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Xylariaceae Endophytic Fungi Metabolites Against *Salmonella*

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1. Introduction

For several years, natural products have been used directly as drugs or have provided the basic chemical architecture for deriving such drugs. Natural products are naturally derived metabolites and/or byproducts from plants, animals or microorganisms. These products have been exploited for human use for thousands of years and plants have been chief source of compounds used for medicine. Besides plants, microorganisms constitute a major source of natural products with desirable bioactive properties. The ultimate purpose of the researchers is looking for new sources of metabolites. The marine organisms, for example, have been studied with more attention in the recent decades. Endophytic fungi appear to be another interesting source of research. Because of what appears to be their contribution to the host plant, the endophytes may produce a plethora of substances that may have potential use to modern medicine, agriculture and industry.

Infections caused by pathogenic microorganisms are responsible for high rates of morbidity and mortality in world (Coelho et al., 2007; Souza et al., 2007). These infections can occur in invasive form, and are an increasing problem due to the increase of their incidence in hospitals, especially in patients who are undergoing cancer treatment, transplantation or are immunosuppressed for other reasons (Oliveira et al., 2001).

The genus *Salmonella* is extremely heterogeneous, comprising almost 2000 serotypes, of which only a few are major human pathogens (Kaufmann et al, 2001). However, despite this apparent complexity, *Salmonella* species are actually quite similar genetically, with the serotype differences based on surface antigen differences such as LPS and flagella (Kaufmann et al, 2001). The two main symptoms of salmonellosis are typhoid or typhoid-like fever and gastroenteritis (Tahergorabi et al, 2011). Salmonella spp. are a leading cause of acute gastroenteritis in several countries, and salmonellosis remains an important public health problem worldwide, particularly in the developing countries (Mead et al., 1999). Food is a transmission vehicle for *Salmonella* that causes about 96% of all salmonellosis cases (Tahergorabi et al, 2011). Gastroenteritis-causing pathogens are the second leading cause of morbidity and mortality worldwide, with children under the age of 5 years at greatest risk

(Guerrant et al, 2001). Such serious infections are most common in children and the elderly (Rotimi et al, 2008).

S. typhi remains an important health threat for humankind, with more than 16 million cases and 600000 deaths annually, worldwide (Kaufmann et al, 2001). Ironically, typhoid fever is declining worldwide, but non-typhoidal *Salmonella* infections are increasing rapidly, due to increased automation in food processing and other factors (Kaufmann et al, 2001). Non-typhoidal species also cause serious disease in immunocompromised individuals. (Rotimi et al., 2008). Up to a decade ago, in many countries, conventional 1st-line antimicrobial agents, such as ampicillin, chloramphenicol, and trimethoprim–sulfamethoxazole, were the drugs of choice for the treatment of lifethreatening salmonella infections (Rotimi et al 2008). However in the past two decades, these species are also becoming increasingly resistant to most antibiotics, which has significantly increased the concern about these food and water-borne pathogens (Threlfall et al., 1997; Therefall, 2002; Wedel et al., 2005).

In recent years, several powerful new technologies have been developed that have significantly enhanced our knowledge of *Salmonella* pathogenesis (Beuzon & Holden, 2001; Kaufmann et al., 2001). Novel approaches for development of new antibiotics have been pursued, such as combinatory chemistry tools but only a few new antibiotics are produced by the pharmaceutical industry nowadays (Coates & Hu, 2007) Despite of the huge expectative on synthetic molecules with effective antimicrobial properties, natural products are still a worth promise.

Thus, the search for new compounds with antimicrobial activity from plants and fungi has been the subject of intense research in recent years (Harvey, 2007; Lee et al., 2007; Hostettmann et al., 2003). This is due mainly to the fact that the plants are widely used in folk medicine to combat various diseases in humans caused by bacteria and fungi (Stefanello et al., 2006; Duarte et al., 2004; Cruz et al., 2007). In this sense, many researchers are aiming to scientifically prove the use of plant extracts as an effective control of infections of the skin (Weckesser et al., 2007), the mouth (More et al., 2008) and other infections caused by a range of Gram-positive and Gram-negative bacteria (Vuuren, 2008; Lee et al., 2007; Chauhan et al.,2007).

2. Endophytic fungi

Endophyte is one which resides in the tissues beneath the epidermal cell layers without causing any immediate, overt negative effects (Stone et al., 2000). It is worthy to note that studies have shown that, nearly 300000 plant species that exist on earth, each individual plant is the host to one or more endophytes, the population of a given endophytic species varies from several to a few hundreds strains (Strobel & Daisy, 2003; Huang et al., 2007; Yu et al., 2010). The endophytes may live in plants air parts, especially in leaves, but can also be found living in intracellular gaps of roots, that is one of the main entrance door for these microorganisms (Azevedo et al., 2001). The endophytic colonization can be positive to host plants. Both fungi and bacteria are the most common microbes existing as endophytes, but the most frequently isolated are fungi.

The endophytes transmission from one generation to another may occur vertically, among seeds, during plants reproduction, or horizontally, where fungi spores are transmitted by air way, water or insects (Carroll, 1988). The microorganism penetration may occur from natural gaps or insects. The roots are the entrance main door (Kobayashi & Palumbo, 2000).

Of the myriad of ecosystems on earth, those having the greatest biodiversity seem to be the ones also having endophytes with the greatest number and most diversity (Strobel et al., 2005). The observation of Moricca & Ragazzi (2008) indicates that the type of interaction between an endophyte and a plant is controlled by the genes of both organisms and modulated by the environment. The fungi produce secondary metabolites compounds that have various biological activities, and have great bioactive potential (Petrini, 1991). The symbiosis among plants and fungi, mainly endophytic fungi, might be an important source of active pharmacologic compounds.

Beyond production of substances that come from secondary metabolism as the majority of antimicrobial, the symbiosis among plants and endophytic fungi can lead to other benefits to host plants as substances that improve growth and host competitively in nature (Hallman et al., 1997; Azevedo et al., 2000). Besides pharmacological potential, these microorganisms can also be highlighted for their capacity to produce interesting substances for farming, as growth plants regulators or insecticides, acting an important role from the ecological point of view (Souza, 2004). Only a handful of plants have ever been completely studied relative to their endophytic biology, consequently is an opportunity to find novel endophytic microorganisms and antimicrobial metabolites produced by them.

2.1 Biological activity from medicinal plants endophytes

The search for substances with pharmaceutical utility was one of the reasons that boosted the endophytic fungi researches. Beyond the studies about endophytic colonization, the characterization of new metabolites produced from a symbiotic association between fungus and host plant lead to isolation of various compounds with commercial importance. A diversity of biosynthetic classes metabolites were isolated from endophytic fungi and most of them showed interested pharmacological effects (Tan et al., 2001; Gunatilaka, 2006)

The development of new agents with pharmacological proprieties still is a great challenge for science and represents and endless research area. In the last 40 years, plenty of metabolites with different carbon skeleton were isolated from fungi. The isolation of cyclosporine in 1970, as a metabolite from *Cylindrocarpon lucidum* and *Tolypocladium inflation* fungi, represented and important step in the immunosuppressive treatment. (Hanson, 2008). Thought the past of the years, another studies were published describing interesting metabolites isolation. Borges et al. (2006) described the presence of derivatives anthraquinones produced by *Phoma sorghina*, and endophytic fungus associated to a medicinal plant *Tithonia diversifolia* (Asteracea). This plant extracts are used for the treatment of malaria, diarrhea, fever, hepatitis and wounds (Gu et al., 2002, Cos et al., 2002). There are also attributed anti-inflammatory, amebic, antispasmodic, antifungal, antibacterial and antiviral activities (Goffin et al., 2002, Cos et al., 2002).

Lu & co-workers (2000) in a research at Nanjing, China, observed the presence of 11 bioactive metabolites produced by *Colletotrichum* sp. and endophytic fungus isolated from *Artemisia annu*a (Asteracea), traditional plant from Chinese medicine. When tested against bacteria, some of there metabolites showed inhibitory activity against gram-negative and positive bacteria, as *Pseudomonas sp*. and *Bacillus subtilis*. Other metabolites were active against pathogenic fungi, as *Candida albicans* and *Aspergillus niger* in the concentration of 200 μg/mL.

2.2 Xylariaceae metabolites biological activity

The Xylariaceae family is considered a great source of a variety of bioactive compounds, showing plenty of chemical structures and biological activity. As an example, can be highlighted the taxol, a diterpene derived that have been used as an effective anti-cancer agent (Stierle et al., 1993). Among fungi that belong to Xylariaceae, the genus *Xylaria* is an important source of new secondary metabolites, with a variety of chemical structures and distinct biological activities.

The chemical investigation of fungi of Xylariaceae family showed as a potential source of biotechnological products, mainly with pharmacological proprieties. The study leadered by Healy et al. (2004) resulted on the isolation of xanthones, compounds isolated from endophytic fungi identified as *Xylaria sp.* The fungus isolated from *Glochidion ferdinandi* plant, and the metabolites extracted showed important pharmacological activities (Peres & Nagem, 1996; Peres et al., 2000) as for example, anti-inflammatory (Lin et al., 1996), antimicrobial (Malet-Cascon et al., 2003), antioxidants (Minami et al., 1994), antifungical (Rocha et al., 1994) e anticancer properties (Ho et al., 2002).

Krohn et al. (2004), developed an similar research, describing the syntesis of xyloketal D, a natural product that belongs to a group of secondary metabolites isolated from *Xylaria* sp. The biotechnological interested for this group is based on inhibitory activity over acethylcholinesterase enzyme.

Based on the abundance of secondary metabolites found in Xylariaceae family, Liu et al. (2008), identified and described the biological activity of 7-amino-4-methylcoumarin, a compound extracted from *Xylaria* sp YX-28 endophytic fungus. The chemical investigation of *Xylaria* (Xylariacea) genus fungus leads to potentials sources of natural products, as Xylarenal A, a terpenoid isolated form *Xylaria persicaria* fermentation (Smith et al., 2002) e xylactam, a nitrogened compound obtained from *Xylaria euglossa* ascomycete (Wang et al, 2005).

The *Hypoxylon, Nodulisporium* and *Daldinia* constitute one of the largest and most important genus of Xylariaceae, and they show a great diversity and production of secondary metabolites (Laessoe et al., 2010; Stadler et al., 2001; Kamisuki et al., 2007). The figure 1 shows some chemical structures isolated from genus *Hypoxylon, Nodulisporium* and *Daldinia.*

2.3 Antimicrobial activity of endophytic fungi metabolites

There is a general call for new antibiotics that are highly effective, possess low toxicity and will have minor environmental impact. This search is driven by the development of resistance infectious microorganisms (e.g. *Staphylococcus, Mycobacterium, Streptococcus*) to existing compounds and by the menacing presence of naturally resistant organisms (Strobel et al., 2005). In support of this idea, metabolites of endophytes have been reported to inhibit a number of microorganisms (Petrini, 1991; Gurney & Mantle, 1993). Many important antifungal and antibacterial chemotherapeutics are either microbial metabolites or their semi-synthetic derivates.

Between the years of 1981 to 2006, the Food and Drug Administration (FDA) had approved 1,184 new drugs among about 609 (51.4%) were natural products related: 55 were natural products, 270 natural product derived by chemical modification (semi-synthetic), 52 were done by synthesis where the active core came from a natural product, and 232 were synthesized by imitating a natural product (Newman & cragg, 2007).

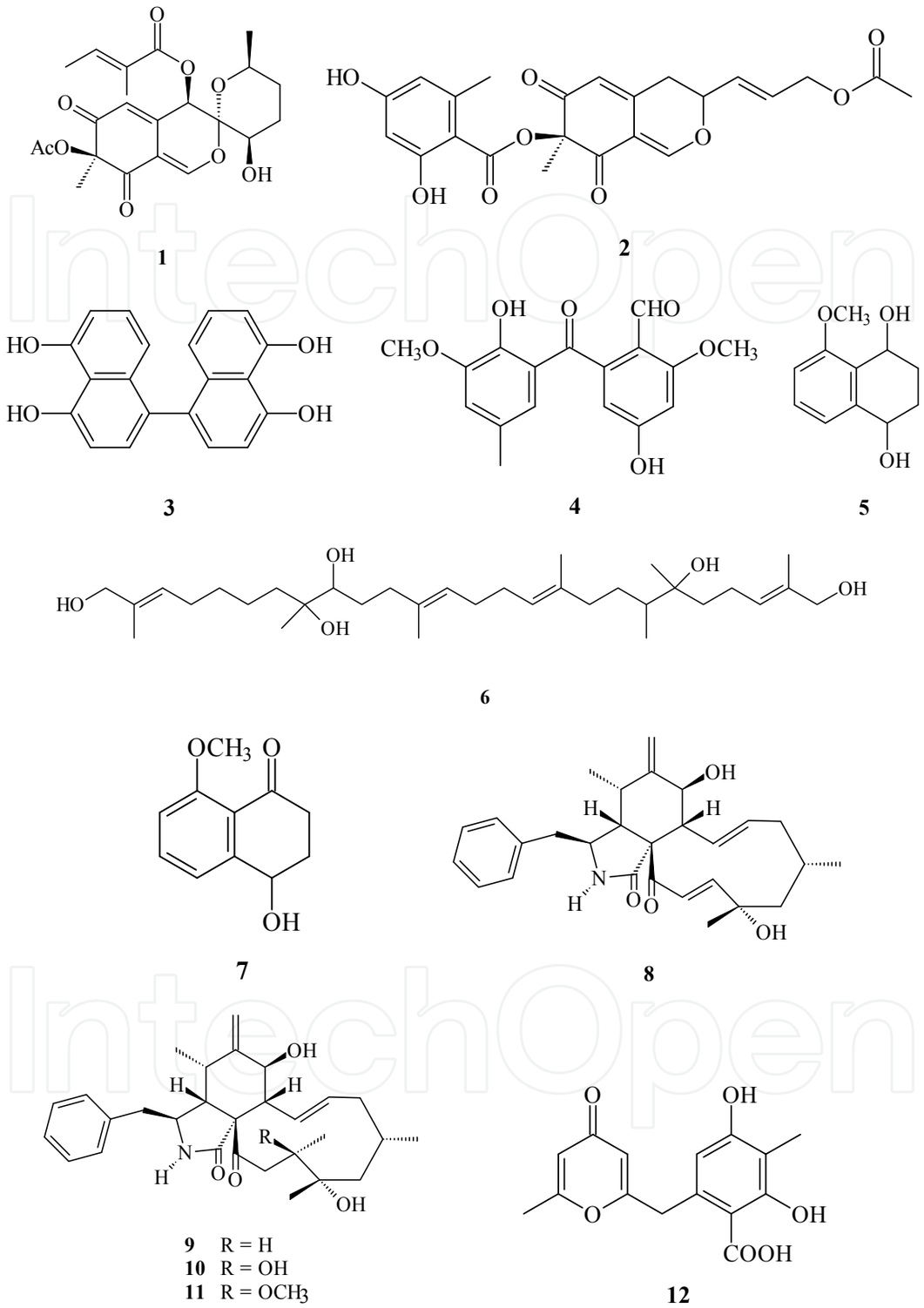


Fig. 1. Chemical structures isolated from species of the genus *Nodosporium, Hypoxylon* and 7 *Daldinia*.1= daldinin C; 2= hypomiltin; 3= BNT; 4= dalninal A; 8= cytochalasin 8 II; 9= cytochalasin I; 10= cytochalasin III; 11= cytochalasin IV; 12= macrocarpon A

Natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful microorganisms including, but not limited to phytopathogens, as well as bacteria, fungi, viruses and protozoan that affect humans and animals (Strobel et al., 2005). Taechowisan et al. (2008), described the biological activity of chemical compounds of *Streptomyces* sp., an endophytic fungus isolated from Alpinia galanga (Zingiberaceae) roots, medicinal plant collected in Nakom Pathom, Tailândia, surrounds. According to the authors, the isolated substances showed antimicrobial activity against the following microorganism: Staphylococcus aureus ATCC25932, *Bacillus subtilis* ATCC6633, *Escherichia coli* ATCC10536, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* ATCC90028 e *Colletrotrichum musae*.Taechowisan et al. (2008), described the biological activity of chemical compounds of *Streptomyces* sp., an endophytic fungus isolated from Alpinia galanga (Zingiberaceae) roots, medicinal plant collected in Nakom Pathom, Tailândia, surrounds. According to the authors, the isolated substances showed antimicrobial activity against the following microorganism: Staphylococcus aureus ATCC25932, *Bacillus subtilis* ATCC6633, *Escherichia coli* ATCC10536, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* ATCC90028 e *Colletrotrichum musae*. Rocha et al. (2010) observed antagonistic activity of endophytic fungi against to conidia of *Microcyclus ulei*, the agent of South American Leaf Blight responsible for the weak development of rubber plantations in Latin America. Endophytic fungi were isolated from *Hevea brasiliensis* (the rubber tree) leaves, cultivars FX3864, CDC312, MDF180, exhibiting distinct resistance levels to the attack by *M. ulei*. Lyophilized culture filtrates obtained from fungal isolates, grown in liquid malt extract medium, were tested *in vitro* and showed activity against germination of *M. ulei* and exhibited marked inhibitory activity on *M. ulei* conidia germination *in vitro*. The lyophilized culture filtrate of eleven foliar endophytic isolates achieved high inhibitory activity on *Microcyclus ulei* conidia germination and belong to seven genera: *Fusarium* sp., *Gibberella* sp., *Glomerella cingulata*, *Microsphaeropsis* sp., *Myrothecium* sp., *Pestalotiopsis* sp. and *Phomopsis* sp.

Davis et al. (2005) also tested antimicrobial activity of endophytic fungi. After chemical analysis of endophytic fungi cultures, *Eupenicillium* sp. Isolated from an endemic plant in Australia, *Glochidium ferdinandi* (Euphorbiaceae), the authors verified the presence of the following compounds: phomoxin B e C, eupenoxide e phomoxin. The isolated compounds were tested against plenty of microorganism associated to nosocomial infections, including *Staphylococcus aureus* drug multi-resistant (MRSA), *Staphylococcus aureus* (NCCLS 29523), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (NCCLS 29212), *Pseudomonnas aeruginosa* (ATCC 27853), *Streptococcus pyogenes* (ATCC 19615), *Acinetobacter anitratus* e *Candida albicans* (ATCC 60193).

The fungus was isolated from a traditional medicinal Chinese plant Ginkgo biloba L. (Ginkogoaceae). The metabolite was identified by NMR and mass spectra. The isolated compound showed activity against Staphylococcus aureus (MIC, 16 µg.mL-1), Escherichia coli (MIC, 10 µg.mL-1), Salmonnela typhia (MIC, 20 µg.mL-1), Salmonnela typhimurium (MIC, 15µg.mL-1), Salmonella enteriditis (MIC, 8,5 µg.mL-1), Aeromonas hydrophila (MIC, 4µg.mL-1), Yerisinia sp. (MIC, 12,5 µg.mL-1), Vibrio anguillarum (MIC, 25 µg.mL-1) Shigella sp. (MIC, 6,3 µg.mL-1), Vibrio parahaemolyticus (MIC, 12,5 µg.mL-1) Candida albicans (MIC, 15 µg.mL-1), Penicilium expansum (MIC, 40 µg.mL-1) e Aspergillus niger (MIC, 25 µg.mL-1).According many researchers (Arnold and Lutzoni 2007; Huang et al., 2008; Tejesvi et al., 2009), endophytes of tropical plants constitute a species-rich ecological

assemblage of fungi and should be included in screening programs for novel metabolites (Suryanarayanan et al., 2009).

Metabolites biologically active have been isolated and characterized (Shulz et al., 2002; Tejesvi et al., 2009; Aly et al., 2010), and could be new molecules for many applications. Shulz and Boyle (2005) and Aly et al. (2010) point that these active metabolites belong to the chemical groups, such as phenols, steroids, flavonoids, quinines, terpenoids, xantones, peptides, cytocatalasins, alkaloids, aliphatic compounds, and phenylpropanoids.

3. Xylariaceae metabolites and *Salmonella* strains

The Xylariaceae endophytics fungus were previously isolated by our research group from a cultivate specimem of *Mikania laevigata*, Asteraceae family (Ribeiro, 2011). This plant constituted in a Brazilian medicinal plant more used for respiratory infections. The endophytics fungus species were submitted to sequencing the rDNA ITS region which resulted in the identification of three strains of *Nodulisporium* sp., three strain of *Hypoxylon* sp., one strain of *Daldinia* sp and four strains unknown. The crude extracts above fungi were obtained by its cultivation on potato dextrose agar at 28 °C in Erlenmeyer flasks (3 × 100 m L), for 7 days. The cultures were filtered to separate broth and mycelia. The mycelia were extracted by reflux with methanol (100 mL) to furnish the respective crude extract (Ribeiro et al, unpublished data)

For antimicrobial test four isolates of *Salmonella* were tested: *S. enteritidis* CCMB 522; *S. carrau* CCMB 523 and two isolated from from food (*Salmonella* sp. CCMB 270 and CCMB 281).

3.1 Determination of minimun inhibitory concentration (MIC)

The broth microdilution susceptibility test was used to determinate the minimum inhibitory concentration (MIC) for bacteria as recommended by CLSI (2003). The extract was dissolved in dimethyl sulfoxide (DMSO) and water (50:50) to reduce the inhibitory potential of DMSO and then the extract were sterilized by filtration through cellulose acetate membrane (0.22 mm). Geometric dilutions were prepared using 96-well, flat-bottom microdilution plate which received 90 mL of DMSO extracts diluted in water in lines A1 to A9 containing 90 mL of previously Mueller-Hinton broth two times concentrated. So, the first wells (A1 to A9) contained crude extracts diluted in a concentration from 1 $mg.mL^{-1}$ until 0.008 $mg.mL^{-1}$ (H1 to H9). The suspension of the micro-organism test was adjusted to 1.5 x 10^8 cells mL^{-1} for bacteria in 0.45% sterile saline. After the dilutions were carried out in all wells, each well received 10 µL of the microbial suspension performing a total volume of 100 mL by well (90 µL of the extract and HCM + 10 µL micro-organism). The plates were incubated at 37 ° C for 24 hours. After the incubation period, were added 30 mL of rezasurin (7-hydroxy-3H-fenoxazina-3-one-10-oxide) at final concentration of 0, 01% for quantitative analysis of microbial growth and determining the relative antimicrobial activity of each sample dilution. All tests were performed in triplicate. Dilutions of the antibiotic chloramphenicol (10 $mg.mL^{-1}$) were used as positive control for comparison of data between independent experiments and as indicators for assessing the relative level of inhibition of the samples tested. Controls of the microbial viability, sterility of the medium, sterility the extract and the potential for inhibition of DMSO on the micro-organisms tested were also carried out. In this work a representative results of MIC values was regarded as equal to or less than 0.5 $mg.mL^{-1}$ of the extracts tested.

3.2 Determination of minimum microbicide concentration (MMC)

After determining the MIC, the minimal microbicidal concentration (MMC) was done. Aliquots of 5 µL of the wells were plated on Mueller Hinton Agar (MHA) and incubated at 37 ° C for 24 hours. The MMC was considered the lowest concentration of the extract which showed no cell growth on the surface of (MHA).

3.3 Xylariaceae extracts against *Salmonella*

Twelve extracts were tested against four strains of *Salmonella.* The results are shown in Table 1.

Extracts		*Salmonella* sp. CCMB 270	*Salmonella* sp. CCMB 281	*Salmonella Enteritidis* CCMB 522	*Salmonella carrau* CCMB 523
Nodulisporium sp. (specie 1)	MIC	0,25	0,5	0,5	0,25
	MMC	0,5	1	-	1
Nodulisporium sp. (specie 2)	MIC	0,5	0,5	0,25	0,5
	MMC	1	1	-	-
Nodulisporium sp. (specie 3)	MIC	0,5	0,5	0,25	0,5
	MMC	1	1	-	-
Hypoxylon sp. (specie 1)	MIC	1	0,5	0,5	0,5
	MMC	1	1	-	-
Hypoxylon sp. (specie 2)	MIC	0,5	0,5	0,5	0,25
	MMC	1	1	-	1
Hypoxylon sp. (specie 3)	MIC	0,5	0,5	0,25	0,5
	MMC	1	1	-	-
Daldinia sp.	MIC	0,5	0,5	N/A	N/A
	MMC	1	1	N/A	N/A
Specie 3 (Unknown)	MIC	0,5	0,5	0,25	0,25
	MMC	1	1	-	-
Specie 8 (Unknown)	MIC	0,5	0,5	0,5	0,25
	MMC	1	1	-	1
Specie 10 (Unknown)	MIC	0,5	0,5	N/A	N/A
	MMC	1	1	N/A	N/A
Specie 12 (Unknown)	MIC	0,25	0,5	0,25	0,5
	MMC	0,5	1	-	-
Specie 14 (Unknown)	MIC	0,5	0,5	0,25	0,5
	MMC	1	1	-	-
CONTROL	CHLORAN	0,31	0,31	0,16	0,31
	DMSO	1	1	1	1

N/A: not applied; CHLORAN: Chloramphenicol; DMSO: dimethylsulfoxide

Table 1. Minimum inhibitory concentration (MIC) and minimal microbicidal concentration (MMC) (mg.mL^{-1}) of Xylariaceae endophytic fungal extracts on *Salmonella* strains

The microdilution technique for determination of minimum inhibitory concentrations (MIC) is often considered as the best methodology for assessing susceptibility or resistance of bacteria to antibiotics (Rivers et al., 1988; Reis, 2006; Alves et al., 2008). According to Ostrosky et al. (2008), MIC has several advantages and one is that this method can be 30 times more sensitive than other methods used in the literature. The DMSO control showed growth inhibition in a dilution corresponding to 1 $mg.mL^{-1}$ extract through MIC determination. Therefore, results were considered representative for MIC values at or below the next lower dilution of the extracts tested (0.5 $mg.mL^{-1}$). DMSO is a substance that facilitates the diffusion (Vieira, 2005), but it was necessary to control the solvent, since this can enhance the activity of the antimicrobial agent (Herschler, 1970; Ribeiro et al., 2001). All the extracts tested (three strain of *Nodulisporium* sp., three strain of *Hypoxylon* sp., one strain of Daldinia sp. and five strain unknown) samples shown antimicrobial activity less than the representative value for DMSO for MIC (Table 1). Most of samples studied demonstrated MMC= 1.0 $mg.mL^{-1}$ for *Salmonella* sp. CCMB 270 and *Salmonella* sp. CCMB 281. These results show that crude extracts had the same antimicrobial effect on salmonella strains studied**.** None of the tested extracts showed activity against all microorganisms, because some MIC values obtained were 1 $mg.mL^{-1}$ and were not considered as representative (Table 1). However, the fact of not showing detectable antimicrobial activity does not mean that the fungal extracts evaluated did not possess bioactive compounds against microorganisms tested.

The strain *Salmonella* enteritidis CCMB 523 seems more sensitive compared to the extracts tested, since it was inhibited at 0.25 $mg.mL^{-1}$ by 6 (60%) of the extracts evaluated (Figure 2). Under the same conditions, *Salmonella* carrau CCMB 523 was inhibited by 4 (40%), *Salmonella* sp. CCMB 270 was inhibited by 2 (20%) of the extracts evaluated, while *Salmonella* sp. CCMB 281 was not inhibited at this concentration by none of the tested extracts.

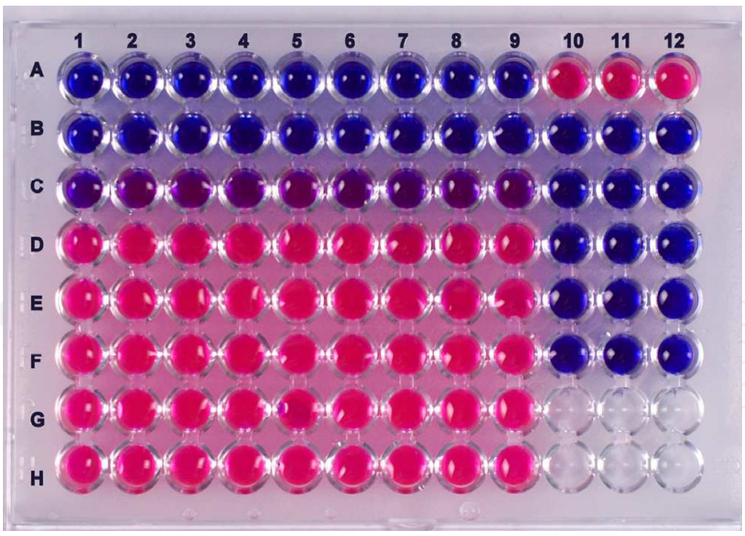


Fig. 2. Determination of minimum inhibitory concentration. Representation of serial dilution of methanolic extracts against *Salmonella enteritidis*. Columns 1-3: **Specie 14 (Unknown)** extract, MIC = 0.25 $mg.mL^{-1}$, Columns 4-6: ***Hypoxylon* sp. (specie 3)** extract, MIC = 0.25 $mg.mL^{-1}$ and Column 7-9: **Specie 12 (Unknown)** extract, MIC = 0.25 $mg.mL^{-1}$. Line A 10-11-12: microbial growing control of microorganism tested. Lines B 10-11-12, C 10-11-12 and D 10-11-12: control of extracts and lines F 10-11-12 and G 10-11-12: Control of the sterility of the culture medium (HCM).

The Gram-negative bacteria are reported to possess resistance to several antibiotics. The complexity of Gram-negative bacteria makes them less susceptible to antimicrobial agents (Tadeg et al., 2005).Variations related to determining the MIC of natural extracts can be attributed to several factors. Thus, there is no standardized method for expressing the results of antimicrobial testing of natural products (Fennel et al., 2004; Ostrosky et al., 2008). So the results can be influenced by microorganisms used for testing, the selected method and the solubility characteristics of each substance (Vanden et al., 1991; Valgus et al., 2007).

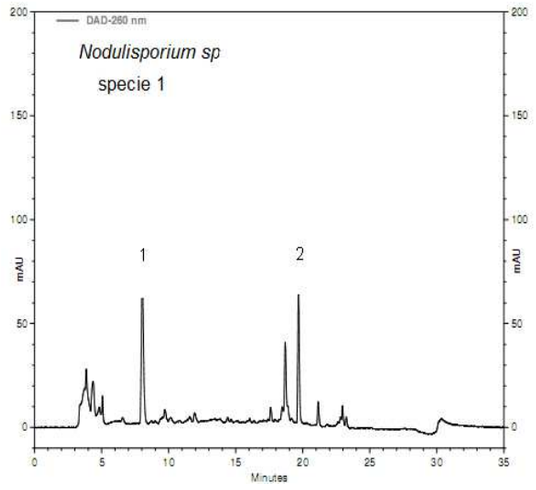
Through the use of the MIC was possible to demonstrate quantitatively the concentration of extracts that inhibited each microorganism, but this test only indicates the concentration able to cause growth inhibition and does not identify whether the inhibition was bactericidal or bacteriostatic. For this, we used the test Microbicide Minimum Concentration (MMC). Observing the results, the *Nodulisporium* sp. (specie 1) and Specie 3 (Unknown) extracts showed microbicidal activity up to 0.5 $mg.mL^{-1}$ against *Salmonella* sp. CCMB 270.

3.4 HPLC-DAD extracts analysis

Chromatographic techniques are used to separate the constituents from a mixture of substances aiming isolation and identification, being used to chemical investigation of crude extracts and identification of secondary metabolites of interested (Strege, 1999). High Performance Liquid Chromatography (HPLC) is one of methods of choice for determination of secondary metabolites profile to fungi isolated from Xylariaceae family. Ascomycets as *Daldinia, Hypoxylon* and *Xylaria* have been extensively studied using this method for chemical profile determination of majority and minority sample components. The analyses are based on retention time and UV absorption spectra. This technique also facilitates uncolored metabolites detection (Stadler et al., 2004).

The crude extract obtained from methanol extraction and tested to biological activity were analysed and monitored by High Performance Liquid Chromatography with diode array detector (HPLC-DAD), Hitachi, Laechrom Elite model, LiCospher 100 RP18 (5 μm) column, with 150 mm x 04 mm dimensions, Merck, equipped with diodo array detector (DAD). The mobile phase was composed of solvent (A) H_2O/H_3PO_4 0.1% and solvent (B) MeOH. The solvent gradient was composed of A (75-0%) and B (25-100%) for 25 minutes. A flow rate of 1.0mL/min was used, and 20μL of each sample was injected. Chromatographic peaks were monitored at 260 nm and characterized by retention time and UV-vis spectrum (200-600 nm). The HPLC-DAD chromatograms of the methanolic crude extracts from mycelium of *Nodulisporium* sp. (three strains), hypoxylon sp. (three strains) and *Daldinia* sp. were showed in Figure 2. The Figure 3 showed the HPLC-DAD chromatograms of the methanolic crude extracts from mycelium of five unknown species of Xylariaceae.

The *Nodulosporium* sp (specie 2) and *Daldinia* sp demonstrated to contain lack compounds in this analysis conditions when *Nodulosporium* (species 1 and 3) and all species de *Hypoxylon* showed two majoritary compound in the respective chromatograms. In the specific case of unknown species was to verify the presence de several compounds in the respective HPLC-DAD chromatograms (Figure 3). In the crude extract of unknown species 3, 10 and 12 the peaks eluted between 15 to 20 minutes, while in species 6 and 10 the peaks were eluted before 10 minutes, showing two majoritary peaks as the same profile for *Hypoxylon* chromatograms.


DAD-260 nm
Nodulisporium sp
specie 1
1
2
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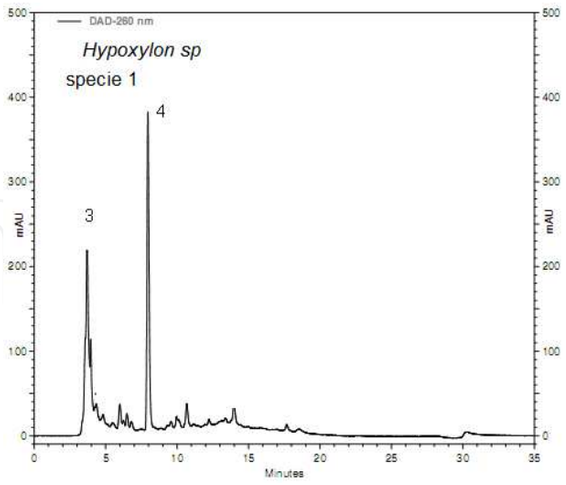
Minutes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Hypoxylon sp
specie 1
3
4
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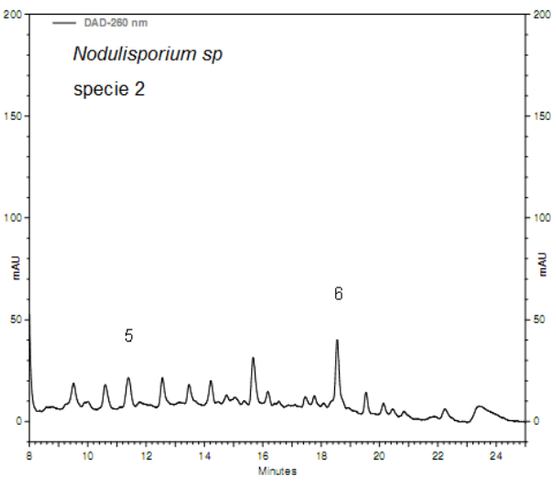
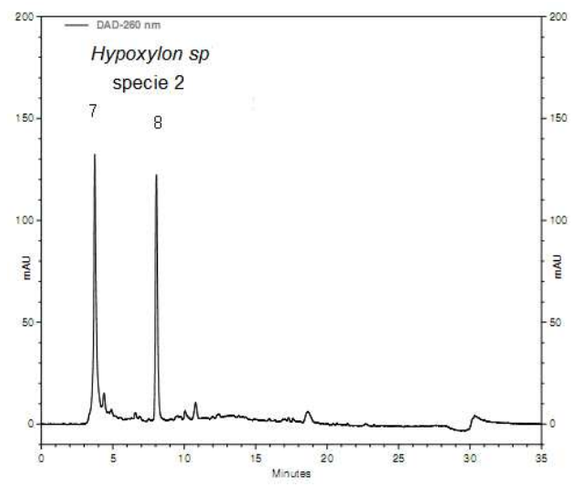
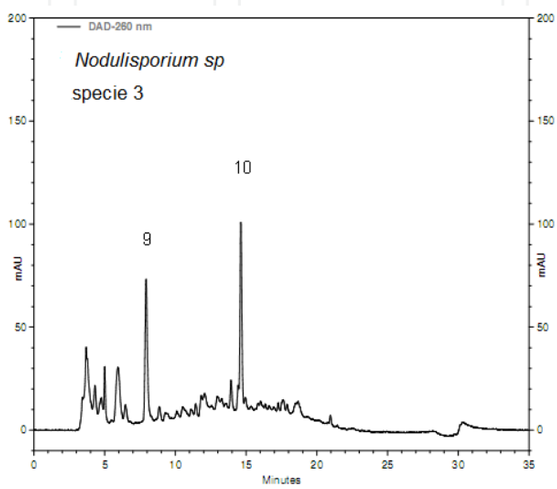
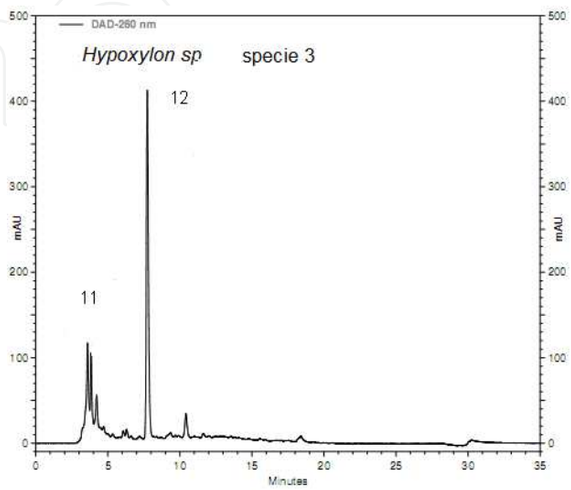

DAD-260 nm
Nodulisporium sp
specie 2
5
6
mAU
Minutes


DAD-260 nm
Hypoxylon sp
specie 2
7
8
mAU
Minutes


DAD-260 nm
Nodulisporium sp
specie 3
9
10
mAU
Minutes


DAD-260 nm
Hypoxylon sp
specie 3
11
12
mAU
Minutes

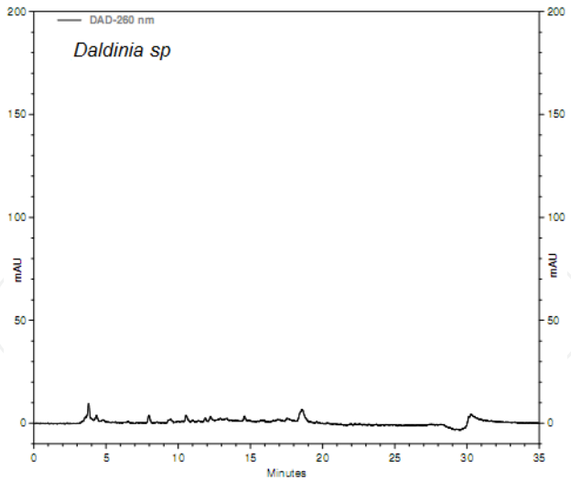
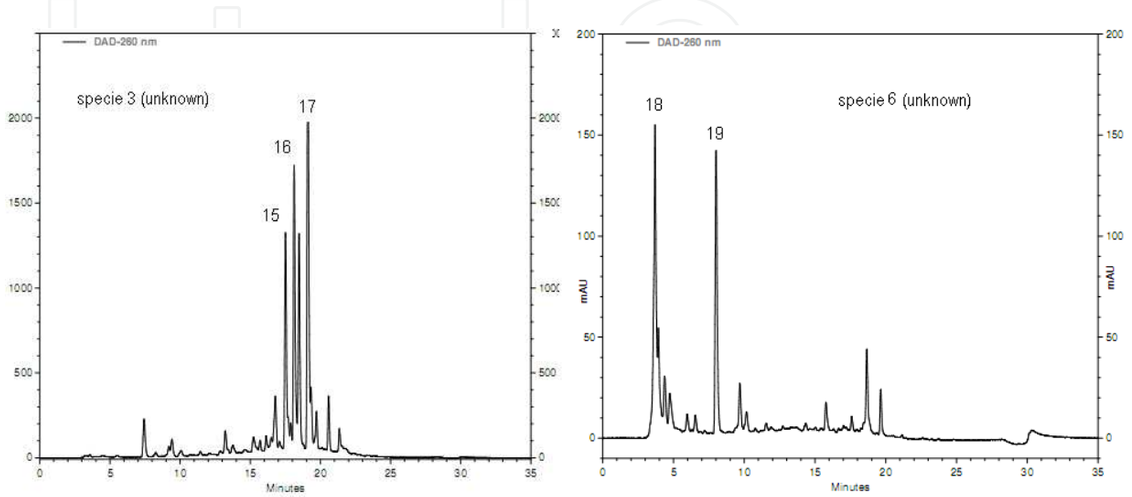


Fig. 3. HPLC-DAD chromatograms of methanolic extracts obtained from *Nodulisporium* sp., *Hypoxylon* sp. and *Daldinia* sp

The *Nodulisporium* chromatograms showed two majoritary compounds eluted in slightly different times but showed similar UV characteristics, suggesting to have the same chromophores (236 and 292 nm). The *Hypoxylon* chromatograms also showed two majoritary compounds eluted in about 3.7 and 7,8 minutes with the same UV profile (260 and 280 nm). On the basis of spectral identification it can be suggest that these compounds might be identified as phenolics.

A possible explanation for the antimicrobial activity of the methanolic extract against salmonella may be the fact that one or some of its constituents caused a significant inhibition of bacterial mobility besides ion permeability alteration on the into bacteria membrane. Antimicrobial activity of phenolic compound toward microorganism, as *salmonella*, is well documented and support this chemical investigation (Orsi et al, 2005; Nohynek et al, 2006). Addition studies are being performed for compounds isolation and identification.



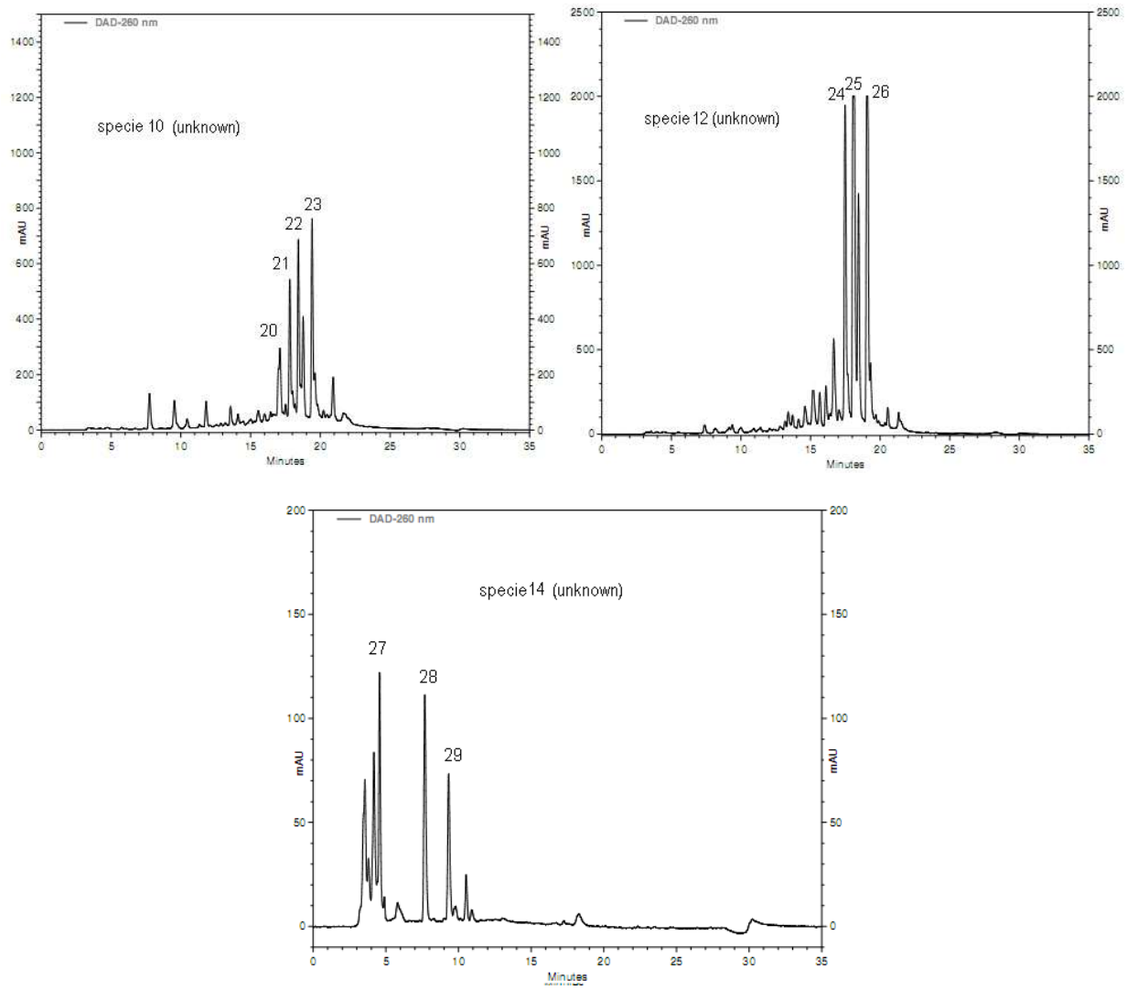


Fig. 4. HPLC-DAD chromatograms of methanolic extracts obtained from five unknown species

4. Conclusion

This work evaluated the antimicrobial activity against four *Salmonella* species of the crude extracts of endophytic fungi. Eleven fungi were isolated from *Mikania laevigata* (Asteraceae), a Brazilian medicinal plant and identified by our group and identified as being from Xylariaceae family. All the extracts of *Nodulisporium* sp., *Hypoxylon* sp., *Daldinia* sp. and unknown species showed similar antimicrobial activity. The HPLC-DAD analysis showed that extracts may contain phenolics compounds comum in others genus.

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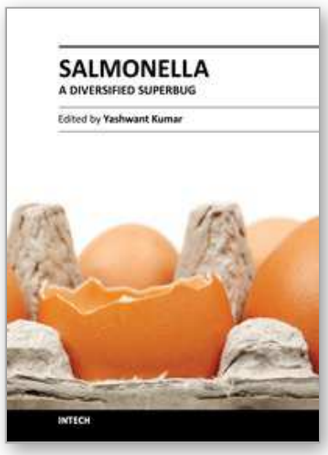

Salmonella - A Diversified Superbug
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Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

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