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Antibacterial activity of 3,3′-dihydroxycurcumin (DHC) is associated with membrane perturbation

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ABSTRACT

Curcumin is a plant diphenylheptanoid and has been investigated for its antibacterial activity. However, the therapeutic uses of this compound are limited due to its chemical instability. In this work, we evaluated the antimicrobial activity of diphenylheptanoids derived from curcumin against Gram-positive and Gram-negative bacteria, and also against *Mycobacterium tuberculosis* in terms of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) values. 3,3′-Dihydroxycurcumin (**DHC**) displayed activity against *Enterococcus faecalis*, *Staphylococcus aureus* and *M. tuberculosis*, demonstrating MIC values of 78 and 156 μg/mL. In addition, **DHC** was more stable than curcumin in acetate buffer (pH 5.0) and phosphate buffer (pH 7.4) for 24 h at 37 °C. We proposed that membrane and the cell division protein FtsZ could be the targets for **DHC** due to that fact that curcumin exhibits this mode of antibacterial action. Fluorescence microscopy of *Bacillus subtilis* stained with SYTO9 and propidium iodide fluorophores indicated that **DHC** has the ability to perturb the bacterial membrane. On the other hand, **DHC** showed a weak inhibition of the GTPase activity of *B. subtilis* FtsZ. Toxicity assay using human cells indicated that **DHC** has moderate capacity to reduce viability of liver cells (HepG2 line) and lung cells (MRC-5 and A549 lines) when compared with doxorubicin. Alkaline comet assay indicated that **DHC** was not able to induce DNA damage in A549 cell line. These results indicated that **DHC** is promising compound with antibacterial and antitubercular activities.

1. Introduction

Curcumin is the main diphenylheptanoid from rhizomes of *Curcuma longa*, commonly named as turmeric. It has been considered a privileged natural product due to its therapeutic potential [1]. Its abilities to interact with multiple targets has recognized role in polypharmacology of complex diseases such as cancer and Alzheimer [2]. Additionally, several clinical studies have supported its safety and tolerability for humans [3].

Curcumin has demonstrated a broad spectrum of *in vitro* antibacterial activity against Gram-positive, Gram-negative and *Mycobacteria* species [4,5]. *In vivo* investigations showed that curcumin was able to eliminate *Helicobacter pylori* from mice stomach [6] and to decrease the mortality of mice infected with methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) [7]. The mode of antibacterial action of curcumin has been reported as a combination of membrane disruption [8] and cell division blockage [9]. Activity of curcumin on the membrane was attributed to its amphiphilicity, which allows its insertion into phospholipid bilayers, increasing the permeability of membranes [8,10]. The ability of curcumin to perturb bacterial cell division has been attributed to a direct action on FtsZ, a prokaryotic cytoskinesis protein.

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Curcumin inhibited FtsZ protofilament assembly and enhanced the GTPase activity of this protein by altering its secondary structure [9,11]. The FtsZ polymerization into protofilaments require GTP as a stabilizer and an energy source, leading to the formation of the Z-ring, a pivotal structure in the assembly of the divisome [12].

Although curcumin has shown to be a promising antibacterial agent, its poor chemical stability has not supported advances of its use in clinical and pharmaceutical studies [13,14]. In addition, curcumin exhibits low oral bioavailability due to its poor absorption and extensive intestinal and first-pass metabolism, resulting in rapid excretion [15]. Wang and collaborators described 90% curcumin decomposed rapidly in buffer systems at neutral-basic pH conditions similar to physiological matrices [16]. However, the addition of glutathione, ascorbic acid or phosphate buffer in culture media increased its stability [16]. Interestingly, the presence of human blood also decreased its degradation [16] which may be correlated with effect of plasma proteins, mainly serum albumin and fibrinogen [17]. Thereby, efforts have been made in order to search for more stable analogs [18,19].

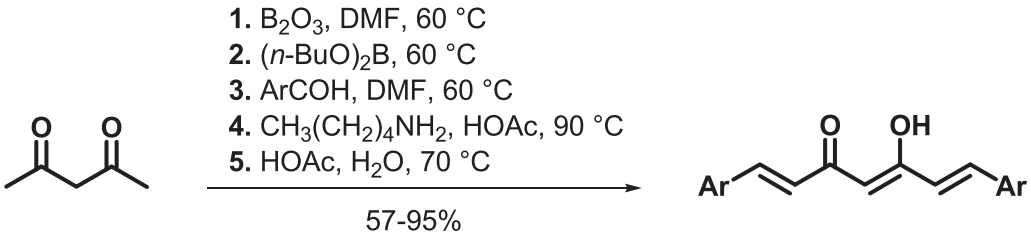
In our previous work, curcumin was used as framework to design antibacterial simplified monoketones, which were more active and stable than curcumin [20]. In the current work, we explored the role of substituents on the aromatic rings of curcumin framework, including electron-donating groups (OH, OMe, Me, $N(Me)_2$ and OAc) and halogens (F, Cl, Br and CF_3) toward antimicrobial activity. We synthesized a series of diphenylhepatanoids based on curcumin framework (curcuminoids) and evaluated their antibacterial and antitubercular activities. The chemical stability of most active curcuminoid (**DHC**) was compared with curcumin in acetate buffer (pH 5.0) and phosphate buffer (pH 7.4) for 24 h at 37 °C. **DHC** was investigated regarding its effects on the membrane and FtsZ of *Bacillus subtilis*. In order to understand toxicity of **DHC** toward human cells, cytotoxicity and alkaline comet assays were carried out.

2. Results and discussion

2.1. Chemistry

One-pot synthesis of curcuminoids **1–12** was achieved by Pabon protocol [21], with minor modifications [22]. Reactions between respective benzaldehydes and acetylacetone were performed through successive boron complexes, resulting in yields of 57–95% (Scheme 1). In general, a clear and unambiguous relationship between the stereoelectronic effect of substituents on aromatic ring of benzaldehyde derivatives and reaction yields cannot be established. Ester **13** was synthesized by acetylation of compound **5** using acetic anhydride and pyridine, with yield of 87%.

Structure of curcuminoids was confirmed by their melting point and ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectral data analyses. Spectra were presented in Supplementary Material. For all curcuminoids, NMR data, including chemical shifts, integrations multiplicities and coupling constants, corresponded to the proposed structures. In ^{1}H NMR spectrum, aromatic hydrogens were observed as signals in range of δ_H 6.50–7.67 ppm. Hydrogens α and β resonated as doublets and clearly indicated the formation of two olefines. Coupling constants values of olefin hydrogens signals ranging from 15 to 16 Hz, corroborating their *trans* configuration. In addition, singlet with resonance at $\delta_H \sim 5.80$ ppm and integration to one hydrogen was attributed to methyne hydrogen of enol moiety [23].



Scheme 1. One-pot synthesis of curcuminoids **1–12.** Ar = phenyl ring substituted by electron-donating groups or electron-withdrawing groups.

2.2. Antibacterial and antitubercular activities

The antibacterial and antitubercular activities of curcumin and curcuminoids **1–13** were evaluated against *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Mycobacterium tuberculosis*. Antimicrobial potency was expressed as Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values in μg/mL. Ciprofloxacin and isoniazid were used as reference antibacterial and antitubercular drugs, respectively (Table 1).

Curcumin displayed activity against *E. faecalis*, *S. aureus* and *B. subtilis*, with MIC values of 78 and 156 μg/mL. However, curcumin demonstrated MIC > 156 μg/mL against *M. tuberculosis*, *E. coli* and *P. aeruginosa*. Although curcumin has been recognized for its broad antibacterial spectrum, some studies reported that its activity against Gram-positive bacteria has been higher than against Gram-negative species [24,25], corroborating our results. Activity of curcumin against *S. aureus* has been extensively reported. A review about curcumin activity against MSSA strains indicated MIC values ranged from 125 to 256 μg/mL by using broth microdilution method. These values varied according to strain types and vehicles (DMSO, EtOH or DMF) [26]. In addition, there are studies demonstrating ability of curcumin to reverse methicillin resistance in MRSA [27] and its efficacy against osteomyelitis in rats induced by MRSA, suppressing bacterial growth [28].

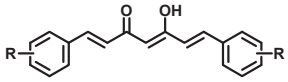
First, in order to investigate preliminary relationships between antibacterial/antitubercular activities and substituents on aromatic rings, we compared the activity of curcumin with the unsubstituted curcuminoid (**1**). Compound **1** was inactive (MIC and MBC values > 156 μg/mL), indicating that substituents OMe and OH at *meta* and *para* positions, respectively, are relevant for antibacterial activity of curcumin against Gram-positive species.

Second, we evaluated the effect of *m*-OMe and *p*-OH separately, testing simplified analogs 3,3′-dimethoxycurcumin (**2**) and 4,4′-dihydroxycurcumin (**3**), respectively. Compounds **2** and **3** had MIC and MBC values > 156 μg/mL, suggesting that antibacterial activity of curcumin can be correlated to simultaneous presence of OMe and OH at *meta* and *para* positions, respectively. Also, compound **3** exhibits a natural occurrence in turmeric (also named as bisdesmethoxycurcumin or curcumin III) [14]. Its antibacterial activity was previously studied by Péret-Almeida and co-authors, who reported their inactivity against *S. aureus*, *E. coli* and *Salmonella choleraesuis* [29], corroborating our current findings.

Third, we evaluated two regioisomers of compounds **2** and **3**, which were designed by replacement of OMe and OH positions, furnishing 4,4′-dimethoxycurcumin (**4**) and 3,3′-dihydroxycurcumin (**5** or **DHC**), respectively. Compound **4** was inactive (MIC and MBC values > 156 μg/mL). On the other hand, **DHC** demonstrated antibacterial activity similar to curcumin against *S. aureus* and *B. subtilis* with MIC values of 156 μg/mL. Furthermore, **DHC** was more potent than curcumin against *E. faecalis* and *M. tuberculosis* with MIC values of 78 and 156 μg/mL, respectively.

Fourth, we evaluated the effect of other electron-donating groups on aromatic rings, including Me (curcuminoid **6**) and $N(Me)_2$

Table 1
Antibacterial and antitubercular activities of curcumin and curcuminoids **1–13** expressed as MIC and MBC values in μg/mL.



Compound	R	Ef		Sa		Bs		Mt
		MIC	MBC	MIC	MBC	MIC	MBC	MIC
Curcumin	*m*-OMe,*p*-OH	156	> 156	156	> 156	78	156	> 156
1	H	> 156	> 156	> 156	> 156	> 156	> 156	> 156
2	*m*-OMe	> 156	> 156	> 156	> 156	> 156	> 156	> 156
3	*p*-OH	> 156	> 156	> 156	> 156	> 156	> 156	> 156
4	*p*-OMe	> 156	> 156	> 156	> 156	> 156	> 156	> 156
DHC or 5	*m*-OH	78	156	156	> 156	78	156	156
6	*p*-Me	> 156	> 156	> 156	> 156	> 156	> 156	> 156
7	*p*-$N(Me)_2$	> 156	> 156	> 156	> 156	> 156	> 156	> 156
8	*p*-F	> 156	> 156	> 156	> 156	> 156	> 156	> 156
9	*p*-Cl	> 156	> 156	> 156	> 156	> 156	> 156	> 156
10	*p*-Br	> 156	> 156	> 156	> 156	> 156	> 156	> 156
11	*p*-CF_3	> 156	> 156	> 156	> 156	> 156	> 156	> 156
12	*m*,*p*-diCl	> 156	> 156	> 156	> 156	> 156	> 156	> 156
13	*m*-OAc	> 156	> 156	> 156	> 156	> 156	> 156	> 156
Ciprofloxacin	—	0.25	0.25	0.015	0.03	0.015	0.015	—
Isoniazid	—	—	—	—	—	—	—	0.04

MIC = minimal inhibitory concentration; MBC = minimum bactericidal concentration; Ef = *Enterococcus faecalis;* Sa = *Staphylococcus aureus*; Bs = *Bacillus subtilis* and Mt = *Mycobacterium tuberculosis*; — not determined.

(curcuminoid **7**), which release electrons by inductive and mesomeric effect, respectively. Curcuminoids **6** and **7** were inactive (MIC and MBC values > 156 μg/mL). The comparison among the curcuminoids **DHC**, **6** and **7** indicated that the electron donation on aromatic rings is not relevant to antibacterial and antitubercular activities. However, the bioactivity seems to be related to acidity of phenol group in **DHC**, because neutral (Me, curcuminoid **6**) and basic ($N(Me)_2$, curcuminoid **7**) groups did not improve antimicrobial potency.

Fifth, we assayed a set of curcuminoids substituted by *p*-F, *p*-Cl, *p*-Br, *p*-CF_3 and *m*,*p*-diCl (compounds **8** to **12**, respectively). All halogenated curcuminoids were not able to inhibit bacterial growth (MIC and MBC values > 156 μg/mL), suggesting electron-withdrawing substituents on aromatic rings did not increase antibacterial and antitubercular activities.

In our previous work, we described that diacetylated curcumin (DAC) was more active than curcumin against *Streptococcus mutans*, MSSA and MRSA planktonic and biofilm forms [30,31]. Thus, we hypothesized that the acetylation of **DHC** could lead to a more potent analog. Unfortunately, ester **13** was inactive (MIC and MBC values > 156 μg/mL), corroborating the relevance of acidic OH at *meta* position (phenol moiety) to antibacterial and antitubercular activities. In general, phenolic compounds are recognized for their antibacterial activity [32,33]. Recent studies have elucidated the importance of the hydroxyl group position in phenolic natural product derivatives concerning antibacterial potency, including cinnamylideneacetophenones [34], gallates, protocatechuates, gentisates and β-resorcylates [35].

Curcuminoids **1–13** were not able to inhibit growth of Gram-negative species, including *P. aeruginosa* and *E. coli*. Gram-negative bacteria demonstrate a complex barrier, including two membranes, which makes them less susceptible to antibacterial agents. As part of this barrier, the cell wall exerts central role in the permeation of compounds due to their complex composition (lipopolysaccharides, phospholipids and proteins) [36,37].

We selected **DHC** for further assays, aiming to evaluate its chemical stability and the elucidation of its molecular targets, which could be related to the mode of antibacterial action exhibited by curcumin. In previous studies by our group, curcumin disrupted the membrane of *B. subtilis*, which was confirmed by microscopy assays. Also, we described that curcumin blocked *B. subtilis* cell division, stimuling the GTPase activity of FtsZ [20].

2.3. Evaluation of *DHC* chemical stability

Although curcumin is a promising antibacterial agent, its clinical application is limited due to its poor stability under physiological conditions [14]. Thus, chemical stability of **DHC** was evaluated at pH 5.0 and 7.4 by monitoring the decrease in its UV–Vis absorption at 37 °C and compared with curcumin. As expected, both compounds were more stable under acidic than basic conditions (Fig. 1). Curcumin was rapidly degraded at pH 5.0 and 7.4, reducing 52% and 63% after 24 h, respectively. However, **DHC** was more stable than curcumin at pH 5.0 and 7.4, reducing only 16% and 24% of its initial concentration after 24 h, respectively. Liu and co-authors also reported a higher stability of **DHC** when compared with curcumin in RPMI media at 25 °C [38], corroborating our current findings.

Wang and collaborators reported feruloylmethane, vanillin and ferulic acid as degradation compounds of curcumin under phosphate

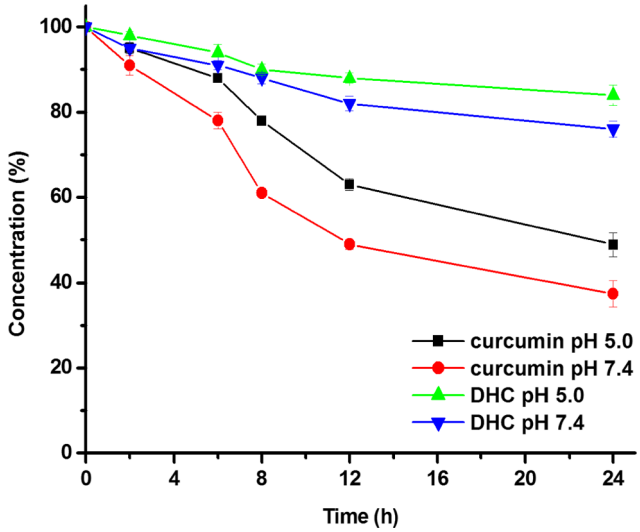


Fig. 1. Chemical stability of **DHC** and curcumin at 13.5 μg/mL (pH 5.0 and 7.4) by monitoring the decrease in their maximum absorbance at 37 °C for 24 h. Data are represented as means ± SEMs and expressed as percentage.

buffer at pH 7.2 (at 37 °C) [16]. These compounds are produced by cleavage of the β-diketone moiety. Thus, stabilizing effects on transition state of these reactions can improve the degradation rate. In this context, curcumin exhibits a hydroxyl group at *para*-position, which resonates to β-diketone moiety, dispersing π-electron density. On the other hand, **DHC** presents a hydroxyl group at *meta*-position, which did not allow that alone electron pairs of hydroxyl group resonate to β-diketone moiety. Thereby, extended conjugation between 4-OH and β-diketone in curcumin structure can be correlated to its degradation. Furthermore, in basic conditions hydroxyl group at *para*-position is deprotonated, facilitating conjugation and degradation of curcumin.

2.4. Effect of **DHC** on the membrane of *B. subtilis*

In order to evaluate the effects of **DHC** on the bacterial membrane, we used *B. subtilis* strain 168. Bacterial cells were exposed to **DHC** at the MIC (78 μg/mL) for 15 and 30 min. Two fluorescent dyes propidium iodide (PI) and SYTO9 were added, which stain cells with damaged membrane (in red) and intact membrane (in blue) [8,39]. Nisin was used as a reference for membrane disruption due to produce membrane pores in *B. subtilis*. Representative fluorescence microscopy images of the negative control (cells treated with 1% DMSO), the positive control (nisin at 5 μg/mL) and **DHC** are shown in Fig. 2. Cells exposed to DMSO and nisin appear labeled in blue and red, respectively. Exposure to **DHC**, in both periods of 15 and 30 min, led to a prevalence of pink-red stained cells, indicating membrane permeability.

Percentages of cells with damaged membrane were quantified from the microscope images and are shown in Fig. 3. Cultures treated with 1% DMSO had approximately 2% of the cells with membrane permeabilized. On the other hand, treatment with nisin demonstrated 99% of the cells stained with PI due to its ability to make pores in the bacterial membrane [40]. Treatment with **DHC** (at the MIC) for 15 min and 30 min displayed damage percentages of 72% and 85%, respectively, evidencing the membrane disruption of *B. subtilis* by **DHC** is time-dependent effect.

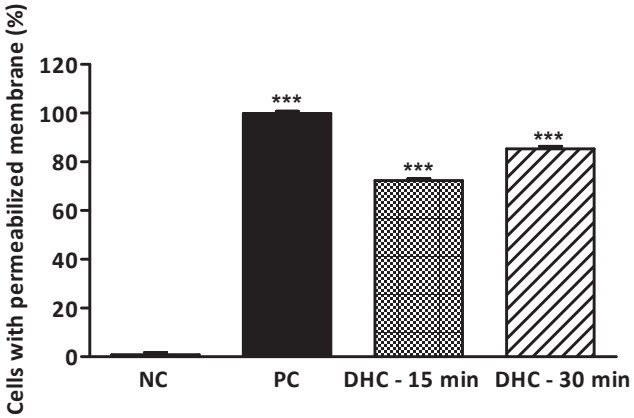


Fig. 3. Percentage of permeabilized cells of *B. subtilis*. **NC:** negative control (cells treated with 1% DMSO); **PC:** positive control (cells treated with nisin at 5 μg/mL, after 30 min); **DHC** (cells treated at MIC, after 15 and 30 min). *** $p < 0.05$, ANOVA with Dunnett's post-test.

Membranes seems to be a target of phenolic compounds [41,42]. They induce changes in the membrane structure and function, including increased permeability [33,43]. Some antibacterial drugs exhibited the membrane as a primary target such as daptomycin [44] and polymyxin B [45] or a secondary one such as telavancin [46]. Furthermore, the combined use of compounds that increase membrane permeability with other antibacterial agents may be a good alternative against bacterial resistance [47,48].

2.5. Effect of **DHC** on the GTPase activity of *B. subtilis* FtsZ

In order to investigate bacterial division as target of **DHC**, we evaluated its ability to affect the GTPase activity of *Bacillus subtilis* FtsZ (BsFtsZ). Compounds that affect the GTP hydrolysis of FtsZ are promising inhibitors of bacterial division, since GTP and GDP levels

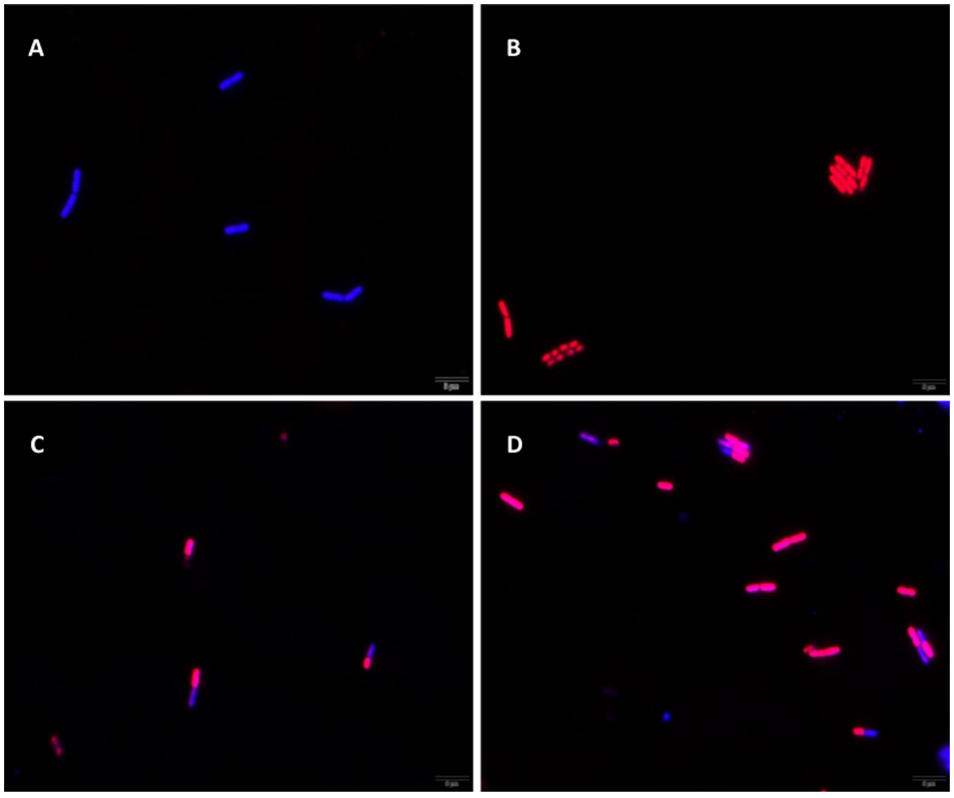


Fig. 2. Fluorescence microscopy of *B. subtilis* strain 168 stained with Live/Dead Baclight kit. **(A)** negative control (cells treated with 1% DMSO); **(B)** positive control (cells treated nisin at 5 μg/mL, after 30 min) **(C) DHC** (cells treated at MIC, after 15 min); **(D) DHC** (cells treated at MIC, after 30 min). Magnification 100 ×; bar 5 μm.

regulate the Z-ring dynamics [49]. We assessed the protein GTP hydrolysis rates during treatment with **DHC** at the MIC (78 μg/mL) and 2 × MIC (156 μg/mL). BsFtsZ was incubated with 1% DMSO and used as negative control. BsFtsZ hydrolysis activity was slightly reduced by **DHC** when compared to the negative control. At the MIC and 2 × MIC values, the percentage of GTPase activity reduction was of 10% and 26%, respectively. Thus, the weak inhibition of bacterial division was less relevant to antibacterial activity than membrane disruption.

2.6. Assessment of *DHC* cyto-genotoxicity and determination of selectivity index

The investigation of new antibacterial agents against eukaryotic cells is an important step for evaluation of their selectivity and safety. Toxicity of **DHC** was evaluated against human cells from liver (tumorigenic HepG2 cell line) and lung (tumorigenic A549 cell and normal MCR-5 cell lines). Hepatocytes are widely used to evaluate the toxicity of bioactive compounds due to biotransformation abilities [50] and lung cells were chosen based on their importance as host site for infections of *E. faecalis*, *S. aureus* and *M. tuberculosis* [51,52]. Toxic effect of curcumin and **DHC** was expressed as the concentration required to reduce the viability of cells at 50% (IC_{50}) (Table 2).

DHC displayed IC_{50} values ranging from 9.6 to 10.6 μg/mL and it was slightly more toxic than curcumin (15.5 μg/mL ≤ IC_{50} ≤ 32.3 μg/mL). Biotransformation capacity of hepatocytes did not affect cytotoxicity of **DHC**, because its IC_{50} value was similar to those of lung cell lines. In addition, no significant difference was observed in the toxic effects of **DHC** against lung lines. Normal human fibroblasts (MCR-5) and adenocarcinoma cells (A549) showed similar sensitivity to **DHC**, indicating altered genes and proteins expression of the tumorigenic lines did not alter the toxicity effect. When compared to doxorubicin (0.03 μg/mL ≤ IC_{50} ≤ 0.9 μg/mL), a compound known for its cytotoxic properties [53], **DHC** had moderade toxicity against human cells. In order to compare the effect towards prokaryotic cells versus eukaryotic cells, the selectivity indexes (SI) of **DHC** were determined as the ratio between IC_{50} and MIC values. We used MIC value of 78 μg/mL and IC_{50} for each human cell line. SI values were of 0.12, 0.13 and 0.12 for HepG2, MRC-5 and A459, respectively. SI values < 1 indicated **DHC** was not selective, displaying higher toxicity against human cells than bacteria.

Several studies have correlated toxicity of curcumin toward human cells to DNA damage, due to its oxidizing properties [54,55]. These effects toward DNA are due to phenolic nature of curcumin and its quinone products, as well as chelatogenic diketone. In order to study genotoxicity of **DHC**, we performed single cell gel electrophoresis assay (alkaline comet assay) with A549 cells, which was the most sensitive line. Comet assay is capable of evaluating damage induced by alkylating, intercalating or oxidizing agents in the DNA of individual cells [56,57]. The DNA of an undamaged nucleus maintains its spherical organization, whereas fragmented DNA migrates across gel (tail) [58]. Hydrogen peroxide (1 mM) was used as reference genotoxic compound due to its strong oxidizing properties and high capacity to induce long comet tail. Lower concentrations than IC_{50} were used to guarantee high percentagens of metabolically viable cells. We selected the concentrations referred to ½ IC_{50} (4.8 μg/mL) and ¼ IC_{50} (2.4 μg/mL) for **DHC**. Also, curcuminin was evaluated at ½ IC_{50} (16.2 μg/mL) and ¼ IC_{50} (8.1 μg/mL).

Tail moment and percentage of DNA in the tail is directly proportional to DNA break [59]. **DHC** demonstrated no significant genotoxic effects compared with the negative control (cells treated with 1% DMSO). Furthermore, the genotoxic parameters of **DHC** were closely similar to curcumin (Fig. 4). Both compounds were three times less able to induce DNA breaks than H_2O_2, indicating weak genotoxic effect toward A549 cell.

Table 2
Toxicity of curcumin and **DHC** against human cells expressed as IC_{50} values (in μg/mL).

Compounds	HepG2 (liver)	MRC-5 (lung)	A549 (lung)
DHC	9.8 ± 0.9	10.6 ± 1.4	9.6 ± 0.4
Curcumin	16.9 ± 0.4	15.5 ± 0.8	32.3 ± 2.1
Doxorubicin	0.5 ± 0.08	0.03 ± 0.002	0.9 ± 0.05

IC_{50} = concentration required to reduce the viability of cells at 50%. HepG2 = liver carcinoma; MRC-5 = normal lung fibroblast; A549 = lung adenocarcinoma.

3. Conclusion

In conclusion, we synthesized a series of curcuminoids as part of our ongoing search for antibacterial agents based on curcumin structure. 3,3′-Dihydroxycurcumin (**DHC**) was found to be an important hit with antibacterial activity against Gram-positive species and *M. tuberculosis*. The mode of action of **DHC** involved membrane disruption, which is an important target for the development of novel antibacterial agents. Furthermore, preliminary *in vitro* investigations of toxicity and stability indicated that **DHC** is potentially safe and stable at pH 5.0 and 7.4. Altogether, our results corroborate the potential of curcumin as a privileged framework for the discovery of compounds for the treatment of bacterial infections and tuberculosis.

4. Experimental

4.1. Chemistry

4.1.1. Material and instruments

All starting materials and reagents were purchased from Merck. Melting points were determined on a Tecnopon PFM-II® apparatus and were uncorrected. NMR spectra were recorded on two spectrometers: Bruker® Avance III 600 MHz (14.1 T) and Bruker® Avance III 400 MHz (9.4 T). Compounds were dissolved in $CDCl_3$ or DMSO-d_6 and its non-deuterated residue was used as internal standard for establishment of chemical shift values (δ_H and δ_C). ^1H and ^{13}C NMR parameters, including δ_H and δ_C, integrations, multiplicities and coupling constants (*J* in Hz) were measured. NMR parameter values were compared to previous descriptions, confirming proposed structures. The ^1H and ^{13}C NMR spectra of curcuminoids **1–13** and their detailed analyses are in Supplementary Material.

4.1.2. Synthesis of curcuminoids *1–12*

Compounds **1–12** were synthesized by Pabon method [21], according to Venkateswarlu and co-authors [22], with minor modifications. Acetylacetone (5.0 mmol, 0.5 mL) was added to suspension of B_2O_3 (5.0 mmol) in DMF (2.0 mL) and stirred for 15 min at 60 °C. Tributyl borate (10 mmol) was added and stirred for 15 min at 60 °C. Respective benzaldehyde derivatives (10 mmol) was added and stirred for 5 min at 60 °C. Mixture of amylamine (0.1 mL) and acetic acid (0.3 mL) in DMF (1 mL) was added to the reaction and heated to 90 °C for 4 h. After cooling, aqueous solution of acetic acid (20%, 50 mL) was added and stirred for 1 h at 70 °C. Reaction medium was poured onto crushed ice from deionized water. Precipitated crude products were filtered, washed with cold water and dried at room temperature. Compounds **1**, **6** and **8–12** were recrystallized in cold ethanol. Compounds **2–5** and **7** were purified by gel filtration column chromatography (LH-20, Sephadex) using ethanol as mobile phase.

4.1.3. Synthesis of curcuminoid *13*

Compound **13** was synthesized by acetylation reaction of **DHC** according to Sardi and co-authors [30], with minor modifications [60]. Acetic anhydride (10 mL) was added to a solution of **DHC** (1 mmol) in pyridine (10 mL) and the reaction mixture was kept under magnetic stirring at 100 °C. After 48 h, the residue was partitioned with ethyl

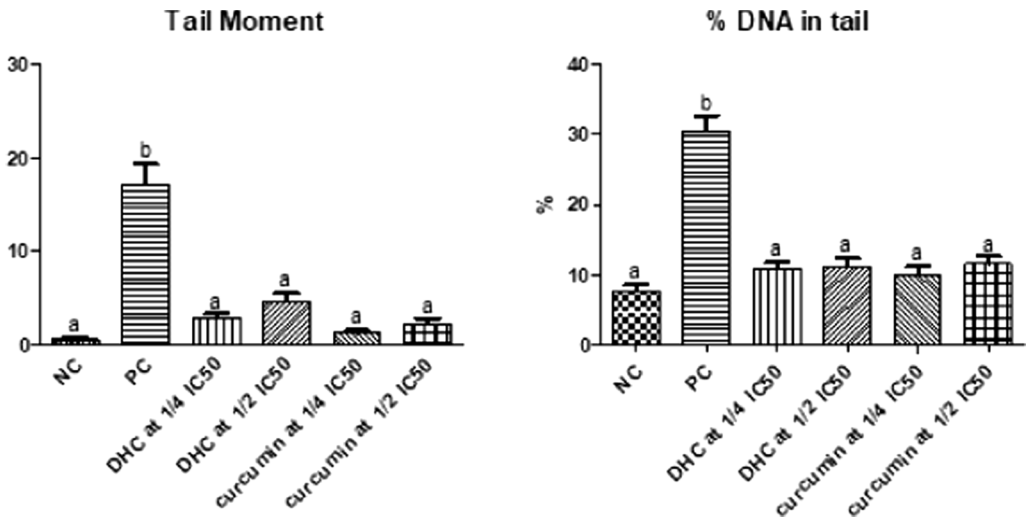


Fig. 4. Genotoxicity of curcumin and **DHC** against A549 cell line measured by tail moment and percentage of DNA in tail using alkaline comet assay. **NC:** negative control (cells treated with 1% DMSO); **PC**: positive control (cells treated with H_2O_2 at 1 mM). ANOVA with Tukey's post-test NC versus treatment. The same letters do not differ significantly at 5% level in comparison to all groups (a), different letters significantly differ at 5% level in comparison to all groups (b).

acetate (3 × 25 mL) and dried at room temperature. The crude product was purified by column chromatography over silica gel using hexane and ethyl acetate (4:1).

4.2. Antibacterial assay

Bacterial species used in this work were *Enterecoccus faecalis* (ATCC 51299), *Staphylococcus aureus* (ATCC 14458), *Bacillus subtilis* strain 168, *Pseudomonas aeruginosa* (ATCC 15442) and *Escherichia coli* (ATCC 10536). MIC and MBC values were determined by the broth microdilution method in 96-well microtiter plates, following protocol of National Committee for Clinical Laboratory Standands [61]. Minor modifications in this protocol and their detailed procedures were reported in previous work by Polaquini and co-authors [34]. Stock solutions (at 10 mg/mL) of curcumin and curcuminoids **1–13** were prepared in DMSO and tested in concentrations ranging from 156 to 2.4 μg/mL. Ciprofloxacin was used as a reference antibacterial drug. Three independent assays were performed.

4.3. Antitubercular assay

Antitubercular activity was performed toward *Mycobacterium tuberculosis* H_{37}Rv strain (ATCC 27294). MIC values were determined by Resazurin Microtiter Assay (REMA), following protocol described by Palomino and co-authors [62]. Minor modifications in this protocol and their detailed procedures were reported in previous work by Polaquini and co-authors [34]. Stock solutions (at 10 mg/mL) of curcumin and curcuminoids **1–13** were prepared in DMSO and tested in concentrations ranging from 156 to 2.4 μg/mL. Isoniazid was used as reference antitubercular drug. Three independent assays were performed.

4.4. Chemical stability assay

Stability of **DHC** (at λ_{max} 398 nm) and curcumin (at λ_{max} 426 nm) was assessed by monitoring their UV–visible absorptions in acetate buffer (pH 5.0) and phosphate buffer (pH 7.4) at 37 °C [19,38]. Compounds were solubilized in DMSO and evaluated at 13.5 μg/mL. The spectra were run against blanks containing buffer solution and DMSO.

4.5. Membrane perturbation assay

The membrane disruption assay was performed as described by Morão and co-authors [20]. The analysis was carried out with the Live/Dead Baclight Bacterial Viability kit (Thermo-Scientific L7012). All analyses were performed at 15 and 30 min. All pictures were taken using an Olympus BX-61 microscope equipped with an OrcaFlash-2.8 monochromatic camera (Hamamatsu) and guided by the CellSens software version 11. Experiments were performed in triplicates, and the quantification analyses considered at least 200 individuals (n = 200) per treatment.

4.6. Anti-FtsZ assay

FtsZ of *Bacillus subtilis* (BsFtsZ) was expressed and purified using the ammonium sulfate precipitation method as described previously [63]. The BsFtsZ GTP hydrolysis rate was determined using the malachite green phosphate assay described by Król and co-authors [63] with minor modifications. Stocks of **DHC** at MIC and 2 × MIC with FtsZ (24 μM), $MgCl_2$ (20 mM) and Triton X-100 (0.02%) prepared in polymerization buffer (Hepes 50 mM, KCl 300 mM, pH 7.5) were stabilized at 30 °C for 5 min. Then, GTP (2 mM) dissolved in the polymerization buffer was added in different time points (the final concentrations were half of the stock solutions). The reactions were kept at 30 °C and developed in accordance with MAK307 (Sigma-Aldrich®) kit. 1% DMSO was used as negative control.

4.7. Cytotoxicity assay

The toxicity against human cells was evaluated according to Silva and co-authors [64]. The cell lines used were HepG2 (human liver carcinoma cell line ATCC HB-8065), MRC-5 (normal human lung fibroblast cell line ATCC CCL-171) and A549 (human lung adenocarcinoma epithelial cell line ATCC CCL-185). For the cytotoxicity assay, 2.5×10^4 cells/mL were seeded into a 96-well cell culture plate with a total volume of 100 μL for 24 h. Cells were treated with curcumin and **DHC** in concentrations ranging from 0.8 to 100 μg/mL. After 24 h incubation, the medium was removed, and 50 μL of resazurin (0.01% w/v) was added to each well, and the plates were incubated at 37 °C for 3 h. The absorbance was measured with a microplate reader in which an excitation (530 nm) and an emission (590 nm) filters were used. Three independents assays were performed. IC_{50} value that represents the concentration required to reduce the viability of cells at 50% was calculated from an analytical curve through a regression analysis. Doxorubicin was used as reference cytotoxic drug.

4.8. Genotoxicity assay

The genotoxicity of curcumin and **DHC** was evaluated using the alkaline version of the comet assay (single cell gell electrophoresis) by Tice and co-authors protocol [56], with minor modifications [64]. For

the comet assay, 2.5×10^4 A549 cells/well were seeded and treated with **DHC** and curcumin in concentrations at ½ IC_{50} and ¼ IC_{50} values for 24 h. After resuspending the cells, the following steps of the assay were carried out: (i) the microscope slides were coated with normal-melting-point agarose; (ii) the treated cell suspension was transferred to a microcentrifuge tube; (iii) 20 µL cells were suspended in 100 µL of a 0.5% low-melting agarose solution at 37 °C; (iv) 100 µL of this suspension were placed on the slides, which had been kept in freezer for 5 min. In summary, 100 µL cells were taken, homogenized with low-melting point agarose, spread on microscope slides, which were pre-coated with normal-melting-point agarose and covered with a coverslip. After 5 min at 4 °C, the coverslip was removed from the slides and they were immersed in cold lysing solution (2.4 M NaCl; 100 mM EDTA; 10 mM Tris, 10% DMSO and 1% Triton-X, pH 10) for 24 h. After the lysis, the slides were placed in electrophoresis chamber, covered with electrophoresis buffer (300 mM NaOH plus 1 mM EDTA, pH > 13) and left for 20 min for DNA to unwind. The electrophoresis ran for 20 min (25 V and 300 mA), after which slides were submerged for 15 min in neutralization buffer (0.4 M Tris–HCl, pH 7.5) and fixed in ethanol. Duplicate slides were stained with SYBR Green and 50 cells per sample were screened in fluorescent microscope equipped with excitation filter of 515–560 nm, barrier filter of 590 nm and objective (magnification × 40). The level of DNA damage was assessed by image analysis system (TriTek CometScore™ 1.5, 2006) and the percentage of DNA in the tail and tail moment were calculated. The results were expressed as the mean ± standard error of three independent experiments performed in duplicate.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103031.

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