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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and in vitro evaluation of antifungal and cytotoxic activities of eugenol glycosides

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Abstract Six eugenol glycosides were prepared in order to assess their antifungal activity against *Candida* species. They were synthesized by glycosylation of eugenol with the appropriate glycosyl bromides followed by deacetylation with sodium methoxide in methanol and were evaluated in vitro for their antifungal activity through a Mueller–Hinton broth microdilution method. The peracetyl glycoside (derivative **4**) was the most promising one since it was able to inhibit growth of *C. albicans*, *C. tropicalis* and *C. glabrata* with IC_{50} values much lower than that of the prototype eugenol. Derivative **4** showed to be 160.0 and 3.4 times more potent than eugenol and fluconazole, respectively, against *C. glabrata* with low cytotoxity (selectivity index of 45). Moreover, it was possible to verify the positive effect of gluco configuration and lipophilicity on antifungal activity, since glucose peracetyl derivatives were more active than the free sugars of galacto configuration.

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Introduction

Fungal diseases represent a serious challenge since ancient times. At the beginning of the twentieth century, bacterial epidemics were a global and important cause of mortality. In contrast, fungal infections were almost not taken into account. Since the late 1960s when antibiotic therapies were developed, a drastic rise in fungal infections was observed, and they currently represent a global health threat. This increasing incidence of infection is influenced by the growing number of immunodeficient cases related to AIDS, cancer, old age, diabetes, cystic fibrosis, organ transplants and other invasive surgical procedures (Vandeputte *et al*., 2012). Treating invasive fungal infections is becoming more and more interesting. In the 1980s, our options to treat patients with deadly fungal infections were restricted by the number and toxicity of available compounds; since then major efforts have resulted in more effective antifungal drugs (Pasqualotto and Denning, 2008).

The use of known antimicrobial structural scaffolds to afford new bioactive derivatives has not been the main strategy employed, mainly for the high prevalence of resistance to these agents. Thus, new chemical entities design is the main focus when searching for new antibacterial and antifungal drug leads, oriented especially to new molecular targets (Bush and Pucci, 2011; Moellering Jr., 2011). In that context natural products, especially plant-derived ones, represent an excellent source of new bioactive compounds since they show innovative structures that may be used as natural pharmacophores to structural derivatization.

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Eugenol is a natural allylphenol used for several medical purposes since many years and has been used alone or associated to other constituents of clove oil, its most important natural source (Pramod *et al*., 2010). Many important biological activities have been attributed to eugenol or to its close analogues, as analgesic and anti-inflammatory (Daniel *et al.*, 2009), anaesthetic (Guenette *et al*., 2006), hypotensive (Lahloua *et al*., 2004), antioxidant (Hidalgo *et al*., 2009), antitumoral (Manikandan *et al*., 2010), anti-parasitic (Machado *et al*., 2011; Ueda-Nakamura *et al*., 2006) and antidepressive-like action (Kabuto *et al*., 2007).

The eugenol activities against microorganisms are also known since many years. The mechanisms of antimicrobial action have been well documented in bacteria and fungi recently (Oyedemi *et al*., 2009; Devi *et al*., 2010). Ahmad *et al*. (2010) studied the effects of eugenol in proton translocation through *Candida* cytoplasmatic membrane. They showed eugenol is able to inhibit fungal ATPase enzyme which is essential to maintain electrochemical proton gradient across cell membrane necessary for nutrient uptake. Recently, Carrasco and his colleagues (2012) have prepared and tested a group of eugenol analogues against a panel of fungal strains. They showed the ability of nitro derivatives in disrupting fungal cytoplasmatic membrane without interacting to ergosterol.

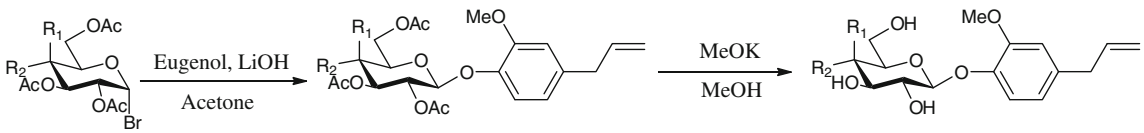
Considering these findings, we describe here our attempts in enhancing eugenol antifungal ability by synthesizing and evaluating some glycoside derivatives, i.e. its peracetylated and deacetylated glucosides, galactosides and lactosides. A series of biological activities has been attributed to natural and synthetic glycosides, since glycosylation has been pointed as an important method for improving the physicochemical and biological properties of the parental compound (Jung and Park, 2007; Zhang *et al*., 2012). Although the saccharide unit itself is generally not active, the presence of carbohydrate moieties may modify greatly the activity of bioactive compounds. This may occur either by modulating solubility or enhancing absorption via fungal membrane transporters for saccharides or even optimizing affinity for receptors since it may be involved in lectin–carbohydrate interactions.

Results and discussion

Chemistry

The compounds were obtained following the synthetic route depicted in Scheme 1.

The synthetic strategy involved the glycosylation of eugenol by first reacting it with three peracetylglycosyl bromides. This method is one of the most usual for this purpose, since it is cheap and easily performed in aqueous alkaline media, despite its variable reaction yields (Jacobson *et al*., 2006). Following, these intermediates obtained (**4**–**6**) were deacetylated by methoxide/methanol solution, condition known as Zemplén method, which resulted in final products **7**–**9** (Roy and Kim, 2003). To the best of our knowledge, eugenol lactoside was not described previously. Eugenol galactoside synthesis was described in a few studies focusing on its use as sanitary deodorant while its glucose derivative, on the other hand, has been known for a long time and was obtained both from synthetic way and natural source extraction (Mulkens and Kapetanidis, 1988). Acetylated glycosides (**4**–**6**) were obtained in good yields (ca. 55 %) as crystalline solids after recrystallisation from isopropyl alcohol. It could be observed in IR spectra ester C=O and vinyl C=C bands in 1,751–1,736 cm^{-1} and 1,642–1,636 cm^{-1}, respectively. All three acetylated glycosides were obtained as single β-anomers. This could be illustrated by the coupling constant of 8 Hz for a singlet at δ 4.8 ppm in ^{1}H-NMR spectra of glycoside **5**, typical to this type of proton. Aromatic hydrogens signals could be observed at δ 7–6.6 ppm range for all products confirming the 1,2,4-trissubstituted ring pattern of eugenol moiety. Acetyl hydrogen signals were found at δ 2.1–1.9 ppm for tetra-acetylated (**4** and **5**) and hepta-acetylated derivatives (**6**), while methoxy hydrogens signals could be verified at δ 3.8 ppm, for all compounds. Zemplén's method of deacetylation provided final glycosides in quantitative yield as crystalline solids. IR spectra of compounds **7**–**9** showed broad O–H stretching bands in 3,360–3,290 cm^{-1} range and no signal relative to C=O acetyl groups. This was certified by ^{1}H and ^{13}C NMR spectra of these substances which showed no acetyl carbon or hydrogen signal.



1: R_1=H; R_2=OAc
2: R_1=OAc; R_2=H
3: R_1=H; R_2=tetra-*O*-acetyl galactosyl

4: R_1=H; R_2=OAc
5: R_1=OAc; R_2=H
6: R_1=H; R_2=tetra-*O*-acetyl galactosyl

7: R_1=H; R_2=OH
8: R_1=OH; R_2=H
9: R_1=H; R_2=galactosyl

Scheme 1 Synthesis of eugenol derivatives

Compounds **4**–**9** were considered pure enough for the biological evaluations described herein, since they gave narrow melting ranges, single-spot in TLC analysis and ascertained identities by IR and NMR spectroscopy techniques. ^{1}H and ^{13}C NMR spectra of eugenol glycosides are shown in Online Resource.

In vitro bioassays

The prototype **EUG** and some of its derivatives presented activity against the fungi (yeasts) evaluated. The results of antifungal assays are reported in Table 1, along with those of reference drug fluconazole.

The action of **EUG** was lower than the standard fungistatic drug fluconazole. Eugenol peracetyl-glucoside **4** was the only one that presented activity against all the yeasts with prominent results against *C. glabrata*, with IC_{50} value 160 times lower than that of **EUG**. The glucosides **4** and **7** were both active against *C. albicans* and showed activities 3- and 2-fold greater than the natural prototype **EUG**, respectively. For *C. tropicalis*, only the derivative **4** was active and showed activity 3-fold greater than that of **EUG**. The modifications carried out on the derivatives **4** and **5** did not contribute to the enhancement of activity modulation against *C. krusei*, since they were 1.1 and 1.67 times less active than **EUG**, respectively. The same negative activity modulation was seen with derivative **4** for *C. parapsilosis.* On the other hand, derivative **4** were about 3.5 times more active than fluconazole, showing that the modifications carried out on this derivative in comparison to the prototype **EUG** were very important for positive activity modulation. Notably, eugenol peracetyl-galactoside was 80 times less potent than its close analogue **4**. This may suggest stereoselectivity in the drug actions on these fungi since in all other cases the glucose derivatives were more active than those of galacto configuration. Lactosides **6** and **9** showed no activity up to the higher concentration tested. The overall action of acetylated glycosides was higher than that of deacetylated derivatives. This is not surprising since they may be acting as high lipophilicity pro-drugs which can more easily cross the fungal cell membrane, followed by intracellular cleavage of the acetyl groups to the free sugars. Deacetylated derivatives themselves are unable to penetrate because they are too polar. Most of these glucose and galactose eugenol derivatives presented fungistatic action and only compounds **4** and **5** for *C. krusei* and compound **4** for *C. glabrata* presented fungicidal activity.

The cytotoxic activities of the most active compounds (**4**, **5** and **7**) were tested in peripheral human blood mononuclear cells obtained from healthy donors and the results are shown in Table 2. The prototype molecule **EUG** showed higher toxicity than their derivatives on these cells with a CC_{50} equal to 90 μM. Eugenol derivatives **4**, **5** and **7** showed an increase in cytotoxic concentration for 50 % of cells values ranging from 1.9 to 3.3 times higher than **EUG** CC_{50}. These data indicate that the structural modifications done on prototype have the potential to reduce the cytotoxic activity of **EUG** in eukaryotic cells. In accordance to our findings, the midpoint cytotoxicity values of eugenol solutions were reported in the region of 100–300 μM by some researchers (Atsumi *et al.*, 2000; Babich *et al.*, 1993) while Prashar and colleagues (2006) have demonstrated that unsolubilized eugenol is highly cytotoxic in concentrations as low as 1.8 μM. It may suggest that the phenol group masking seen in these glycosides could be responsible for this lower toxicity. The selectivity index values of <1 for some of tested microorganisms may indicate that the observed antifungal activity may be a result of non-specific toxicity. On the other hand, the selectivity index of derivative **4** (45.3) showed the in vitro inhibitory potential of this derivative to act as potential antifungal molecule to treat *C. glabrata* infections. *C. glabrata* is considered a

Table 1 Antifungal activity of eugenol and its derivatives (in μM)

Compound	Microorganism									
	C. albicans ATCC 10231		*C. tropicalis* ATCC 750		*C. krusei* ATCC 6258		*C. parapsilosis* ATCC 22019		*C.glabrata* ATCC 90030	
	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}
4	121.5	–[a]	202.0	–	202.0	202.0	202.0	–	3.8	202.0
5	–	–	–	–	306.7	306.7	–	–	306.7	–
6	–	–	–	–	–	–	–	–	–	–
7	184.0	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–	–
EUG	365.8	–	609.8	–	182.9	–	182.9	–	609.8	–
FLC[b]	3.3	–	3.3	–	104.5	–	13.1	–	13.1	–

[a] No significant activity

[b] Fluconazole

Table 2 Cytotoxic activity (CC_{50}) and selectivity index for antifungal compounds **4**, **5** and **7** on test microorganisms

Compound	CC_{50} (μM)	Selectivity index				
		C. albicans ATCC 10231	*C. tropicalis* ATCC 750	*C. krusei* ATCC 6258	*C. parapsilosis* ATCC 22019	*C. glabrata* ATCC 90030
4	172.0	1.42	0.85	0.85	0.85	45.3
5	262.0	ND[a]	ND	0.85	ND	0.85
7	300.0	1.63	ND	ND	ND	ND
EUG	90.0	0.25	0.15	0.49	0.49	0.15

[a] Not determined

common commensal in gastrointestinal and genitourinary tracts, but can turn into an opportunistic fungal pathogen in immunocompromised patients. This species has both innate and acquired resistance against antifungal drugs, due to its ability to modify ergosterol biosynthesis, mitochondrial function, or antifungal efflux. This resistance allows for its relative overgrowth over other susceptible species and may contribute to the recent emergence of *C. glabrata* infections in chronically immunocompromised populations (Li *et al*., 2007).

Conclusions

A set of six eugenol glycosides were synthesized by conventional methods and they were evaluated in vitro as antifungal agents against *Candida* sp. Derivatives **4**, **5** and **7** had variable anti-*Candida* activity. Lactoside derivatives **6** and **9** showed no activity up to the higher concentration tested. The peracetylglucose derivative **4** presented the most promising antifungal action since it was less cytotoxic and more potent than eugenol in this antimicrobial evaluation. Moreover, derivative **4** was 3.5 times more potent than fluconazole against *C. glabrata*. Deacetylated analogue **7** and galacto stereoisomer **5** were less active than derivative **4** on *Candida* species suggesting the influence of lipophilicity and configuration issues on observed activity. Finally, all the derivatives were less cytotoxic than eugenol, indicating that glycosylation was effective in order not to be harmful to eukaryotic cells.

Materials and methods

Physical measurements

Melting point of the compounds was determined on Microquímica MOAs 301 apparatus and was uncorrected. IR spectroscopy was performed by Spectrum One, Perkin-Elmer. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on Bruker Avance DPX-200 or Avance-DRX-400. The specific optical rotation $[\alpha]_D$ were measured on Perkin Elmer 341, at 20 °C. Reaction courses and product mixtures were monitored by thin-layer chromatography (TLC) on silica gel-G TLC plates (Merck).

Synthesis of compounds

*Synthesis of peracetylglycosyl bromides (**1–3**)*

The α-D-glycosyl bromides (intermediates **1**–**3**) were prepared according to literature methods by reacting peracetyl glucose, galactose or lactose with hydrobromide acid in acetic anhydride at low temperature (Mitchell *et al*., 2001).

*Synthesis of peracetylated glycosides (compounds **4–6**)*

A solution of the corresponding glycosyl bromide (6.15 mmol) in acetone (20 mL) was added to a solution of eugenol (18 mmol) in 1.0 mol/L lithium hydroxide (10 mL) and the solution stirred for 2 h at room temperature. The completion of reaction was monitored by TLC technique, when acetone was removed by ventilation and the resulting suspension was extracted with dichloromethane (3 × 50 mL). The crude product was washed with 10 % sodium hydroxide (3 × 30 mL), water and dried by anhydrous sodium sulphate. After filtration and removal of the solvent under reduced pressure, the crude product was recrystallised from isopropyl alcohol, affording compounds **4–6** (Scheme 1, Table 1).

*Synthesis of deacetylated glycosides (compounds **7–9**)*

The peracetylated glycosides (0.7 mmol) were solubilized in a solution of MeOK in MeOH (20 mL, 1.0 mol/L) and stirred at room temperature for 30 min. After the completion of the reaction, noticed by TLC, the mixture was neutralized with IRA-120 resin. The resin was filtered off and washed with methanol. The collected filtrate was concentrated in vacuum to afford glycosylated derivatives **7–9** (Scheme 1; Table 1).

*4-allyl-2-methoxyphenyl-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside) (**4**)* White crystals, yield: 55 %; m.p. 117–119 °C; $[\alpha]_D$ −16.0° (*c* 0.5 CH_2Cl_2); IR; υ_{max} in cm^{-1}: 3050 (ar. C–H), 2969, 2901 (sp^3 C–H), 1751 (C=O),

1636 (C=C), 1595, 1512, 1466 (ar. C=C); ^{1}H NMR ($CDCl_3$, 200 MHz) δ ppm: 7.0 (1H, Ar–H), 6.69–6.64 (m, 2H, Ar–H), 5.9–5.8 (1H, Allylic), 5.2–4.8 (m, 6H, sp^3, Allylic), 4.3–4.0 (m, 2H, sp^3), 3.8–3.7 (m, 4H, sp^3), 3.3 (2H, sp^3), 2.05–2.01 (12H, sp^3); ^{13}C NMR ($CDCl_3$, 50 MHz) δ ppm: 170.4–169.1 (4C, C=O), 150.2 (1C, ar.), 144.1 (1C, ar.), 136.9 (1C, ar.), 136.5 (1C, allylic), 120.3 (1C, ar.), 120.0 (1C, ar.), 115.7 (1C, allylic), 112.7 (1C, ar.), 100.7 (1C, sugar), 72.3 (1C, sugar), 71.6 (1C, sugar), 70.9 (1C, sugar), 68.1 (1C, sugar), 61.6 (1C, sugar), 55.6 (1C, sp^3), 39.6 (1C, sp^3), 20.3–20.3 (4C, sp^3); Mol. formula: $C_{24}H_{30}O_{11}$; Mol. Weight: 494.4.

*4-allyl-2-methoxyphenyl-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside) (**5**)* White crystals, yield: 56 %; m.p. 125–127 °C; $[\alpha]_D$ −11.5° (*c* 0.5 CH_2Cl_2); IR; υ_{max} in cm^{-1}: 2972, 2881 (sp^3 C–H), 1736 (C=O), 1642 (C=C), 1604, 1588, 1508, 1467 (ar. C=C); ^{1}H NMR ($CDCl_3$, 200 MHz) δ ppm: 7.0 (1H, Ar–H), 6.7–6.6 (m, 2H, Ar–H), 6.0–5.8 (1H, Allylic), 5.5–5.4 (m, 2H, Allylic, sp^3), 5.1–5.0 (m, 3H, sp^3, Allylic), 4.86 (1H, anomeric, $J = 8$ Hz), 4.2–4.1 (m, 2H, sp^3), 3.98–3.92 (1H, sp^3), 3.8 (3H, sp^3), 3.34 (2H, sp^3), 2.1–2.0 (12H, sp^3); ^{13}C NMR (DMSO, 50 MHz) δ ppm: 170.1–169.3 (4C, C=O), 150.2 (1C, ar.), 144.3 (1C, ar.), 136.9 (1C, ar.), 136.4 (1C, allylic), 120.3 (1C, ar.), 119.8 (1C, ar.), 115.7 (1C, allylic), 112.7 (1C, ar.), 101.3 (1C, sugar), 70.56 (1C, sugar), 70.50 (1C, sugar), 68.4 (1C, sugar), 66.6 (1C, sugar), 60.9 (1C, sugar), 55.7 (1C, sp^3), 39.6 (1C, sp^3), 20.5–20.3 (4C, sp^3); Mol. formula: $C_{24}H_{30}O_{11}$; Mol. Weight: 494.4.

*4-allyl-2-methoxyphenyl-(2,3,6,2′,3′,4′,6′-hepta-O-acetyl-β-D-lactoside) (**6**)* Pale yellow crystals, yield: 51 %; m.p. 51.2–53,7 °C; $[\alpha]_D$ −19.7° (*c* 0.5 CH_2Cl_2); IR; υ_{max} in cm^{-1}: 2972, 2901 (sp^3 C–H), 1741 (C=O), 1638 (C=C), 1594, 1509 (ar. C=C); ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm: 6.9 (1H, Ar–H), 6.7–6.6 (m, 2H, Ar–H), 5.9–5.8 (1H, Allylic), 5.3–4.8 (m, 8H, sp^3, allylic), 4.1–4.0 (m, 4H, sp^3), 3.9–3.8 (m, 3H, sp^3), 3.7–3.6 (m, 1H, sp^3), 3.7 (s, 3H, sp^3), 3.3 (2H, sp^3, $J = 6$ Hz), 2.1–1.9 (21H, sp^3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ ppm: 170.6–169.3 (7C, C=O), 150.7 (1C, ar.), 144.6 (1C, ar.), 137.4 (1C, ar.), 136.9 (1C, allylic), 120.8 (1C, ar.), 120.2 (1C, ar.), 116.2 (1C, allylic), 113.3 (1C, ar.), 101.2 (1C, sugar), 101.0 (1C, sugar), 76.5 (1C, sugar), 73.0 (1C, sugar), 72.9 (1C, sugar), 71.6 (1C, sugar), 71.2 (1C, sugar), 70.9 (1C, sugar), 69.3 (1C, sugar), 62.2 (1C, sugar), 61.1 (1C, sugar), 60.6 (1C, sugar), 56.1 (1C, sp^3), 39.9 (1C, sp^3), 20.8 (7C, sp^3); Mol. formula: $C_{36}H_{46}O_{19}$; Mol. Weight: 782.4.

*4-allyl-2-methoxyphenyl β-D-glucopyranoside (**7**)* Brown crystals, yield: 54 %; m.p. 109–110 °C; $[\alpha]_D$ −38.4° (*c* 0.5 MeOH); IR; υ_{max} in cm^{-1}: 3295 (OH), 3077 (ar. C–H), 2975, 2917 (sp^3 C–H), 1638 (C=C), 1595, 1512, 1465 (ar. C=C); ^{1}H NMR (DMSO, 200 MHz) δ ppm: 7.0 (1H, Ar–H), 6.8 (1H, Ar–H), 6.6 (1H, Ar–H), 6.0–5.8 (1H, Allylic), 5.1–4.8 (m, 6H, sp^3, Allylic, OH), 4.5 (1H, OH), 3.7–3.1 (m, 10H, sp^3, Allylic, OH); ^{13}C NMR (DMSO, 50 MHz) δ ppm: 148.8 (1C, ar.), 144.8 (1C, ar.), 137.9 (1C, allylic), 133.4 (1C, ar.), 120.2 (1C, ar.), 115.5 (1C, allylic), 115.4 (1C, ar.), 112.8 (1C, ar.), 100.1 (1C, sugar), 76.9 (1C, sugar), 76.8 (1C, sugar), 73.2 (1C, sugar), 69.6 (1C, sugar), 60.5 (1C, sugar), 55.5 (1C, sp^3), 38.8 (1C, sp^3); Mol. formula: $C_{16}H_{22}O_7$; Mol. Weight: 326.24.

*4-allyl-2-methoxyphenyl β-D-galactopyranoside (**8**)* White crystals, yield: 55 %; m.p. 163–165 °C; $[\alpha]_D$ −34.6° (*c* 0.5 MeOH); IR; υ_{max} in cm^{-1}: 3350 (OH), 3001 (ar. C–H), 2975, 2938, 2903 (sp^3 C–H), 1638 (C=C), 1594, 1514, 1464 (ar. C=C); ^{1}H NMR (DMSO, 200 MHz) δ ppm: 7.0 (1H, Ar–H), 6.7 (1H, Ar–H), 6.6 (1H, Ar–H), 6.0–5.8 (1H, Allylic), 5.1–5.0 (m, 3H, sp^3, OH), 4.87–4.80 (m, 2H, sp^3, Allylic), 4.6 (1H, OH), 4.5 (1H, Allylic), 3.7–3.2 (m, 11H, sp^3, Allylic); ^{13}C NMR (DMSO, 50 MHz) δ ppm: 148.9 (1C, ar.), 144.9 (1C, ar.), 137.9 (1C, allylic), 133.3 (1C, ar.), 120.2 (1C, ar.), 115.5 (1C, allylic), 115.4 (1C, ar.), 112.8 (1C, ar.), 100.7 (1C, sugar), 75.4 (1C, sugar), 73.5 (1C, sugar), 70.2 (1C, sugar), 68.1 (1C, sugar), 60.3 (1C, sugar), 55.6 (1C, sp^3), 39.1 (1C, sp^3); Mol. formula: $C_{16}H_{22}O_7$; Mol. Weight: 326.24.

*4-allyl-2-methoxyphenyl β-D-lactoside (**9**)* Brown crystals, yield: 50 %; m.p. 190–192 °C; $[\alpha]_D$ −27.5° (*c* 0.5 MeOH); IR; υ_{max} in cm^{-1}: 3358 (OH), 2971, 2918 (sp^3 C–H), 1638 (C=C), 1593, 1512, 1460 (ar. C=C); ^{1}H NMR (DMSO, 400 MHz) δ ppm: 6.9 (1H, Ar–H), 6.7 (1H, Ar–H), 6.5 (1H, Ar–H), 5.89–5.79 (1H, allylic), 5.23 (1H, sp^3), 4.99–4.92 (4H, allylic, sp^3), 4.83 (2H, OH), 4.66 (1H, OH), 4.56–4.40 (2H, sp^3), 4.1 (1H, OH), 3.64 (3H, sp^3), 3.53–3.19 (m, 14H, sp^3, OH, allylic). ^{13}C NMR (DMSO, 50 MHz) δ ppm: 148.9 (1C, ar.), 144.7 (1C, ar.), 137.8 (1C, ar.), 133.6 (1C, allylic), 120.2 (1C, ar.), 115.57 (1C, allylic), 115.55 (1C, ar.), 112.9 (1C, ar.), 103.8 (1C, sugar), 99.8 (1C, sugar), 80.2 (1C, sugar), 75.5 (1C, sugar), 75.0 (1C, sugar), 74.8 (1C, sugar), 73.2 (1C, sugar), 73.0 (1C, sugar), 70.5 (1C, sugar), 68.1 (1C, sugar), 60.4 (1C, sugar), 60.1 (1C, sugar), 55.6 (1C, sp^3), 39.0 (1C, sp^3); Mol. formula: $C_{22}H_{32}O_{12}$; Mol. Weight: 488.32.

In vitro bioassays

Antifungal activity evaluation

Eugenol (**EUG**) and its derivatives **4**, **5**, **6**, **7**, **8** and **9** were evaluated in vitro for their antimicrobial activities against the fungi through a Mueller–Hinton broth microdilution method and with the methodology and interpretative

criteria proposed by document M27A3 (CLSI, 2008). The stock solutions of all the compounds were prepared in DMSO 1 % at final concentration and tested at concentrations (μg/mL) 100, 62.5, 31.2, 15.6, 7.8, 3.9, 1.95, 0.48, 0.24 and 0.06, respectively. The standard drug fluconazole was applied as control of fungistatic action at concentration (μg/mL) 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125, respectively. The microplates were incubated at 37 °C and for 24 h for fungi. Results were visualized and analyzed by spectrophotometry at 530 nm in an Anthos Zenyth 200rt Microplate Reader. The inhibitory concentration of microbial growth was determined at 50 % (IC_{50}) (fungistatic action) and 100 % (IC_{100}) (fungicidal action) in μM and compared for each compound and microorganism. 20 μL-aliquots of each well corresponding to the IC_{50} and IC_{100} values and to the growth control well were plated onto Sabouraud-chloramphenicol agar medium. The absence of growth confirmed the fungicidal action while the growth reduction when compared to the growth control well confirmed the fungistatic action of the compound.

Cytotoxicity assay

The cytotoxicity of compounds **4**, **5**, **7** and **EUG** (concentration range 100–0.78 μg/mL) to peripheral human blood mononuclear cells (PBMCs) was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Dias *et al*., 2012). 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) and the cells were incubated again for an additional 4-h 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 $[(A - B)/A \times 100)]$, 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 IC_{50}/CC_{50}.

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