has attracted interest due to the growing list of newly described structures and biological activities (Chang et al., 1999; Cunha et al., 2009). Acetogenins along with the lanostane-type triterpene polycarpol have been suggested as potential chemotaxonomic markers for this family (Leboeuf et al., 1982; Goulart et al., 1986; Jung et al., 1990).

Polycarpol displays a growing list of biological activities described in the literature, such as antifilarial (Nyasse et al., 2006), antineoplastic (Matos et al., 2006), antitrypanosomal (Ngantchou et al., 2009) and more recently anti-inflammatory (Saadawi et al., 2012). Although triterpenes possess proven antimicrobial activities (Haraguchi et al., 1999; Katerere et al., 2003; Djoukeng et al., 2005; Angeh et al., 2007) a lack of studies describing the antibacterial activities of polycarpol is observed. The continuous search for new antimicrobial drugs is stimulated by the increasing appearance of antibiotic-resistant organisms, such as individuals of the *Staphylococcus*, *Pseudomonas*, *Enterococcus*, and *Pneumococcus* genera (Pacheco et al., 2012).

Unonopsis, *Bocageopsis* and *Onychopetalum* are botanically close genera distributed through neotropical regions. The morphological similarities among these genera were expressed by Fries, when he placed them in the informal "Unonopsis-Gruppe". Recently this close relationship was supported by phylogenetic researches (Maas et al., 2007). *Unonopsis* is the largest genus among these three, comprising approximately 50 species. On the other hand, *Bocageopsis* and *Onychopetalum* comprise few species, four and two, respectively (*B. mattogrossensis*, *B. canescens*, *B. multiflora* and *B. pleiosperma*, *O. amazonicum* and *O. periquino*) (Maas et al., 2007). *Unonopsis* is also the most explored from the chemical and

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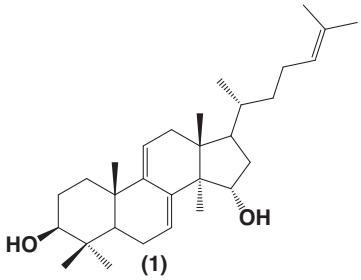
biological points of view (Siqueira et al., 1998; Waechter et al., 1999; Silva et al., 2012b,c, 2014). The chemical information regarding the *Bocageopsis* and *Onychopetalum* genera is still limited, being focused directed to the study of the essential oils and alkaloid compositions (Almeida et al., 1976; Oliveira et al., 2014; Soares et al., 2015). In this work, the presence of the triterpene polycarpol (**1**) in the aerial parts (trunk barks and twigs) of Amazonian *Bocageopsis*, *Onychopetalum* and *Unonopsis* species was investigated by thin layer chromatography (TLC) and mass spectrometry (MS) approaches. The chemotaxonomic significance of polycarpol was discussed for these three genera and for the Annonaceae family. In addition, polycarpol was submitted to *in vitro* assays to evaluate its antimicrobial activity against *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 1228), *Pseudomonas aeruginosa* (ATCC 27853), *Enterobacter faecalis* (Ef), *Bacillus subtilis* (Bs), *Escherichia coli* (ATCC 10538 and 10799), *Candida albicans* (ATCC 10231 and 1023), *Candida parapsilosis* (ATCC 22019), *Candida tropicalis* (ATCC 157 and ct), *Candida glabrata* (ATCC 30070) and *Candida dubliniensis* (ATCC 778157).



Materials and methods

Plant material

The aerial parts (twigs and trunk barks) of *Unonopsis duckei* R.E. Fr., (voucher no. 3289), *Onychopetalum amazonicum* R.E. Fr. (voucher no. 218341) and *Bocageopsis pleiosperma* Maas (voucher no. 183125) were collected in the Reserva Florestal Adolpho Ducke, at the municipality of Manaus, Brazil, from individuals previously identified by specialists. The voucher specimens are deposited in the herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA). The same parts for *U. floribunda* (voucher no. 6701) and *U. rufescens* (voucher no. 3767) were collected in the Distrito Agropecuário da SUFRAMA in the same city, from individuals previously identified by specialists. The voucher specimens are deposited in the botany collection of PDBFF/INPA (Projeto Dinâmica Biológica de Fragmentos Florestais). The plant material (twigs and trunk barks) of *U. stipitata* (voucher no. 8250) was collected in the campus of the Universidade Federal do Amazonas (UFAM). A voucher specimen was deposited in the Herbarium of the institution. The specimen collected on the campus of UFAM was identified by Prof. Antonio Carlos Webber from the Departamento de Biologia of the UFAM. The SISBIO license number for the collection is 34677-1.

Extraction and confirmation of the polycarpol

The extraction of polycarpol from *U. duckei*, *U. floribunda*, *U. rufescens*, *U. stipitata*, *O. amazonicum* and *B. pleiosperma* was made according to the adapted method described for *U. guatterioides* (Silva et al., 2012a), briefly: 10 g of powdered material (twigs and trunk barks) were macerated for three days with hexane (80 ml). The extract was concentrated at reduced pressure. The precipitate formed was washed with hexane and re-crystallized in ethyl acetate. Approximately 1 mg of the each solid was separated for TLC and MS analysis, being polycarpol (**1**) identified by comparison with an authentic standard (Silva et al., 2012a).

Table 1
Antimicrobial activity *in vitro* of polycarpol with their MIC values (μg ml^{-1}).

Microorganism	Polycarpol MIC[a]	Positive controls[b] MIC
Staphylococcus aureus (ATCC 6538)[c]	25	25
Staphylococcus epidermidis (ATCC 1228)[c]	50	50
Bacillus subtilis (Bs)[d]	–[e]	50
Escherichia coli (ATCC 10538)[c]	50	50
Escherichia coli (ATCC 10799)[c]	50	50
Pseudomonas aeruginosa (ATCC 27853)[c]	–	>500
Enterobacter faecalis (Ef)[d]	–	50
Candida albicans (ATCC 10231)[c]	250	12.5
Candida albicans (ATCC 1023)[c]	250	12.5
Candida parapsilosis (ATCC 22019)[c]	–	12.5
Candida tropicalis (ATCC 157)[c]	–	12.5
Candida tropicalis (ct)[d]	–	12.5
Candida glabrata (ATCC 30070)[c]	–	12.5
Candida dubliniensis (ATCC 778157)[c]	250	12.5

[a] MIC minimum inhibitory concentration in μg ml^{-1}.
[b] Positive control: chloramphenicol for bacteria strains and ketoconazole for yeast strains.
[c] Standard strain.
[d] Field strain.
[e] (–) without inhibition of development. Compounds evaluated in the range of 10 and 500 μg ml^{-1}.

Biological assay

The pure standard of polycarpol was evaluated for its antimicrobial activity using the broth microdilution method (96-well microtiter plates), as previously described (Salvador et al., 2002; Barros et al., 2009; Costa et al., 2010; Bataglion et al., 2014) to give concentrations between 10 and 500 μg ml^{-1}. The minimal inhibitory concentration (MIC) was calculated as the lowest concentration showing complete inhibition of a tested strain. In these tests, chloramphenicol and ketoconazole were used as experimental positive control, while the solution propyleneglycol-sterile distilled water (5:95, v/v) served as the negative control. Each sensitivity test was performed in duplicate for each microorganism and repeated three times. The strains utilized in the assays are shown in Table 1.

Instruments and materials

TLC analysis were run on a 0.25 mm tick aluminum-backed silica-gel 60 plates type F-UV$_{254\ and\ 366}$ from Merck (Darmstadt, Germany). The spots were exposure to ultra-violet (UV) light at 254 and 366 nm, as well as revelation with vanillin–sulphuric acid reagent. The MS analyses were performed using a LCQ Fleet ion-trap mass spectrometer (Thermo LCQ Fleet – San Jose, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source operating in positive ion mode. All mass spectra were acquired in a continuous monitoring mode (Thermo LCQ Fleet Tune application). The TLC and MS experiments were conducted in comparison with an authentic polycarpol standard previously isolated from *U. guatterioides* and characterized by nuclear magnetic resonance experiments (NMR) (Silva et al., 2012a). All solvents used for chromatographic and MS experiments were high performance liquid chromatography (HPLC) grade purchased from Tedia (Fairfield, OH, USA), and the water purified by using a Milli-Q system (Millipore, Bedford, MA, USA).

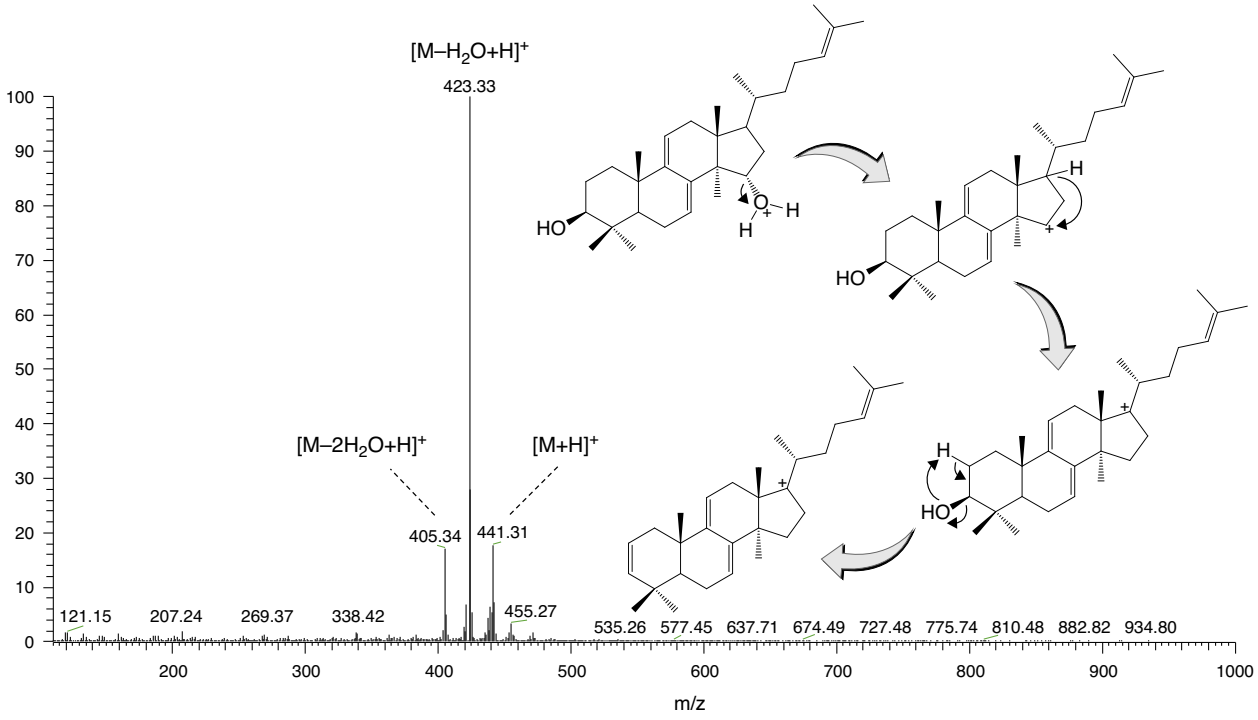


Fig. 1. Mass spectrum and in source thermal dissociation proposal for the sequential losses observed for APCI ionization of polycarpol.

Results and discussion

Confirmation of polycarpol by TLC and MS analyses

From the aerial parts of *U. duckei*, *U. floribunda*, *U. rufescens*, *U. stipitata*, *O. amazonicum* and *B. pleiosperma* white solids were obtained after extraction and re-crystallization procedures. All the samples were dissolved in ethyl acetate and analyzed by TLC in comparison with an authentic polycarpol standard (Silva et al., 2012a). The samples and the standard presented the same retention factor (R_f 0.6) when eluted with a hexane–ethyl acetate (7:3) mixture and revealed with vanillin–sulphuric acid reagent (blue spot) and under UV light (254 nm). The observation of an intense blue spot in all samples is in agreement with results expected for triterpene compounds (Oleszek et al., 2008). The samples when subjected to MS analysis in full scan mode, presented three major ions at *m*/*z* 441 $[M+H]^+$, 423 (−18 Da) and 405 (−18 Da) (Fig. 1). The ion at *m*/*z* 441 corresponds to the protonated molecule expected for polycarpol, while the *m*/*z* 423 and 405 are consistent with sequential neutral losses ($-H_2O$). Hydroxilated triterpenes present this dissociative behavior due to the application of high desolvation temperatures in APCI experiments (Koolen et al., 2013). All samples presented the same three major ions when subjected to MS analysis. These evidence along with TLC observations assisted the confirmation of polycarpol (**1**) in aerial parts (twigs and trunk barks) of *U. duckei*, *U. floribunda*, *U. rufescens*, *U. stipitata*, *O. amazonicum* and *B. pleiosperma*, being this substance reported for the first time in this *Unonopsis* species as well in *Onychopetalum* and *Bocageopsis* genus.

Antibacterial activity

The polycarpol was evaluated *in vitro* for antimicrobial activity against eleven strains of microorganism (Table 1). This substance presented significant antimicrobial activity against *S. aureus* (ATCC 6538), *S. epidermidis* (ATCC 1228) and *E. coli* (ATCC 10538 and ATCC 10799) with MIC values between 25 and 50 μg ml^{-1}. For the strains of *B. subtilis*, *E. faecalis* and *P. aeruginosa* were not observed inhibition of the development. These observations are in agreement with literature data, since lannostane triterpenes isolated from the vegetal species and fungi when assayed against gram-negative and gram-positive bacteria, present several active structures (Liu et al., 2010a,b; Mosa et al., 2014). Oleanane, ursane, lupane, friedelane, fernane and others miscellaneous-type triterpenes also present several active compounds against gram-negative bacteria, mainly *P. aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, and *Salmonella typhi*, and gram-positive, comprising *S. aureus*, *B. subtilis*, *Bacillus cereus*, and *S. faecalis*. The tentative understanding of the relationships between the chemical structures of triterpenes and the antibacterial activities indicates that the activity may be related to the presence of an oxygenated group at C-3 (Pacheco et al., 2012). In polycarpol this position is occupied by a hydroxyl group, however another substituents, like carbonyls, *O*-glycosides, esters (mainly acetyl moieties), or hydroxylamines can be present in this position (Pacheco et al., 2012).

The significant antibacterial activities observed for polycarpol and described in this work for the first time needs further investigations, which could help in the search for new antimicrobial drugs.

Against the tested fungi, polycarpol showed inhibition on the growth of *C. albicans* (ATCC 10231 and ATCC 1023) and *C. dubliniensis* (ATCC 778157) with MIC values of 250 μg ml^{-1}, being this inhibitions considerate weak when compared to the positive control (ketoconazole, MIC 12.5 μg ml^{-1}). In general the antifungal activity of lanostane triterpenes is not well explored, being found only few works reporting this type of study (Kitagawa et al., 1981, 1985, 1989; Krohn et al., 1992; Hosoe et al., 2000). In most cases satisfactory activities are related to unusual lanostane-triterpenes (*e.g.* oligosides-lanostane, oligoglycosides-lanostane and lactone-lanostane triterpenes) (Kitagawa et al., 1981, 1985, 1989; Krohn et al., 1992).

Chemotaxonomic significance of polycarpol

The chemotaxonomic importance of the polycarpol for the Annonaceae family was firstly recognized by Leboeuf et al. (1982) during a systematic research with several species belonging to neighboring families, such as Lauraceae, Monimiaceae and Menispermaceae. At this time, polycarpol was not found, being suggested for the first time in literature as chemotaxonomic marker for Annonaceae. This idea was supported over the years (Goulart et al., 1986; Jung et al., 1990). Polycarpol was recently isolated from *Duguetia glabriuscula* (Pereira et al., 2003), *Duguetia furfuraceae* (Silva et al., 2007), *Artabotrys madagascariensis* (Murphy et al., 2008), *Piptostigma preussi* (Ngantchou et al., 2009) and *Artabotrys spinosus* (Sichaem et al., 2011), all species from Annonaceae family, which enhances the initial proposition of this compound as a chemotaxonomic marker for this family. Considering its broad occurrence at genera level, is appropriate to restrict the term "chemotaxonomic marker" at a family level, being suggested the use of the term "chemical marker" to genera level.

In *Unonopsis* genus, polycarpol has been reported in *Unonopsis glaucopetala* (Jayasuriya et al., 2005), *Unonopsis guatterioides* (Touché et al., 1981; Silva et al., 2012a), *Unonopsis spectabilis* (Laprévote et al., 1987) and *Unonopsis pacifica* (Arango et al., 1988). The re-current observation of polycarpol in all these species subjected to phytochemical approaches, as well as in *O. amazonicum* and *B. pleiosperma* is an important chemical evidence not only to confirm the botanical proximity of these genera (Maas et al., 2007), but also to reinforce polycarpol as a chemical marker for these genera. The insufficient phytochemical knowledge regarding *Onychopetalum* and *Bocageopsis* genera still not does allows a profound evaluation of the chemical similarities between them and *Unonopsis*. In this sense, phytochemical investigations directed at better understanding of the chemical relationships among these three close-related genera are indispensable in the future.

Conflict of interest

The authors declare no conflicts of interest.

Authors contributions

FMAS, HHFK (PhD students), BRL, ERS (MSc students), RAM (PhD student) and FASF (CI student) contributed equally in collecting plant samples, confirmation of the compound structure and written of the manuscript. WRC and MJS contributed to *in vitro* biological assays. ADLS, MLBP and AQLS supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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