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Antimicrobial activity of extracts and various fractions of chloroform extract from the lichen *Laurera benguelensis*

Nedeljko T. MANOJLOVIC[1*], Perica J. VASILJEVIC[2] and Zoran S. MARKOVIĆ[3]

[1] *Department of Pharmacy*, *Medical Faculty*, *University of Kragujevac*, *34000 Kragujevac*, *Serbia*
[2] *Department of Biology*, *Faculty of Science*, *University of Nis*, *Visegradska 33*, *Nis*, *Serbia*
[3] *Department of Biochemical and Medical Sciences*, *State University of Novi Pazar*, *Novi Pazar 36300*, *Serbia*

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The antimicrobial activity of the benzene, chloroform, acetone and methanol extracts and some organic fractions isolated from the lichen *Laurera benguelensis* growing in Thailand was investigated against the bacteria *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis*, and the fungi *Candida albicans*, *Mucor mucedo* and *Trichoderma harzianum* using the agar diffusion technique. The results showed that the extracts were effective against all tested microorganisms. The extracts demonstrated inhibition zones ranging from 3.5 to 10.3 mm against investigated bacteria and from 4.0 to 14.0 mm against investigated fungi. Chloroform fractions showed inhibition zones ranging from 5.0 to 15.0 mm for bacteria and from 8 to 13 mm for fungi. It appears that the antimicrobial activity of *Laurera benguelensis* is mainly related to the presence of lichexanthone. This is the first time that the antimicrobial activity of the lichen *L. benguelensis* is reported; also, this paper has a chemotaxonomic significance due to the fact that the literature referring to the secondary metabolites present in *Laurera* is rather limited. The results of the study provide scientific basis for the use of the lichen extracts as an accessible source of natural antimicrobial substances in the pharmaceutical industry.

Key words: *Laurera benguelensis*, Trypetheliaceae, lichexanthone, antimicrobial activity, extracts.

INTRODUCTION

Lichens produce a diverse range of primary and secondary metabolites (Hale, 1983). Slow growth and often harsh living conditions, make production of protective metabolites a necessity to lichens, and many secondary constituents are believed to serve as antigrowth, antimicrobial or antiherbivore agents (Hale, 1983; Rankovic *et al.*, 2008). A large number of lichen species have been proved to be a source of these metabolites for food and pharmaceutical industries. The lichen secondary metabolites show a wide range of potentially useful biological activities (Yamamoto *et al.*, 1993; Bačkor *et al.*, 1998; Land & Lundstrom, 1998; Shahi *et al.*, 2001). Most lichen substances with antibiotic activity are phenolic metabolites (e.g. usnic acid and the anthraquinone, endocrocin) (Hale 1983; Marcano *et al.*, 1999).

Lichens produce some characteristic anthraquinone and xanthone derivatives, which have yet to be found in higher plants (Santesson, 1970; Nakano *et al.*, 1972; Søchting, 1997, 2001; Balderrama *et al.*, 2001; Eichenberger *et al*., 2007). Anthraquinones and xanthones are also important constituents of plants, microorganisms and insects.

In the literature, the information about isolation of xanthones from the family Trypetheliaceae is limited. Until now, only 1,5,8-trihydroxy-6-methoxy-3-methyl-anthraquinone, has been found in other species of the genus *Laurera* (Stensio & Wachtmeister, 1969). It is an ingredient of many medicines of plant origin, and it possesses a broad spectrum of biological activities including antibacterial, antiinflammatory, antitumorous, purgative, astringent, antiviral (Cyong *et al.*, 1987; Muzychkina, 1998), antioxidant and antifungal (Agarwal *et al.*, 2000; Yen *et al.*, 2000; Manojlovic *et al.*, 2002, 2008).

* Corresponding author: tel.: +38 134364854, fax: +38 134 306800, e-mail: ntm@kg.ac.rs

Laurera benguelensis (Müll. Arg.) Zahlbr. is a tropical microlichen (pyreno-mycete) which belongs to the family Trypetheliaceae. Its color is gray to light-yellow. This lichen is distributed in tropical regions (Vongshewarat *et al.*, 1999). Until now, *L. benguelensis* metabolites have not been explored, and there is lack of information about their chemical activity. On the other hand, the biological activities of this lichen have never been reported.

In the present study, we have studied the antimicrobial properties of the benzene, chloroform, acetone and methanol extracts and the main xanthone metabolite of this tropical lichen.

MATERIALS AND METHODS

Lichen species studied

Lichen material was collected from the Kancanaburi Province (10 km south of the Erawan National Park) in Thailand during July 2006. The studied lichen was identified by Prof. Dr. Boonpagob, Department of Biology, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand as *Laurera benguelensis* (Müll. Arg.) Zahlbr. (voucher specimen, RU-22160). Lichens have been maintained at the lichen herbarium of the Ramkhamhaeng University, Thailand.

Preparation of lichen extracts

The lichen material was air dried at room temperature (26 ± 1˚C) for one week, after which it was grinded to a uniform powder. Benzene, chloroform, acetone and methanol extracts of the lichen *Laurera benguelensis* were prepared in a Soxhlet apparatus using 20 g of the lichen in 200 ml of each solvent for 8 hrs. All the extracts were filtered through a Whatmann filter paper No. 42 (125 mm) and concentrated using a rotary evaporator. The extracts were used for antimicrobial and chemical studies. All the extracts were analyzed by TLC on silica gel using benzene and benzene-acetone mixtures as eluent and detected under UV (254 nm) by spraying with a methanolic solution of magnesium acetate. The chloroform extract (140 mg) was fractionated on a silica gel column. The column was eluted with benzene, benzene-acetone (20:1, 10:1, 5:1 and 1:1) and acetone, as eluents to yield three fractions (CH-1, CH-2 and CH-3). Solvents for extraction and chromatography were chosen because of good solubility of metabolites and their selective separation. The experiments with toxic solvents were carried out in digestor.

HPLC studies

High-performance liquid chromatography (HPLC) analysis carried out on Agilent 1200 Series HPLC instrument with C18 column (C18; 25 cm × 4.6 mm, 10 m) and UV Spectophotometric detector with solvent methanol-water-phosphoric acid (80:20:0.9, v:v:v). Methanol was of HPLC grade (Merck, Darmstadt, Germany). Phosphoric acid was analytical grade reagent. Deionized water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA). The sample injection volume was 10 µl. The flow rate was 1.0 ml min^{-1}. The constituents of the extracts were identified by comparison of their retention times and absorption spectra (200-600 nm), with those of samples previously isolated from lichens and also compared with literature data (Huneck & Yoshimura, 1996; Manojlovic *et al.*, 2005).

Microorganisms

The bacterial strains of *Bacillus subtilis* (IHP10), *Escherichia coli* (IHP12) and *Staphylococcus aureus* (IHP16) and the fungal strains of *Candida albicans* (IHP 22), *Trichoderma harzianum* (IHP33) and *Mucor mucedo* (IHP36) were used for the antimicrobial investigations. All fungi and bacteria were obtained from the culture collection maintained at the Institute of Health Protection, Kragujevac and Vranje, Serbia.

Antibacterial and antifungal activity

The antibacterial activity of the extracts and fractions of the *L. benguelensis* was tested against the abovementioned Gram-positive and Gram-negative bacteria by the disc agar diffusion method (Berghe & Vlietinck, 1991; Cappuccino & Sherman, 1998). The bacteria were grown on Mueller-Hinton agar media (pH = 7.3). Agar media were poured into the plates to a uniform depth of 5 mm and allowed to solidify. The microbial suspensions at 5×10^6 cfu ml^{-1} were streaked over the surface of the media using a sterile cotton swab to ensure confluent growth of the organism. The discs used were Whatman No. 1 papers, 6 mm in diameter. Thirty µg of extracts per disc were spotted on the filter paper discs, which were then aseptically applied to the surface of the agar plates at well-spaced intervals. The plates were incubated at 37˚C for 24 hrs and the observed growth inhibition zones, including disc diameters, were measured. Con-

trol discs impregnated with standards were used alongside the test discs in each experiment. The above listed fungi were cultured in modified Sabouraud agar and suspensions at 5×10^6 cfu ml^{-1} were used. Streptomycin (2 μg per disc) and fluconazole (2 μg per disc) were used as standards for the antibacterial and antifungal activities, respectively. Antimicrobial activity was determined by measuring the diameter of the zone of inhibition around the disk.

RESULTS AND DISCUSSION

Laurera benguelensis (Mull. Arg.) Zahlbr. belongs to the family Trypetheliaceae which is distributed widely in the tropical regions. The lichen was collected from the steam bark of *Azadirachta excelsa* (Jack) Jacobs. Generally, lichens that belong to the genus *Laurera* are chemically poorly investigated. Until now, only 1,5,8-trihydroxy-6-methoxy-3-methyl-anthraquinone has been found in the lichen *Laurera purpurina* (Stensio & Wachtmeister, 1969). The information about their biological activity is very poor.

The present paper deals with the antimicrobial activity of extracts from the species *Laurera benguelensis*, a lichen growing on sun-exposed branches of trees in the Kanchanaburi province of Thailand. Benzene, chloroform, acetone and methanol extracts from the lichen were prepared and screened for their antimicrobial activity. The agar disc diffusion method was used to evaluate the antimicrobial activity by measuring the inhibition zone against the test microorganisms. The results of this testing are shown in Table 1. The inhibition zones of the extracts varied between 3.5 ± 0.5 mm and 10.3 ± 0.3 mm for the bacteria and between 4.0 ± 0.7 mm and 14.0 ± 0.3 mm for the fungi. The *L. benguelensis* chloroform extract displayed the highest level of activity against both bacteria (10.3 ± 0.3 mm) and fungi (14.0 ± 0.3 mm) tested.

In addition, the most active extract (chloroform) was eluted on chromatographic column in succession with benzene, benzene acetone (20:1, 10:1, 5:1 and 1:1) and acetone, as eluents to yield three fractions (CH-1, CH-2 and CH-3). Figures 1 and 2 show the HPLC chromatograms of standards and the fractions of the chloroform extract of *L. benguelensis*. These fractions contain different amounts of metabolites which were monitored using the HPLC-UV method.

The chromatogram of the CH-1 fraction shows that this fraction contains mainly the xanthone derivative lichexanthone ($t_R = 16.5$ min) (Fig. 1). This xanthone has the same retention time and UV spectrum as the standard sample of lichexanthone (1-hydroxy-3,6-dimethoxy-8-methylxanthone). The structure and UV spectrum of lichexanthone are shown in Fig. 3. Lichexanthone is of interest because it possesses a biological activity and can be used in the pharmaceutical industry.

The fraction CH-1 contains only lichexanthone, while the fractions CH-2 and CH-3 do not contain this compound. The fraction CH-2 contains the xan-

TABLE 1. Antibacterial and antifungal activity of the extracts of the lichen *Laurera benguelensis*. Data are average (± sd) diameters (in mm) of inhibition zones from three independent observations. BE = benzene extract; CH = chloroform extract; AC = acetone extract; ME = methanol extract; ST = standard (2 μg streptomycin per disc for bacteria and 2 μg fluconazole per disc for fungi)

	Zones of inhibition[a] Extracts				
	BE	CH	AC	ME	ST
Bacteria					
B. subtilis	8.3 ± 0.3	10.0 ± 0.5	7.0 ± 0.5	3.5 ± 0.5	11.0 ± 0.5
S. aureus	4.0 ± 0.5	9.7 ± 0.3	6.0 ± 0.3	4.5 ± 0.3	25.0 ± 1.0
E. coli	5.5 ± 0.3	10.3 ± 0.3	6.0 ± 0.3	3.6 ± 0.5	17.0 ± 1.0
Fungi					
M. mucedo	4.3 ± 0.3	14.0 ± 0.3	9.0 ± 0.3	4.0 ± 0.7	22.3 ± 0.3
C. albicans	5.5 ± 0.5	10.0 ± 1.0	8.0 ± 0.5	4.5 ± 0.5	10.3 ± 0.3
T. harzianum	6.0 ± 0.3	13.0 ± 0.5	8.5 ± 0.5	4.5 ± 0.3	20.0 ± 1.0

[a]diameter of inhibition zones of the extracts from *L. benguelensis* in the agar diffusion test with 30 μg per disk (Ø in mm, paper disk Ø 6 mm)

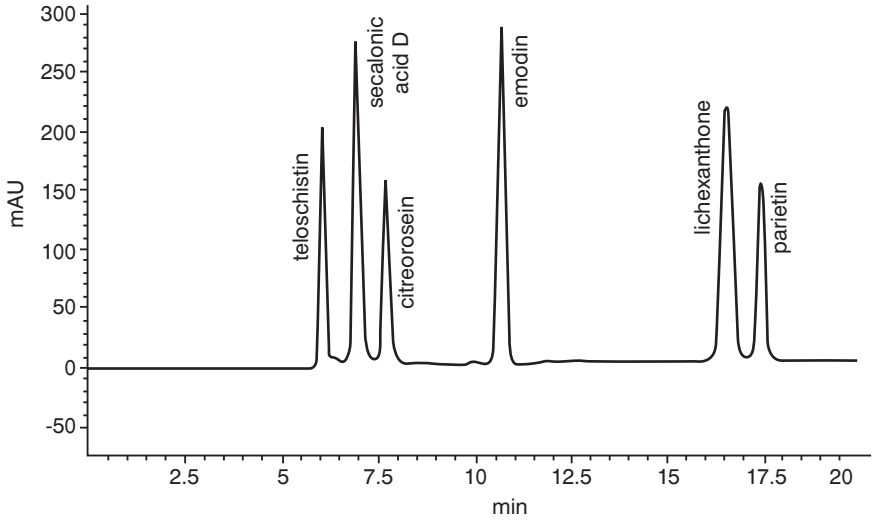

FIG. 1. HPLC chromatogram of mixed standards.

thone derivative secalonic acid D and the anthraquinone metabolites teloschistin (AQ1; $t_R = 5.9$ min), emodin (AQ2; $t_R = 9.7$ min) and parietin (AQ3; $t_R = 17.5$ min) in smaller amounts. On the other hand, the fraction CH-3 contains the anthraquinone derivatives teloschistin (AQ1), emodin (AQ2), citreorosein (AQ4; $t_R = 7.9$ min) and xanthorin (AQ5; $t_R = 15.5$ min). HPLC-UV analysis showed the presence of anthraquinone metabolites as the most abundant metabolites in the fraction CH-3. The structure of these compounds was determined by comparison of their t_R values with the chromatogram of the standard substances. In addition, the comparison of their UV-Vis absorption spectra with those of standard substances, previously isolated from lichens, was also performed. The absorbance spectral data also corresponded to those in the literature (Yoshimura *at al*., 1994a, b; Huneck & Yoshimura, 1996). In nature, parietin (1,8-dihydroxy-3-methoxy-6-methylanthraquinone) can be also found in plants belonging to the genera *Rhamnus* and *Rheum*, and in some lichens belonging to the family Teloschistaceae. Teloschistin (1,8-dihydroxy-3-hydroxymethyl-6-methoxyanthraquinone, orange needles) is a compound which has been found mainly in some lichens which belong to the family Teloschistaceae (*Caloplaca, Xanthoria* and *Teloschistes*) (Søchting, 1997, 2001). Parietin possesses some biological activities, including antibacterial and antifungal ones (Agarwal *et al.*, 2000; Manojlovic *et al.*, 2005). Emodin (1,3,8-trihyroxy-6-methylanthraquinone), a well-known light-orange, biologically active substance, is an ingredient of some pharmaceutical drugs and can be found in nature in the plants *Rheum*, *Rhamnus*, in some fungi and in some lichens belonging to the family Teloschistaceae. Citreorosein (1,3,8-trihyroxy-6-hydroxymethylanthraquinone) is a compound which has been found in some lichens of the family Teloschistaceae (Muzychkina, 1998). The detection of emodin in a small amount supports previous evidence that it is an intermediate of the secalonic acid biosynthesis.

Xanthone derivatives are widespread in nature, commonly occurring in a number of higher plant families and fungi. They are of importance because of their pharmacological properties. Secalonic acid D (pale yellow crystal), a mycotoxin, was previously isolated from several food-born fungi (Steyn, 1970; Andersen *et al.*, 1977). Secalonic acid D is also a metabolite of *Penicillium oxalicum* and *Aspergillus aculeatus* and it possesses a broad spectrum of biological activities (Wang & Poya, 1996; Hanumegowda *et al.*, 2002; Dhulipala *et al*., 2004).

In addition, the fractions CH-1, CH-2 and CH-3 were screened for their antimicrobial activity using the paper disk diffusion method. All the fractions were used for antimicrobial screening under the same conditions as mentioned above. The testing results of the fractions are shown in Table 2.

The fractions showed inhibition zones ranging from 5.0 to 15.0 mm for bacteria and from 8 to 13 mm for fungi. The fraction CH-1 showed a promising antimicrobial activity, while the fractions CH-2 and CH-3 showed a lower activity against the tested microorganisms. It appears that the antimicrobial activ-

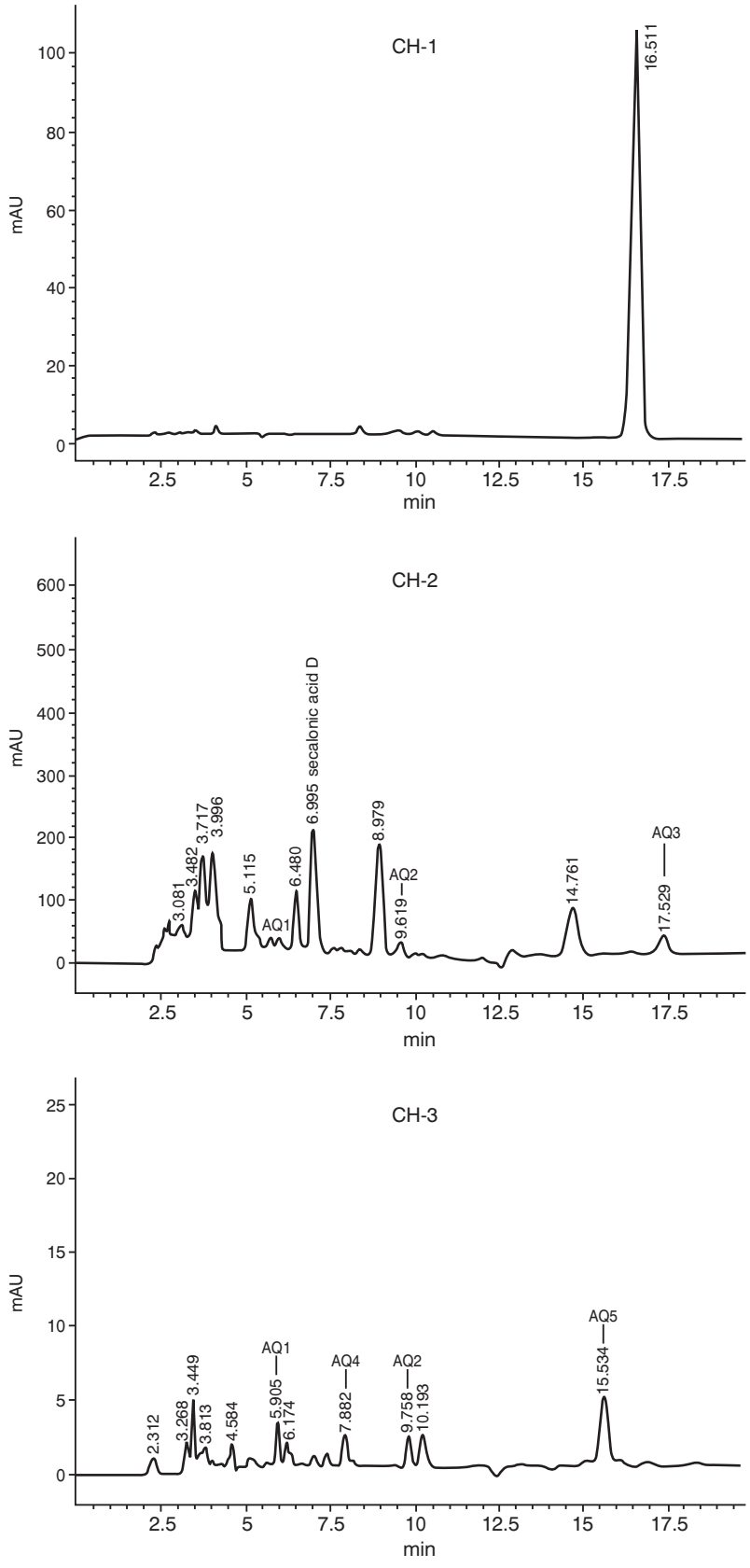

FIG. 2. HPLC chromatograms of three fractions (CH-1, CH-2 and CH-3, respectively) of the chloroform extract of *Laurera benguelensis*; AQ1 = teloschistin; AQ2 = emodin; AQ3 = parietin; AQ4 = citreorosein; AQ5 = xanthorin.

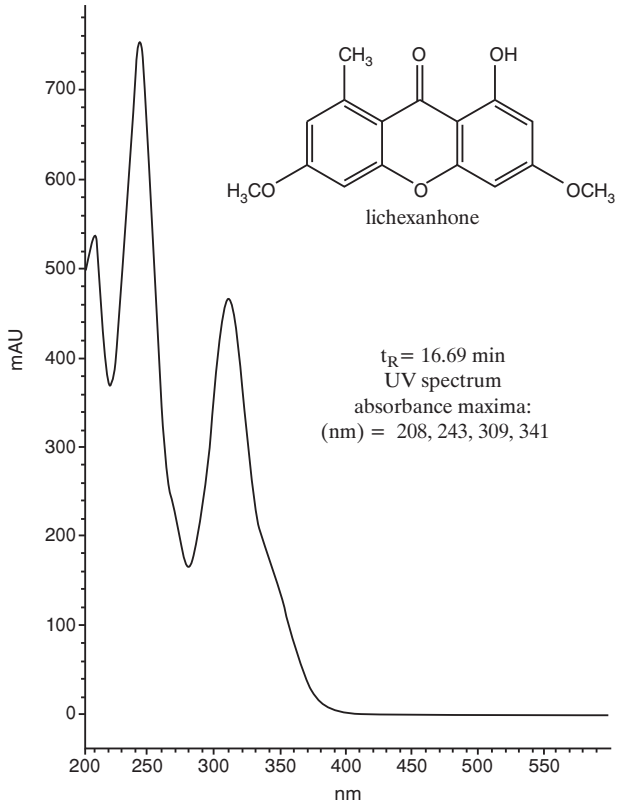

FIG. 3. The structure of the isolated lichexanthone and its UV spectrum. The UV spectrum was recorded from the HPLC chromatogram. UV traces were recorded at 254 nm and UV spectrum from 200 to 600 nm.

TABLE 2. Antibacterial and antifungal activity of the fractions of the chloroform extract of the lichen *Laurera benguelensis*. Data are average (± sd) diameters (in mm) of inhibition zones from three independent observations. CH-1, CH-2 and CH-3 = first, second and third fractions of the chloroform extract, respectively; ST = standard (2 μg streptomycin per disc for bacteria and 2 μg fluconazole per disc for fungi)

	Zones of inhibition[a]			
	CH-1	CH-2	CH-3	ST
Bacteria				
B. subtilis	13.0 ± 1.0	10.0 ± 0.3	7.0 ± 0.5	11.0 ± 0.5
S. aureus	14.0 ± 1.0	6.0 ± 0.3	6.0 ± 0.3	25.0 ± 1.0
E. coli	15.0 ± 0.3	11.0 ± 0.5	5.0 ± 0.5	17.5 ± 0.5
Fungi				
M. mucedo	11.0 ± 0.5	10.5 ± 0.5	10.0 ± 0.5	22.0 ± 1.0
C. albicans	12.0 ± 0.5	10.0 ± 0.5	8.0 ± 0.3	10.5 ± 0.3
T. harzianum	13.0 ± 0.3	9.0 ± 0.3	10.0 ± 0.3	20.0 ± 0.5

[a]diameter of inhibition zones of the extracts from *L. benguelensis* in the agar diffusion test with 30 μg per disk (Ø in mm, paper disk Ø 6 mm)

ity of *Laurera benguelensis* is mainly related to the presence of lichexanthone.

Our current findings show a remarkable activity of benzene, chloroform, acetone and methanol extracts against Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria and fungi (*Candida albicans*, *Trichoderma harzianum* and *Mucor mucedo*). The antimicrobial activity of the extracts from the lichen *L. benguelensis* may be due to the xanthons and other phenolic constituents like anthraquinones. We found that *L. benguelensis* is very rich in lichexanthone, which is the main constituent of the chloroform extract. Also, anthraquinone compounds may also exert an antimicrobial activity.

On the basis of the results it is suggested that the extracts of the lichen *L. benguelensis* could be used as an accessible source of natural antimicrobial substances in the pharmaceutical industry. Further work should be focused on the isolation and purification of other active components of the crude extracts of the studied lichen.

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