

Communication

# The Antibacterial Potential of Zn(II)–Cannabinoid Acid Equilibrium Systems Against Gram-Positive and Gram-Negative Microorganisms

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## Abstract

Interest is growing in the antimicrobial potential of the non-psychoactive cannabinoids, such as cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA), and their metal systems. The present study follows on from previous potentiometric and ESI-MS studies confirming the formation of stable zinc(II) species with cannabinoid acids in an alcohol–water environment under physiological conditions. The antibacterial action of the compounds was assessed against reference Gram-negative strains (*Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*) and Gram-positive strains (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*) using minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays. Their cytotoxicity was also evaluated in vitro using mouse fibroblasts. The tested compounds were found to demonstrate pronounced selectivity against Gram-positive bacterial strains compared to Gram-negative bacteria. The antibacterial efficacy of the free ligands was enhanced by the presence of Zn(II) in the solution: the equilibrium mixtures exhibited greater inhibitory activity against *E. faecalis* and *S. epidermidis*, with MIC and MBC values being non-cytotoxic toward L929 fibroblasts under the tested in vitro conditions. However, Gram-negative bacteria were only found to be susceptible at elevated concentrations, which also induced cytotoxic effects. Among the free ligands, CBGA exhibited slightly stronger antibacterial activity than CBDA.

**Keywords:** antibacterial activity; cannabidiolic acid; cannabigerolic acid; cytotoxicity; zinc(II) systems

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## 1. Introduction

The pharmacological properties of *Cannabis sativa* have attracted growing interest due to its potential therapeutic applications. It has been reported that cannabis-derived compounds can relieve chronic pain and muscle spasms, reduce nausea during chemo-

therapy, improve sleep and ease the spasticity associated with multiple sclerosis and fibromyalgia [1,2].

The psychoactive compound of *C. sativa*, tetrahydrocannabinol (THC), and its non-psychoactive component, cannabidiol (CBD), are the two most well-known and frequently used cannabinoids in medicine. However, as CBD is significantly inactivated by binding to serum [3], the lesser-known cannabinoids and their acidic precursors, whose pharmacological potential remains unclear, have become the subjects of increasing study [4].

Cannabidiolic acid (CBDA, Figure 1) is a significant naturally occurring cannabinoid, which acts as the biosynthetic precursor of CBD by decarboxylation [1]. Although its health-related properties are still under investigation, it has demonstrated significant antioxidant activity comparable to that of vitamin E [4,5]. While many cannabinoids are lipophilic, limiting their bioavailability and therapeutic efficacy [6], CBDA is more soluble in water and other polar solvents [7,8].

Research suggests that CBDA may offer a number of therapeutic benefits. It may contribute to the alleviation of memory impairments, improve brain function and prevent Alzheimer's disease, and bestow anticonvulsant effects [6]. It has also been found to exert anti-inflammatory and antilipogenic effects in a *Propionibacterium acnes*-induced inflammation and lipogenesis model [9]. In vitro evidence suggests that it may have positive anti-cancer properties and enhance the effectiveness of chemotherapeutic agents. Furthermore, unlike CBD, CBDA is effective in reducing motion-induced vomiting [10,11].

Another naturally occurring cannabinoid is cannabigerolic acid (CBGA, Figure 1), the acidic precursor of cannabigerol (CBG); however, despite its unique structure and biological significance, it has been the subject of limited research to date [12,13]. CBGA occurs at a key intersection of the metabolic pathways, and is the primary precursor to many other cannabinoids found in *Cannabis*, including CBDA and CBD [14]. The structure of CBGA differs from that of CBG due to the presence of a carboxyl group; this additional group affects the polarity of the molecule and its interactions with biosynthetic enzymes in the plant, particularly cannabinoid synthases, which direct its conversion into downstream bioactive cannabinoids [15].

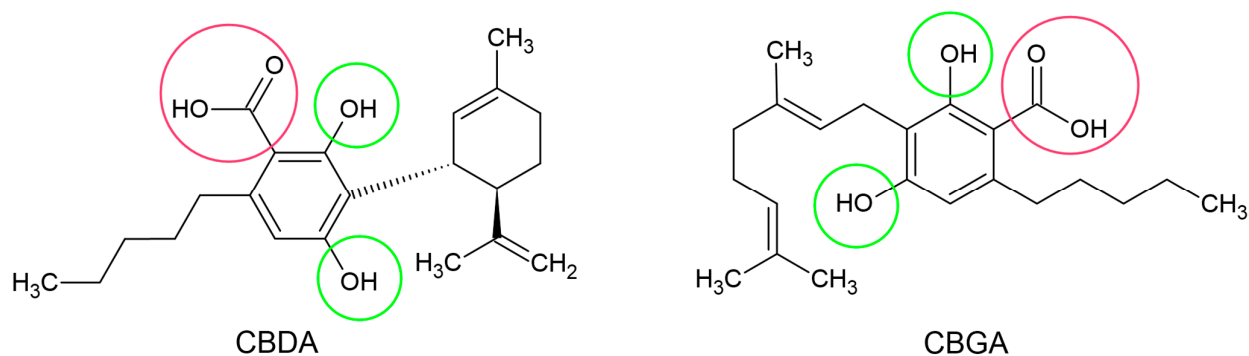
CBG has attracted more pharmacological interest than CBGA due to its more precisely characterized spectrum of biological activity [12,16]. However, both CBG and CBGA exhibit neuroprotective properties in Parkinson's disease [17]. CBGA has demonstrated anticonvulsant potential in murine seizure models, confirming the therapeutic action of cannabis against epilepsy; indeed, CBGA has been found to exhibit multimodal activity, affecting several targets relevant to epilepsy [18]. As a dual PPAR $\alpha$ / $\gamma$  agonist, CBGA has the potential to be used in the treatment of metabolic disorders such as diabetes and dyslipidemia. Suzuki et al. found CBGA to strongly inhibit transient receptor potential melastatin 7 (TRPM7) activity, indicating therapeutic potential in diseases where TRPM7 plays an important role, such as cancer, stroke and kidney disease [17]. In a mouse model of acute nephropathy, CBGA prevented kidney damage and suppressed mRNA expression of inflammatory cytokines. It has also demonstrated antiviral properties by inhibiting SARS-CoV2 infection [19]. Taken together, these data confirm that CBGA has a variety of biological activities beyond its role as a metabolic precursor.

In addition to their diverse biological activities, neutral cannabinoids such as CBD and CBG and their acid forms have demonstrated antibacterial activity, particularly against Gram-positive bacteria [1,19–22]. This has also been noted against antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) [23]. Studies have shown that the presence of phenolic hydroxyl groups is important for combatting bacteria: their methylation eliminates activity against Gram-positive and Gram-negative bacteria [3]. The addition of cannabinoids enhanced the effectiveness of bacitracin against Gram-positive bacteria, suggesting that this combination could be used as an innovative treat-

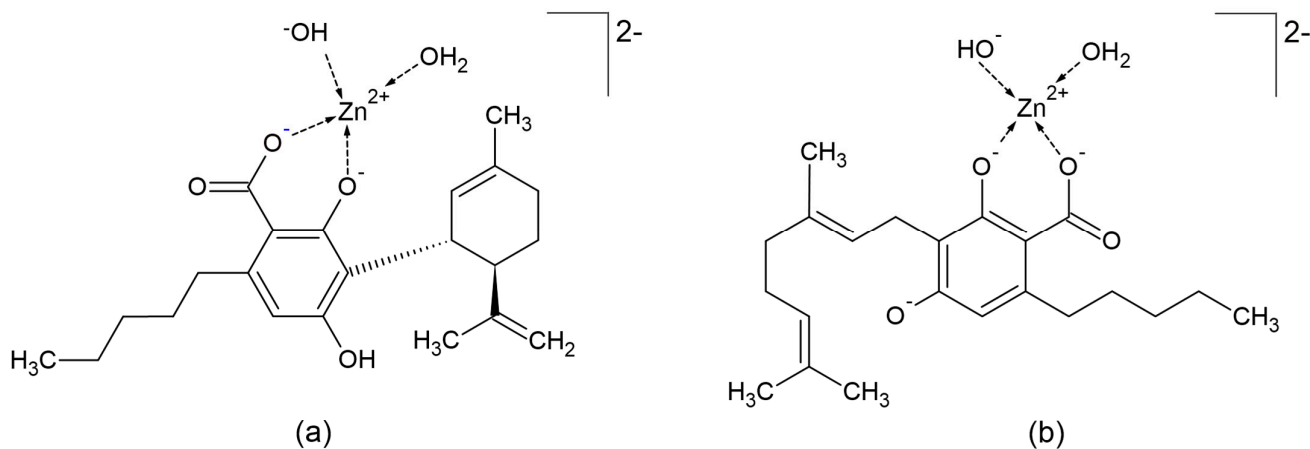
ment for antibiotic-resistant infections in clinical settings; however, the method was found to be ineffective against Gram-negative bacteria [24].

One well-established strategy for enhancing the biological activity of ligands is complexation. Coordination with biologically relevant metal ions, such as zinc(II), can increase antimicrobial potency by improving lipophilicity and membrane permeability [25]. Furthermore, zinc ions themselves exhibit antibacterial properties and play a key role in many physiological processes, making them attractive candidates for pharmaceutical applications [26–28]. Zinc is important for strengthening the antioxidant defenses of the body; it stimulates the removal of free radicals, and it prevents damage to cell membranes. It also acts as a cofactor for various enzymes. In addition, it is a structural component of superoxide dismutase and plays a key role in the expression of metallothionein and in the regulation of glutathione peroxidase, as well as important parts in carbohydrate metabolism and insulin action [29].

Our previous chemical investigations into the protolytic properties of cannabinoid acids, their coordination interactions with Zn(II) ions, the proposed coordination models, stability constant values, and pH-dependent speciation profiles have been expanded in the current work to include the biological aspect, providing new data on the antibacterial potential of the studied systems. Earlier potentiometric and mass spectrometric studies have confirmed the formation of stable zinc(II) species with CBDA and CBGA in alcohol–water equilibrium mixtures under physiological conditions [11], Figure 2. Biological tests of the ligands and their systems containing Zn(II) have found them to demonstrate inhibitory and bactericidal properties against *H. pylori*, while also combating the growth of human gastric cancer cells. Despite these promising results, there is a lack of extensive research evaluating the antibacterial activity of CBDA and CBGA, both in their free form and as metal systems. The aim of this study was to investigate the antibacterial activity of CBDA, CBGA and their zinc(II) systems against selected reference strains of Gram-positive and Gram-negative bacteria. It also evaluated the cytotoxic effects on L929 fibroblasts of these compounds to determine biologically safe concentration ranges. This integrated approach will improve the general understanding of the potential of cannabinoid acids and their zinc(II) systems as selective antimicrobial agents.



**Figure 1.** Structures of cannabinoid acids; CBDA as the [LH<sub>3</sub>] form and CBGA as the [L'H<sub>3</sub>] form.



**Figure 2.** Suggested coordination modes of the complexes: (a)  $[\text{ZnLH-1}]^{2-}$  in the  $\text{Zn(II)}\text{-CBDA}$  system and (b)  $[\text{ZnL'H-1}]^{2-}$  in the  $\text{Zn(II)}\text{-CBGA}$  system.

## 2. Materials and Methods

### 2.1. Materials

Cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA) (Figure 1) were purchased commercially from Covalent CC, LLC (Las Vegas, NV, USA) in high-purity grade (99.95%) and further purified by column chromatography before being used in our study. Zinc(II) nitrate hexahydrate, carbonate-free 0.1 M NaOH solution, Tris-HCl, sodium chloride and ethanol were purchased from Fluka (Buchs, Switzerland), J.T. Baker (Deventer, The Netherlands), Sigma-Aldrich (St. Louis, Missouri, USA), Chempur (Piekary Śląskie, Poland) and POCH (Gliwice, Poland), respectively. High-purity argon was supplied by Linde (Dublin, Ireland).

### 2.2. Preparation of Equilibrium Systems

The free ligand solutions and the corresponding  $\text{Zn(II)}$  equilibrium systems were prepared in a 50/50 (*v/v*) ethanol/water mixture, in accordance with the procedure described previously [11]. Potentiometric measurements were carried out using a Titrand 905 automatic titration system (Metrohm, Herisau, Switzerland) equipped with a combined glass electrode (Metrohm LL Biotrode). High-purity argon was continuously passed over the solution surface to prevent carbon dioxide absorption. The concentrations of CBDA and CBGA solutions were  $2.0 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ . The  $\text{Zn(II)}\text{-CBDA}$  and  $\text{Zn(II)}\text{-CBGA}$  equilibrium systems were prepared at a metal concentration of  $2.5 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1}$  and a ligand-to-metal molar ratio of 2:1. All solutions were prepared in Tris-HCl/NaCl buffer and potentiometrically titrated to pH 7.2 with carbonate-free 0.1 M NaOH solution.

### 2.3. Electrospray Ionization Mass Spectrometry (ESI-MS) Measurements

Mass spectrometric data were obtained using a Varian 500-MS LC hexapole ion-trap mass spectrometer (Varian, Inc., Palo Alto, CA, USA) with an accuracy of 0.1 for *m/z* values. ESI-MS experiments were carried out in both positive- and negative-ion modes for the CBDA and CBGA ligands using a 50/50 (*v/v*) methanol/water mixture. No background electrolyte was added. The concentration of CBDA as well as CBGA was  $2.0 \times 10^{-3} \text{ M}$ . The samples were introduced into the ESI-MS source by continuous infusion using an instrument syringe pump at a rate of  $10 \mu\text{L}\cdot\text{min}^{-1}$ . The ESI source was operated at 5.00 kV, and the capillary heater was set to  $350^\circ\text{C}$ . The cone voltage was within the range 40,120 V.

## 2.4. Biological Assays

In all bioassays, the two ligands, i.e., CBDA and CBGA, and their zinc(II) systems were administered as ethanol/water solutions with a ligand-to-metal molar ratio of 2:1. The pH was maintained at 7.2 with Tris-HCl/NaCl buffer. The concentrations of the Zn(II)-ligand species in both systems were calculated using HySS2009 software [30].

### 2.4.1. Cytotoxicity Assay

The cytotoxicity of the ligands and their metal systems was determined based on exposure to L929 mouse fibroblasts. Briefly, the cells were cultured at 37 °C in 5% CO<sub>2</sub> in RPMI-1640 with 10% FBS, 100 U/mL gentamicin and 100 µg/mL amoxicillin (all from Biowest, Nuaille, France). The cell density was  $2 \times 10^5$  cells/mL, following ISO 10993–5 guidelines [31], using the MTT reduction test as previously described [32].

The test samples were diluted in complete culture medium to the following concentrations:  $2.0 \times 10^{-3}$  M,  $1.0 \times 10^{-3}$  M,  $0.5 \times 10^{-3}$  M,  $0.25 \times 10^{-3}$  M,  $0.1 \times 10^{-3}$  M,  $0.05 \times 10^{-3}$  M,  $0.025 \times 10^{-3}$  M, and  $0.01 \times 10^{-3}$  M. As the solutions were diluted, the ethanol concentration in the mixture decreased until it reached non-cytotoxic levels [33]. One set of cell cultures was prepared in untreated medium to serve as the positive control (PC), while another set was exposed to 0.03% H<sub>2</sub>O<sub>2</sub>, resulting in 100% cell death, to act as the negative control (NC).

Absorbance was measured spectrophotometrically at 570 nm using a Multiskan EX plate reader (Thermo Scientific, Waltham, MA, USA). In each case, the MTT reduction assay was performed in three independent experiments. MTT reduction relative to untreated cells (%) = (absorbance of treated cells/absorbance of untreated cells  $\times$  100%)  $\times$  100%.

### 2.4.2. Assessment of Antimicrobial Activity

The antimicrobial efficiency of CBDA, CBGA, Zn-CBDA and Zn-CBGA systems was determined using a microdilution assay against the following reference strains: Gram-negative bacteria *Pseudomonas aeruginosa* ATCC (American Tissue Type Collection) 27853, *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 29906 and Gram-positive bacteria *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228. All tests were performed with the ligands at the following concentrations:  $0.1 \times 10^{-3}$  M,  $0.05 \times 10^{-3}$  M,  $0.025 \times 10^{-3}$  M and  $0.01 \times 10^{-3}$  M. According to previously described methods, the broth microdilution assay determined the antibacterial activity of tested formulations according to European Committee on Antimicrobial Susceptibility (EUCAST) recommendations [34]. The antimicrobial activity of the formulations was evaluated based on their minimal inhibitory concentration (MIC) or minimal bactericidal concentration (MBC). All tests were performed as three independent experiments.

### 2.4.3. Selectivity and Comparative Activity Analysis

To further assess the relationship between antibacterial activity and cytotoxicity against L929 mouse fibroblasts, selectivity indices (SIs) were calculated using both minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values according to the following equations:  $SI_{MIC} = IC_{50}/MIC$  and  $SI_{MBC} = IC_{50}/MBC$ , where  $IC_{50}$  represents the concentration causing a 50% reduction in L929 fibroblast viability, as determined by the MTT assay. SI values greater than 1 indicate that antibacterial activity occurs at concentrations below the  $IC_{50}$ , while higher SI values show greater in vitro selectivity towards bacterial cells compared to mammalian cells.

To compare the antibacterial activity of the free ligands and the corresponding Zn(II) systems, the fold reduction in MIC values was calculated as: fold reduction =  $MIC_{ligand}/MIC_{Zn-system}$ , where  $MIC_{ligand}$  refers to the MIC of CBDA or CBGA and  $MIC_{Zn-system}$  refers to the MIC of the respective Zn(II)–CBDA or Zn(II)–CBGA system against the same bacterial strain.

Additionally, antibacterial concentrations were directly linked to fibroblast viability by comparing MIC and MBC values with the corresponding concentration–response curves obtained from the MTT assay. Cell viability at antibacterial concentrations was estimated from the experimental cytotoxicity data and used to assess the in vitro selectivity profile of the investigated compound.

#### 2.4.4. Statistical Analysis

Normality of continuous variables was evaluated using the Shapiro–Wilk test. Since none of the datasets met the assumption of normal distribution, non-parametric statistical methods were applied throughout. Continuous data are presented as median values with corresponding ranges. Between-group comparisons were conducted using the Mann–Whitney U test. Statistical significance was set at  $p < 0.05$ .

Cytotoxicity measurements were treated as continuous quantitative variables and analyzed using the aforementioned statistical framework. MIC and MBC values were determined following standard microbiological protocols as endpoint readouts from independent experimental repeats. These data were not subject to inferential statistical analysis and are reported descriptively. MIC and MBC values were considered microbiological endpoint measurements and were not analyzed using the same statistical procedures as the continuous cytotoxicity data obtained from the MTT assay. Selectivity indices and fold-reduction values were calculated directly from the experimentally determined MIC, MBC, and IC50 values.

All statistical analyses were conducted using Statistica 13 PL software (StatSoft, Kraków, Poland). Graphical visualization was performed using GraphPad Prism 10.0 (GraphPad Software Inc., San Diego, CA, USA).

### 3. Results and Discussion

The present study evaluated the antibacterial properties of cannabidiolic acid (CBDA), cannabigerolic acid (CBGA) and their zinc(II) systems against reference strains of Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*, and *Proteus mirabilis*) and Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Enterococcus faecalis*). Analysis of the positive-ion ESI–MS spectra of CBDA, recorded under physiological pH conditions, revealed the presence of the deprotonated ligand species  $[LH]^{2-}$  detected as the following adducts:  $[LH + 3H]^+$  ( $m/z = 359.4$ ),  $[LH + 2H + Na]^+$  ( $m/z = 381.4$ ),  $[LH + H + 2Na]^+$  ( $m/z = 403.4$ ) and  $[2LH + 2H + 3Na]^+$  ( $m/z = 783.8$ ) (Supplemental Figure S1). In the negative-ion mode (Supplemental Figure S2), the  $[LH]^{2-}$  species was identified as the adducts  $[LH + H]^-$  ( $m/z = 357.4$ ) and  $[2LH + 2H + Na]^-$  ( $m/z = 737.8$ ). Similarly, the ESI–MS spectra of CBGA recorded in both positive- and negative-ion modes (Supplemental Figures S3 and S4) confirmed the existence of the  $[L'H]^{2-}$  in the form of the following adducts:  $[L'H + 2H + Na]^+$  ( $m/z = 383.4$ ),  $[L'H + H + 2Na]^+$  ( $m/z = 405.4$ ),  $[L'H + 3Na + HNO_3]^+$  ( $m/z = 490.4$ ),  $[L'H + 4Na + HNO_3 + NO_3]^+$  ( $m/z = 575.4$ ),  $[2L'H + 2H + 3Na]^+$  ( $m/z = 787.8$ ),  $[L'H + H]^-$  ( $m/z = 359.4$ ),  $[L'H + Na + HNO_3]^-$  ( $m/z = 444.4$ ),  $[2L'H + 2H + Na]^-$  ( $m/z = 741.8$ ). The ESI–MS results obtained for both ligands are consistent with the previously reported potentiometric and spectrophotometric findings [11]. Furthermore, the mass spectra of the Zn(II)-CBDA and Zn(II)-CBGA systems, reported previously in [11], likewise support the presence of Zn(II)-bound species in both equilibrium systems.

The antibacterial activity of the ligands and their zinc(II) systems is represented as minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), with the data being compared with broad-spectrum reference antibiotics in Table 1. To account for differences in molecular weight, the MIC and MBC values were expressed in molar units, allowing a direct comparison of the antibacterial activities of the free ligands and their corresponding Zn(II) systems. Their cytotoxicity was assessed against L929

mouse fibroblasts, as recommended by ISO safety standards (Figure 3). The effect of all compounds on fibroblast viability was assessed by the MTT reduction test [11]. For all tested compounds administered at concentrations below  $0.05 \times 10^{-3}$  M, L929 cell viability remained above 70%, ensuring compliance with ISO standards. At this concentration, the approximate ratio of the ethanol–water mixture was 1:99 (*v/v*), making the solutions non-toxic in vitro. The calculated  $IC_{50}$  values for the tested systems in the L929 cell line were  $0.109 \times 10^{-3}$  M for CBDA,  $0.108 \times 10^{-3}$  M for CBGA, and  $0.106 \times 10^{-3}$  M for both Zn–CBDA and Zn–CBGA systems. Based on our previous potentiometric studies described in [11], after dilution of the solutions, the investigated systems should be treated as equilibrium mixtures, in which the  $[ZnLH^{-1}]^{2-}$  and  $[ZnL'H^{-1}]^{2-}$  complexes, for the CBDA- and CBGA-based solutions, respectively, coexist with non-complexed Zn(II) ions. Therefore, the observed biological effects should not be attributed exclusively to a single complex species.

**Table 1.** Antimicrobial activity of CBDA, CBGA, Zn–CBDA and Zn–CBGA systems, shown as minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC).

| Microorganism                                   | MIC/MBC (×10 <sup>-3</sup> M) |      |      |      |         |      |         |      |                                 |        |
|-------------------------------------------------|-------------------------------|------|------|------|---------|------|---------|------|---------------------------------|--------|
|                                                 | CBDA                          |      | CBGA |      | Zn-CBDA |      | Zn-CBGA |      | G                               | A      |
|                                                 | MIC                           | MBC  | MIC  | MBC  | MIC     | MBC  | MIC     | MBC  | MIC = MBC (×10 <sup>-3</sup> M) |        |
| Gram-negative bacteria                          |                               |      |      |      |         |      |         |      |                                 |        |
| <i>Pseudomonas aeruginosa</i><br>ATCC 27853     | >0.1                          | >0.1 | >0.1 | >0.1 | 0.1     | 0.1  | 0.1     | 0.1  | <0.008                          | -      |
| <i>Escherichia coli</i><br>ATCC 25922           | >0.1                          | >0.1 | >0.1 | >0.1 | 0.1     | 0.1  | 0.1     | 0.1  | <0.004                          | -      |
| <i>Proteus mirabilis</i><br>ATCC 29906          | >0.1                          | >0.1 | >0.1 | >0.1 | 0.1     | 0.1  | 0.1     | 0.1  | -                               | <0.001 |
| Gram-positive bacteria                          |                               |      |      |      |         |      |         |      |                                 |        |
| <i>Enterococcus faecalis</i><br>ATCC 29212      | 0.1                           | 0.1  | 0.05 | 0.05 | 0.01    | 0.05 | 0.01    | 0.05 | <0.26                           | -      |
| <i>Staphylococcus aureus</i><br>ATCC 29213      | >0.1                          | >0.1 | >0.1 | >0.1 | 0.1     | 0.1  | 0.01    | 0.1  | <0.002                          | -      |
| <i>Staphylococcus epidermidis</i><br>ATCC 12228 | 0.05                          | 0.05 | 0.05 | 0.05 | 0.01    | 0.05 | 0.01    | 0.05 | <0.002                          | -      |

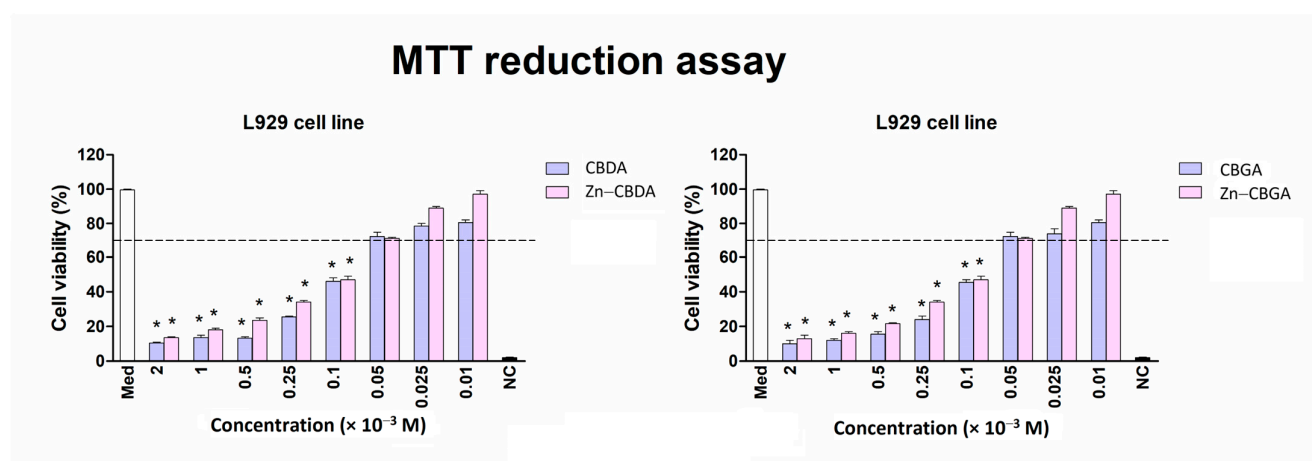
G—Gentamicin; A—Amoxicillin; (-) not tested.

Against Gram-negative bacteria, CBDA and CBGA demonstrated higher MIC and MBC values ( $>0.1 \times 10^{-3}$  M; comparable to values reported in the literature amounting to  $>0.18 \times 10^{-3}$  M [1]) than their Zn–CBDA and Zn–CBGA systems ( $0.1 \times 10^{-3}$  M; Table 1). The higher antibacterial activity of the Zn(II) systems compared with free ligands may be related to their ability to form chelates, which could alter their physicochemical properties, including lipophilicity, and thereby affect their permeability through the bacterial cell membrane. However, because the investigated solutions represent equilibrium mixtures, the relative contribution of individual species cannot be determined from the present data [35,36]. Nevertheless, at these concentrations, all tested compounds showed cytotoxic effects toward L929 cells (cell viabilities below 50%, Figure 3).

The tested agents exhibited high MIC/MBC values for the Gram-negative bacteria, which may be attributed to the bacterial membrane structure. The outer leaflet of the outer membrane is mainly populated with lipopolysaccharides and proteins and only small

amounts of phospholipids; this arrangement would only permit limited diffusion of hydrophobic molecules. Also, the outer membrane is resistant to neutral and anionic compounds [1]. Potentiometric studies of CBDA and CBGA in ethanol-water systems only identified anionic forms of the ligands at physiological pH (as  $[LH]^{2-}$  and  $[L'H]^{2-}$  ions; Figure 1) [11]. Similarly, the speciation diagrams for both zinc(II) systems indicated anionic hydroxide complexes with dissociated molecules of ligand and water ( $[ZnLH-1]^{2-}$  and  $[ZnL'H-1]^{2-}$ ).

The tested compounds showed no activity against Gram-negative bacteria despite activity against *H. pylori* [11], likely due to species-specific differences in outer membrane structure, permeability, and stress mechanisms. The lipopolysaccharide of *H. pylori* is atypical—under-acylated, less toxic—leading to different membrane stability and permeability than typical Gram-negatives [37,38]. It has a weaker outer membrane barrier and distinct efflux systems, possibly allowing more small-molecule accumulation [39]. *H. pylori* is highly adapted to the gastric environment with mechanisms like urease-mediated acid resistance and oxidative stress management, which may increase susceptibility to compounds targeting membrane integrity or redox balance [37]. Conversely, classical Gram-negatives have stronger permeability barriers and efficient efflux systems that limit drug buildup [40]. The selectivity for *H. pylori* reflects its reduced membrane robustness and vulnerabilities, not broad Gram-negative activity. Phylogenetically related bacteria like *Campylobacter jejuni* share some traits but differ in efflux and envelope composition [41,42]. Future studies including *Campylobacter* spp. could clarify if activity is *H. pylori*-specific or extends to related bacteria.



**Figure 3.** The effect of CBDA, CBGA, Zn-CBDA and Zn-CBGA systems on mouse fibroblast viability, measured as the percentage of cells capable of reducing MTT. NC was the negative control (cells treated with 0.03%  $H_2O_2$ ), and PC was the positive control (cells in medium alone). Results are expressed as mean  $\pm$  standard deviation (SD), with  $N = 3$ . The dashed black line indicates 70% viability, i.e., the threshold for cytotoxicity in vitro. Statistical significance was determined using the nonparametric Mann–Whitney U-test, with  $p < 0.05$  considered significant (\* unstimulated versus stimulated cells).

For Gram-positive bacteria, the free ligands demonstrated MIC/MBC values of at least  $0.1 \times 10^{-3}$  M against *E. faecalis* and *S. aureus*. Of the two, CBGA exhibited stronger inhibitory and bactericidal effects towards *E. faecalis*, with MIC and MBC values of around  $0.05 \times 10^{-3}$  M; at this concentration, the ligand was still non-toxic against fibroblasts. This relationship was also confirmed in previous studies conducted by Blaskovich et al., in which the MIC values ranged from  $0.045 \times 10^{-3}$  M to  $0.090 \times 10^{-3}$  M for CBDA and from  $0.006 \times 10^{-3}$  M to  $0.011 \times 10^{-3}$  M for CBGA [3]. Both simple ligands also had strong antibacterial activity against the *S. epidermidis* reference strain.

The Zn(II) systems required high MIC and MBC values against *S. aureus*, which also resulted in cytotoxic effects against fibroblasts. However, the MIC value of the Zn–CBGA system was  $0.01 \times 10^{-3}$  M, which did not elicit cytotoxic effects, with fibroblast viability remaining above 80–90%. Both Zn(II) systems demonstrated effective inhibition (MIC— $0.01 \times 10^{-3}$  M) and bactericidal properties (MBC— $0.05 \times 10^{-3}$  M, with fibroblast viability close to 70%) against *E. faecalis* and *S. epidermidis*, most likely by disrupting the bacterial membrane [3]; the concentrations were non-toxic to fibroblasts. In the case of *E. faecalis*, the MIC/MBC values were up to one order of magnitude lower than those of the reference antibiotic (Table 1).

A comparison of the antibacterial activity of the free ligands and their respective Zn(II) systems revealed a significant reduction in MIC values against Gram-positive bacteria following the addition of Zn(II). For *E. faecalis*, the MIC decreased from  $0.1 \times 10^{-3}$  M to  $0.01 \times 10^{-3}$  M for the CBDA-based preparation and from  $0.05 \times 10^{-3}$  M to  $0.01 \times 10^{-3}$  M for the CBGA-based preparation, representing 10-fold and 5-fold reductions, respectively. Likewise, for *S. epidermidis*, both Zn(II) systems exhibited a 5-fold reduction in MIC compared to the corresponding free ligands. Since the samples studied represent equilibrium mixtures rather than individual molecular species, these observations should be interpreted as properties of the Zn(II)–cannabinoid acid systems.

To further evaluate the relationship between antibacterial activity and cytotoxicity against L929 mouse fibroblasts, the selectivity indices (SIs) were calculated from the  $IC_{50}$  values determined for the tested compounds and the corresponding MIC and MBC values obtained against the investigated bacterial strains (Table 2). The highest  $SI_{MIC}$  value (10.60) was obtained for both Zn(II) systems against Gram-positive bacteria, except for the Zn–CBDA system against *S. aureus*, suggesting a favorable in vitro selectivity profile toward the tested bacterial strains relative to L929 fibroblasts and 10.6-fold more active against bacteria than against mammalian host cells. While the Zn(II)–CBDA and Zn(II)–CBGA systems exhibited high SI values based on MIC against *E. faecalis* and *S. epidermidis*, the corresponding SI values calculated using MBC decreased to approximately 2.12. This suggests that the antibacterial selectivity of the examined systems is most significant at concentrations that inhibit bacterial growth, whereas bactericidal activity occurs closer to the cytotoxicity limit.

**Table 2.** Selectivity Index based on MIC ( $SI_{MIC}$ ) and MBC ( $SI_{MBC}$ ).

| Microorganism         | $SI_{MIC}$ |       |         |         | $SI_{MBC}$ |       |         |         |
|-----------------------|------------|-------|---------|---------|------------|-------|---------|---------|
|                       | CBDA       | CBGA  | Zn–CBDA | Zn–CBGA | CBDA       | CBGA  | Zn–CBDA | Zn–CBGA |
| <i>P. aeruginosa</i>  | ≈1.09      | ≈1.08 | 1.06    | 1.06    | ≈1.09      | ≈1.08 | 1.06    | 1.06    |
| <i>E. coli</i>        | ≈1.09      | ≈1.08 | 1.06    | 1.06    | ≈1.09      | ≈1.08 | 1.06    | 1.06    |
| <i>P. mirabilis</i>   | ≈1.09      | ≈1.08 | 1.06    | 1.06    | ≈1.09      | ≈1.08 | 1.06    | 1.06    |
| <i>E. faecalis</i>    | 1.09       | 2.16  | 10.60   | 10.60   | 1.09       | 2.16  | 2.12    | 2.12    |
| <i>S. aureus</i>      | ≈1.09      | ≈1.08 | 1.06    | 10.60   | ≈1.09      | ≈1.08 | 1.06    | 1.06    |
| <i>S. epidermidis</i> | 2.18       | 2.16  | 10.60   | 10.60   | 2.18       | 2.16  | 2.12    | 2.12    |

The tested compounds were found to be more effective against Gram-positive bacteria than Gram-negative bacteria, which is consistent with earlier reports [21,22]. The selective action of the Zn–CBDA and Zn–CBGA systems against Gram-negative or Gram-positive bacteria suggests they may have potential as narrow-spectrum agents; such compounds could have various uses, including protecting natural microflora. Their lower activity against Gram-negative bacteria may be due to the lipopolysaccharide-protein structure of the outer membrane [1]. According to Mudarmah et al., the antimicrobial effects of zinc(II) complexes are not fully elucidated, but they are likely mediated by multiple mechanisms [43]. These may include membrane destabilization with a consequent increase in permeability, interactions with nucleic acids, and inhibition of respiratory enzymes. Since

$\text{Zn}^{2+}$  ions do not readily penetrate the hydrophobic lipid bilayer, their transport into bacterial cells may depend on a suitable lipophilic ionophore. Furthermore, in addition to serving as zinc ionophores, the ligands may chelate other divalent metal ions, thereby disrupting bacterial metal homeostasis. It should also be noted that both zinc deficiency and zinc overload are detrimental to cellular homeostasis. Another possible mechanism of action of zinc(II) systems involves the induction of reactive oxygen species (ROS), which can damage membranes, lipids, proteins, and DNA, ultimately resulting in bacterial cell death.

Our findings are in line with those of previous studies [3], i.e., that CBGA exhibits slightly stronger antimicrobial potential against Gram-positive bacteria than CBDA. This may be due to the slight structural differences between the two substances. For example, the ring in CBDA forms an alkyl chain in CBGA [21], which may allow the molecules to interact differently with bacterial membrane receptors. Aqawi et al. [12,44] report that CBG induces hyperpolarization in the cell membrane, decreasing its fluidity while increasing its permeability. The presence of an aromatic carboxyl group in the CBGA molecule slightly increases its antibacterial activity against Gram-positive bacteria compared to CBG. In turn, the decarboxylation of CBDA enhances antibacterial properties of CBD [3].

#### 4. Limitations of the Study

Several limitations of this study should be recognized. First, under the biological assay conditions, the investigated Zn(II)–CBDA and Zn(II)–CBGA solutions should be regarded as equilibrium systems rather than single, structurally defined Zn(II) complexes. Based on our previous potentiometric studies, these systems consist of equilibrium mixtures containing zinc(II) hydroxide complexes with cannabinoid acid ligands, as well as non-complexed Zn(II) ions; therefore, the observed biological effects cannot be attributed to any individual species. Second, no Zn(II)-only control was included in the present study, preventing a direct assessment of the relative contributions of free Zn(II), free ligands, and Zn(II)–ligand species to the observed antibacterial activity. Third, cytotoxicity was evaluated only in the L929 fibroblast cell line, and therefore the obtained selectivity indices should be interpreted as preliminary indicators of in vitro selectivity. Finally, the antibacterial activity was assessed exclusively against reference bacterial strains. Consequently, the present results do not allow conclusions regarding efficacy against resistant clinical isolates or the ability of the investigated systems to overcome bacterial resistance mechanisms. Further studies involving structural characterization under biologically relevant conditions, additional mammalian cell models, Zn(II)-only controls, and resistant clinical isolates are required to better define the therapeutic potential and mechanism of action of these Zn(II)–cannabinoid acid systems.

#### 5. Conclusions

Cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA) possess antibacterial activity, particularly against Gram-positive bacteria. These properties were found to be enhanced in systems containing zinc(II), as evidenced by stronger effects against *Enterococcus faecalis* and *Staphylococcus epidermidis*. The observed increase in antibacterial activity may be associated with the chelation of Zn(II) ions by cannabinoid acids, which could modify the physicochemical properties of the systems, including lipophilicity and bacterial membrane permeability. However, due to the equilibrium nature of the studied systems, the relative contribution of individual species present in solution to the observed biological effect cannot be determined. The Zn(II)–CBDA/CBGA systems, and the free ligands to a certain extent, exhibited inhibitory and bactericidal properties at concentrations that were non-cytotoxic to

L929 fibroblasts. Notably, the concentrations effective against *E. faecalis* were up to one order of magnitude lower than those required for the reference antibiotic.

However, all tested substances only demonstrated activity against Gram-negative bacteria at higher concentrations, which were cytotoxic against fibroblasts. The reduced susceptibility of Gram-negative bacteria is likely due to their protective outer membrane and lipopolysaccharide layer, which restrict penetration by external agents. Conversely, antibacterial activity against selected Gram-positive strains was achieved at concentrations that did not reduce L929 cell viability below the threshold set by ISO standards. In addition, both free CBGA and Zn-CBGA systems demonstrated slightly stronger antibacterial properties than CBDA, which may be attributed to the subtle structural differences between them.

Overall, our findings suggest that zinc(II) equilibrium systems of CBDA and CBGA could be promising candidates for further research as selective antimicrobial agents that target Gram-positive bacteria. However, these findings should be viewed with some limitations in mind, including the equilibrium nature of the studied Zn(II) systems, the lack of controls with Zn(II) alone, the use of only one mammalian cell line for cytotoxicity testing, and the testing of antibacterial activity solely against reference strains. Consequently, additional studies are needed to identify the active species under biological conditions, clarify the mechanisms of action, assess activity against clinically relevant resistant isolates, and evaluate safety in more advanced biological models.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app16147031/s1>, Figure S1: ESI-MS spectra for cannabidiolic acid (CBDA) in positive ion-mode, CCBDA =  $2.0 \times 10^{-3}$  M. Figure S2: ESI-MS spectra for cannabidiolic acid (CBDA) in negative ion-mode, CCBDA =  $2.0 \times 10^{-3}$  M. Figure S3: ESI-MS spectra for cannabigerolic acid (CBGA) in positive ion-mode, CCBGA =  $2.0 \times 10^{-3}$  M. Figure S4: ESI-MS spectra for cannabigerolic acid (CBGA) in negative ion-mode, CCBGA =  $2.0 \times 10^{-3}$  M.

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**Conflicts of Interest:** Author Susana Santos is employed by EXMceuticals Portugal Lda. The authors declare that this affiliation did not influence the conduct, analysis, or interpretation of this study, and that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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