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Abstract

New Mannich base-type eugenol derivatives were synthesized and evaluated for their anticandidal activity using a broth microdilution assay. Among the synthesized compounds, 4-allyl-2-methoxy-6-(morpholin-4-ylmethyl) phenyl benzoate (**7**) and 4-{5-allyl-2-[(4-chlorobenzoyl)oxy]-3-methoxybenzyl}morpholin-4-ium chloride (**8**) were found to be the most effective antifungal compounds with low IC_{50} values, some of them well below those of reference drug fluconazole. The most significant IC_{50} values were those of **7** against *C. glabrata* (1.23 μM), *C. albicans* and *C. krusei* (both 0.63 μM). Additionally, the synthesized compounds were evaluated for their *in vitro* cytotoxic effects on human mononuclear cells. As result, the cytotoxic activity of eugenol in eukaryotic cells decreased with the introduction of the morpholinyl group. Given these findings, we point out compounds **7** and **8** as the most promising derivatives since they showed potency values greater than those for eugenol and fluconazole and they also presented high selectivity indexes.

Introduction

In recent years, a significant increase in the incidence of opportunistic fungal infections have been observed, especially among immunocompromised patients, especially those undergoing chemotherapy, long treatment with antimicrobial agents and also patients with immunosupressed conditions.The high toxicity, low bioavailability, the development of resistance by many microorganisms and late diagnosis are the main causes of treatment failure in these patients (1,2). In intensive care units around the world much effort has been invested to reduce and control nosocomial and community-acquired infections (3). Although a relatively small number of *Candida* species are pathogenic for humans, candidiasis are considered the main causes of hospital infection, mainly due to modern medical procedures specially the use of toxic and immunosuppressive drugs that can suppress the immune system and alter the normal bacterial flora. These events can facilitate the development of both skin infections and serious systemic diseases (4-6).

Eugenol is a natural allyl phenol found in the essential oil of cloves (*Syzygium aromaticum*), sassafras (*Ocotea odorifera*) e cinnamon (*Cinnamomum zeylanicum*) and has attracted attention of a lot of researchers, either because of its wide range of biological activities, either by chemical versatility of its structure (7). Among the biological activities already described for eugenol one can cite analgesic and anti-inflammatory (8), anesthetic (9), hypotensive (10), antioxidant (11), antitumoral (12), anti-parasitic (13), and antifungal action (14).

On the other hand, compounds known as Mannich bases were found to possess potent biological activities. They come from Mannich reaction, a condensation/aminomethylation involving a substrate containing an active hydrogen (as alkyl ketones, phenols, N-H heterocycles, aldehydes, etc), a primary or secondary amine and formaldehyde (or, occasionally, other aldehydes), resulting in the creation of the Mannich bases (15, 16). These compounds are biologically active due to their basic character, allowing its protonation under physiological conditions and favoring interactions between ligand and receptor, besides improving its water solubility (17). Literature survey reveals that Mannich bases are known for its several activities, among them anticancer (18), anti-inflammatory (19), antimalarial (20), antiviral (21), antitubercular (22) and antimicrobial, especially antifungal (23, 24, 25, 26, 27, 28, 29). It is also worth noting that piperazine or morpholine bioisosteric rings play a crucial role in antimicrobial activity, as in drugs like linezolid, eperezolid and itraconazole (23). The results presented in these studies indicate that the Mannich base subunit seems to be directly involved in the antimicrobial good results.

Our research group recently reported the anti-*Candida* activity of a glucoside derivative of eugenol (14). Following our interest in eugenol derivatives and in view of the potential of Mannich bases as antimicrobials we now devoted our attention to exploring the antifungal activity of some Mannich bases of this natural product. So, we describe herein for the first time, the synthesis and anti-*Candida sp.* activity of a morpholine-based Mannich base of eugenol and of the esters thereof.

Results and discussion

Chemistry

Preparation of the target compounds is shown in Scheme 1. The esters **3**-**5**, known eugenol derivatives, were obtained as described according to literature with minor adaptations (30, 31) and were synthesized to assess the influence of the Mannich base subunit on anticandidal action of derivatives **2** and **6**-**8**.

Eugenol Mannich base **2** was synthesized as previously reported (32) with some modifications which enhanced product yield and facilitated its isolation. Its identity was checked by IR, NMR and mass analysis. The ^{1}H-NMR peaks at δ 3.73-3.72 and at δ 3.65 correspond respectively to the morpholinyl ring eight protons and to those protons of

methylene group which bridges both rings. The derivatives **6**-**8** are new compounds and were obtained in moderate yields by reaction of **2** with respective anhydrides or acyl chloride using 4-dimethylaminopyridine as a catalyst. We were able to easily obtain these *O*-acylated derivatives using an acyl chloride or anhydride, in spite of what reported Mazzei et al (20) who achieved only methylene bis-derivatives when they treated Mannich bases of hydroxycoumarin with anhydrides. The esters **7** and **8** were isolated as their hydrochloride salts. NMR spectra showed clearly the expected peaks related to each inserted *O*-acyl chain, despite the other peaks related to the rest of the structure. High resolution mass spectrometry analysis showed the expected mass values for compounds **2** and **6**-**8.** All of the new compounds were isolated as crystalline solids after acid-base extraction or by grinding raw product with light petroleum or by recrystallization from ethanol.

Octanol/water partition coefficients ($LogP_{o/w}$) were calculated using QikProp program (33) for eugenol and its derivatives. Morfolinyl group was clearly able to reduce the logP values of derivatives **2** and **6-8** in relation to those of eugenol and simple esters, as shown in Table 1. This finding may be important for the solubility profile of the derivatives during biological evaluation and probably directly affects the antimicrobial potential of these compounds.

Scheme 1: The synthesis of derivatives **2**-**8**: a)morpholin, formaldehyde, toluene, 95°C. b)Ac_2O (derivatives **3** and **6**) or Bz_2O (derivatives **4** and **7**) or 4-ClC_6H_4COCl (derivatives **5** and **8**), DMAP, CH_2Cl_2, r.t.

Antifungal and cytotoxic activity

Antimicrobial activity of phenolic components as eugenol against *Candida* spp. have been reported in some studies with good antifungal potential (34, 35). Studies that evaluate the antifungal activity of eugenol and its analogues have led to derivatives with moderate activity against *Candida* species (36, 37) and indicated that the structural modification of eugenol can generate optimized compounds. In this direction, supported by the biological potential of eugenol and the reports of Mannich bases with antimicrobial action, we proposed to obtain new derivatives of eugenol endowed with antifungal potential by associating the morpholinyl group to it. In addition to that, we decided to explore the effect of having the

phenolic hydroxyl group of these products free or masked as esters of different polarities and volumes.

Most of the derivatives synthesized from eugenol showed significant antifungal activities when evaluated against ATCC species of *Candida* spp., while the action of eugenol was lower than that of the standard drug fluconazole on these same species (Table 1). The Mannich bases **2**, **7** and **8** showed IC_{50} values lower than those observed for fluconazole against most of evaluated *Candida* species. Some of the derivatives exhibited values of minimum inhibitory concentrations well below in relation to eugenol when tested against species of non-*albicans Candida*. Derivatives **7** and **8** were respectively 166 and 90 times more potent against *C. krusei* than that fungistatic drug. This is quite important when one considers naturally fluconazole-resistant species as *C. krusei*. The addition of the morpholinyl group seemed to be responsible for a significant impact on action against these yeasts, since derivatives **2**, **7** and **8** were all superior in activity compared to eugenol. Derivatives **7** and **8** showed IC_{50} values generally quite small, like those in the range of 10.2 to 0.57 μM. The only exception was the derivative **4** which does not possess the morpholinyl subunit but showed interesting activity against some species, but not as good as those of its morpholinyl analogue.

It is interesting to note that while the 4-chlorobenzoic ester of eugenol (**5**) was not active up to the highest concentration evaluated, its morphlolinyl analogue (**8**) reached IC_{50} values substantially lower than those of eugenol and fluconazole. Lal et al (38) reported that similar modification was able to lead to a significant increase in the action of the antifungal mulundocandin. This may be due to a balance between high lipophilicity and ionization potencial for this compound which is important for this biological activity.

Table 1: *In vitro* antifungal activity (IC_{50}, μM) and $LogP_{o/w}$ for eugenol (**1**) and its derivatives (**2**-**8**)

table 1 and below its footnotes

[a] Calculated Log $P_{o/w}$ values using *QikProp* program

[b] Fluconazol

[c] Not active up to 100 $\mu g.mL^{-1}$

The cytotoxic activities of the most active compounds (**2**, **4**, **7** and **8**) were tested in peripheral human blood mononuclear cells obtained from healthy donors and the results are shown in Table 2. As can be seen most of eugenol derivatives showed higher CC_{50} values than this parent compound.'

Mannich base derivative **2** was safer than eugenol and exhibited selective toxicity towards all evaluated yeasts. Derivative **7** was 200-400 times less cytotoxic than eugenol while its 4-chlorobenzoyl analogue **8** proved to be 1.4 to 316 times safer. These data indicate that modifications done on the prototype have the potential to reduce the cytotoxic activity of eugenol in eukaryotic cells and that the morpholinyl group may be involved in this. Moreover, these observations corroborate that the anticandidal activities found may be due to specific interactions in fungal cells, which is important in new antifungal drugs development.

Table 2: Cytotoxic activity (CC_{50}, μM) and selectivity index for antifungal compounds **2**, **4**, **7** and **8** on test microorganisms

table 2 and below its footnotes

[a] Not determined

[b]:The selective index was expressed as the ratio CC_{50}/IC_{50}.

Nosocomial infections can affect as many as 30% of patients admitted to an intensive care unit and knowledge of the causative pathogen of infection is crucial in initiating and managing the correct antimicrobial treatment (39). It is known that *Candida* spp. are among the leading causes of nosocomial blood infections in world and the choice of studying antifungal activity against *Candida* spp. was due to its great importance in the epidemiology of fungal infections (40). It was observed a progressive increase in infections caused by non-*albicans Candida* spp. and *C. parapsilosis* was the most frequent species, followed by *C. tropicalis* and *C. glabrata* (41). It is suggested that the rise in the incidence of these infections is associated with antimicrobial resistance and the restricted number of available antifungal drugs (42). Observations like this support and confirm the importance of studying new alternatives or complementary therapies to combat candidiasis.

Conclusion

We reported herein the synthesis and antifungal evaluation of a morpholine-based Mannich base of eugenol and the esters thereof. Results reveal clearly that the Mannich base derivatives showed to be promising antifungal agents against *Candida* spp. since most of them were more potent than fluconazole. Derivatives **7** and **8** are the most expressive compounds for further optimization since they showed potency values generally greater than those for eugenol and fluconazole, while they also presented high selectivity indexes.

Experimental

Chemistry

Thin-layer chromatography (TLC) on silica gel-G TLC plates (Merck) were used to monitor reactions course. Melting points of the compounds were obtained on Microquímica MOAs 301 melting-point apparatus and are uncorrected. IR spectra were recorded on Shimadzu a FTIR-Affinity-1 spectrometer. NMR spectra were recorded on a Bruker AC-300 spectrometer (300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR spectra). Chemical shifts are expressed as values relative to TMS as internal standard. High resolution mass spectra were acquired using a LCMS-IT-TOF mass spectrometer and the samples were solubilized in MeOH + 0.1% formic acid, following manual injection.

*Synthesis of 4-allyl-2-methoxy-6-(morpholin-4-ylmethyl)phenol (**2**)*

A solution of eugenol (10mL, 65.16mmol), morpholin (11.24mL; 122mmol) and formaldehyde (9.7mL; 49.8mmol) was stirred in toluene (100mL) at 95°C during 24h. After the completion of the reaction, noticed by TLC (hexane/ethyl acetate 3.5:6.5 v/v), the mixture was extracted with 1 mol.L^{-1} HCl (3x30mL). The pH of this aqueous phase was then raised to 6 by addition of saturated $NaHCO_3$ solution and then it was extracted with dichloromethane (5x30mL). The organic layer was dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure to give the product **2** (7.64g, 45%) as a white solid, m.p. 48-49°C. IR (cm^{-1}) v 2970, 2816, 1595, 1494, 1458, 1234. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.62 (s, 1H), 6.41 (s, 1H), 5.98-5.84 (m, 1H), 5.07-5.00 (m, 2H), 3.83 (s, 3H), 3.73-3.72 (m, 4H), 3.65 (s,2H), 3.25 (d, J = 6.7 Hz, 2H), 2.55 (m, 4H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 147.6, 144.7, 137.6, 130.5, 120.4, 115.4, 111.4, 66.6, 61.4, 55.7, 52.7, 39.6. HRMS-ESI: m/z calcd. for $C_{15}H_{21}NO_3$ $(M+H)^+$ 264.1594. Found 264.1487.

*Synthesis of derivatives **3-5***

These compounds were synthesized as previously reported and their identities were confirmed by comparing their infrared and nuclear magnetic resonance spectra to those in the literature (30, 31).

*General procedure for the synthesis of derivatives **6-8***

To a solution of **2** (0.5g, 1.9mmol) in dichloromethane (25mL), the appropriate acylating agent (2.28mmol) and 4-(dimethylamino)pyridine (0,23mmol) were added. The reaction mixture was stirred at room temperature for a period of 1,5-48h and the progress of the reaction was checked by TLC (hexane/ethyl acetate 3.5:6.5 v/v). After its completion ice cold water was added and the organic phase was washed with 0.5 mol.L^{-1} NaOH (6x10mL) followed by water until pH 7. The organic phase was then dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure which led to the directly to the desired products or after crystallization procedures.

*4-allyl-2-methoxy-6-(morpholin-4-ylmethyl)phenyl acetate (**6**)*

From acetic anhydride. White solid; yield: 56%; mp 64-65°C; IR (cm^{-1}) v 3003, 2970, 2870, 1761, 1597, 1490, 1197, 1112. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.78 (s, 1H), 6.72 (s, 1H), 6.02-5.88 (m, 1H), 5.13-5.08 (m, 2H), 3.80 (s, 3H), 3.68-3.65 (m, 4H), 3.40-3.35 (m, 4H), 2.41 (s, 4H), 2.30 (s, 3H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 168.4, 151.0, 138.0, 136.9, 136.7, 122.2, 115.9, 111.4, 66.6, 57.8, 55.6, 53.2, 39.8, 20.4. HRMS-ESI: m/z calcd. for $C_{17}H_{23}NO_4$ $(M+H)^+$ 306.1700. Found 306.1599.

*4-allyl-2-methoxy-6-(morpholin-4-ylmethyl)phenyl benzoate (**7**)*

From benzoic anhydride. White solid after treatment with light petroleum; yield: 49%; mp 84-85°C; IR (cm^{-1}) v 3074, 2976, 2864, 1732, 1602, 1494, 1460, 1269, 1109. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.23-8.20 (m, 2H), 7.64-7.60 (m, 1H), 7.53-7.48 (m, 2H), 6.82-6.78 (m, 2H), 6.05-5.91 (m, 1H), 5.15-5.08 (m, 2H), 3.80 (s, 3H), 3.48-3.38 (m, 6H), 2.34 (s, 4H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 164.3, 151.6, 138.3, 137.4, 137.0, 133.2, 130.1, 129.8, 128.4, 122.5, 116.1, 111.9, 66.5, 58.2, 22.9, 53.2, 40.1. HRMS-ESI: m/z calcd. for $C_{22}H_{25}NO_4$ $(M+H)^+$ 368.1856. Found 368.1803.

*4-{5-allyl-2-[(4-chlorobenzoyl)oxy]-3-methoxybenzyl}morpholin-4-ium chloride (**8**)*

From 4-chlorobenzoyl chloride. White solid after recrystallization from ethanol. yield: 70%; mp 200-201°C; IR (cm^{-1}) v 3014, 2980, 2362, 1749, 1597, 1494, 1261, 1124. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 13.19 (s, 1H), 8.12-8.07 (m, 2H), 7.54-7.47 (m, 3H), 6.89 (s, 1H), 6.04-

5.90 (m, 2H), 5.17-5.10 (m, 2H), 4.32-4.24 (m, 2H), 4.12 (d, J = 4.6 Hz, 2H), 3.89 (dd, J_2 = 13 Hz, J_3 = 3.2 Hz, 2H), 3.44 (d, J = 6 Hz, 2H), 3.32 (d, J = 13 Hz, 2H), 2.89-2.76 (m, 2H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ163.5, 151.1, 140.9, 140.3, 137.9, 136.1, 131.6, 129.2, 126.5, 124.3, 120.9, 116.9, 114.4, 63.5, 56.0, 54.2, 51.2, 39.8. HRMS-ESI: m/z calcd. for $C_{22}H_{25}Cl_2NO_4$ $(M\text{-}Cl)^+$ 402.1467 Found 402.1257.

Antifungal activity evaluation

Eugenol **1** and its derivatives **2-8** were evaluated *in vitro* for their activities against the fungi through a Mueller Hinton broth microdilution method and with the methodology and interpretative criteria proposed by document M27A3 (43). The stock solutions of all the compounds were prepared in DMSO 1% (v/v) at final concentration and tested at the following concentrations ($\mu g.mL^{-1}$): 100; 62.5; 31.2; 15.6; 7.8; 3.9; 1.95; 0.48; 0.24 and 0.06. The standard drug fluconazole was applied as control of fungistatic action at the following concentration (µg/mL): 64; 32; 16; 8; 4; 2; 1; 0.5; 0.25; 0.125; 0.0625 and 0.03125. The microplates were incubated at 37^{o}C for 24h. Results were visualized and analyzed by spectrophotometry. The inhibitory concentration of microbial growth was determined at 50% (IC_{50}) in µM and compared for each compound and microorganism. The tests were all done in duplicates and the results obtained from the replicas were coincident.

Cytotoxic activity evaluation

The cytotoxicity to peripheral human blood mononuclear cells (PBMCs) of eugenol and their derivatives **2**, **4**, **7** and **8** (concentration range 100 to 0.78 µg/mL) was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (44). The PBMCs were obtained from healthy volunteers by Ficoll-Hypaque density gradient centrifugation. The cell suspension of PBMCs at a concentration of 2.4×10^6 cells/mL was distributed in a 96-well plate, 90 µL in each well with 10 µL of test compounds at different concentrations, incubated at 37 °C in an incubator at 5% CO_2 for 48 h. After, it was added 10 µL of MTT dye ($5\ mg.mL^{-1}$) and the cells were incubated again for an additional 4 hours period. Then, the medium was carefully removed and added to 100 µL of DMSO for solubilization of formazan crystals. The plates were shaken for 5 min and absorbance for each sample was measured in a spectrophotometric microplate reader at 560 nm. The percentage of cytotoxicity was calculated as [(A-B)/Ax100)], where A and B are the absorbances of control and treated cells, respectively. Data were analyzed using linear regression to obtain values for CC_{50} (cytotoxic concentration for 50%). Selectivity indexes were expressed as the ratio CC_{50}/IC_{50}.

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Conflict of interest

The authors declare no conflict of interest.

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Compound	$LogP_{o/w}$[a]	Microrganism				
		C. albicans ATCC10231	*C. tropicalis* ATCC750	*C. krusei* ATCC 6258	*C. parapsilosis* ATCC 22019	*C. glabrata* ATCC 90030
1	2.675	365.80	609.8	182.9	182.9	609.8
2	2.349	0.88	380.0	28.5	28.5	0.88
3	2.621	291.0	485.3	145.6	145.6	485.3
4	3.938	0.86	86.0	1.75	14.0	14.0
5	4.579	-[c]	-[c]	-[c]	-[c]	-[c]
6	2.075	983.0	-[c]	-[c]	-[c]	-[c]
7	3.208	0.63	-[c]	0.63	10.2	1.23
8	3.717	74.7	0.57	1.17	74.7	4.67
FLC[b]	0.450	3.27	3.27	104.5	13.1	13.1

Compound	CC_{50}	Selectivity index[b]				
		C. albicans ATCC 10231	*C. tropicalis* ATCC 750	*C. krusei* ATCC 6258	*C. parapsilosis* ATCC 22019	*C. glabrata* ATCC 90030
1	549.13	1.5	0.9	3.0	3.0	0.9
2	1030.80	1170.0	2.7	36.0	36.0	1170.0
4	459.28	534.0	5.3	262.4	32.8	32.8
7	378.50	600.8	-[a]	600.8	37.1	307.7
8	161.85	2.17	283.9	138.3	2.2	34.7