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Efficacy of a novel ferrocenyl diaryl butene citrate compound as a biocide for preventing healthcare-associated infections

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Abstract

The antiseptic and disinfectant potential of a formulation containing the tamoxifen analogue 1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-ferrocenyl-but-1-ene citrate was assessed according to European standards and pharmacopeia in comparison with a commercial antiseptic product containing hexamidine diisethionate, chlorhexidine digluconate and chlorocresol as active ingredients. The formulation met the phase 1 requirement of reducing by 5 cycles the counts of microorganisms frequently involved in healthcare-associated infections, namely *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 6538, *Enterococcus hirae* ATCC 10541 and *Candida albicans* ATCC 10231. It also killed a clinical isolate of *Acinetobacter baumannii* highly resistant to antibiotics and antiseptics. In Phase 2/step 2 tests, it reduced counts of *E. coli* ATCC 10536 by 4 log cycles within 60 seconds on hands (standard EN 1499). The novel formula is a potent biocide, and this demonstration could lead to the development of a new commercial antiseptic.

Keywords: organometallic; tamoxifen; 1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-ferrocenyl-but-1-ene citrate; antiseptic; disinfectant; European standards

1. Introduction

Healthcare-associated infections (HAIs) continue to be a major public health concern worldwide due to the associated morbidity, mortality, non-negligent costs and emergence of multi-resistant bacteria. According to a recent survey in the USA, approximately one of every 25 acute-care inpatients develops at least one HAI, which led to 75,000 fatalities in 2011.¹ According to the European Centre for Disease Prevention and Control (ECDC), 7.1% of European inpatients were diagnosed with HAI.² In 2013, the most common types of HAIs in Europe were respiratory tract infections (33.6%), symptomatic urinary tract infections (22.3%), and skin infections (21.4%).³ The most common bacterial nosocomial infections are due to *Escherichia coli* (23%), *Staphylococcus aureus* (20%), *Pseudomonas aeruginosa* (11%), and *Enterococcus* spp (6%). Antibiotic resistance is acquired usually in association with excessive and inappropriate usage (unnecessary prescriptions, inadequate doses and/or treatment time, etc.). HAIs increase the danger of multi-resistant bacteria emerging from routine antibiotic therapy, which is becoming more and more dependent on continuous screening for new antibacterial agents. The use of detergents and disinfectants in hospitals is being intensified due to the discovery of new infectious agents (HIV, hepatitis viruses, prions...) and the necessity of stopping HAIs.

According to the European Committee for Standardization, an antiseptic is defined as a “substance or preparation allowing the treatment of living tissues by killing and/or inhibiting bacteria, fungi or spores and/or inactivating viruses in order to prevent or limit the danger of infection for these tissues” (CEN/TC 216: Chemical Disinfectant and Antiseptic standards). Products suitable for application to healthy skin are henceforth labeled as disinfectants. Antiseptics belong to many chemical families, including quaternary ammonia, chlorhexidine, phenolics, salicylanilides and carbanilides, alcohols, aldehydes (formaldehyde, glutaraldehyde)

and glyoxal, oxidizers (halogens and derivatives, peroxide), organic acids, organometallics and colorants.⁴ Some disinfectants also belong to these families (chlorinated derivatives, quaternary ammonia, etc.), but are usually aldehydes, in particular formaldehyde.⁵ In Europe, the normative evaluation of antiseptics is based on bactericidal standards EN 1040, EN 13727, and possibly fungicidal standards such as EN 1275, except in the case of hand-washing products, for which only bactericidal and antiviral standards are considered (EN 1499 and EN 1500, EN 14476).⁶ An antiseptic product candidate must first have demonstrated efficacy against bacteria and fungi in phase 1 tests. Its antimicrobial efficacy is then examined under representative conditions in the presence of interfering substances (phase 2/step 1). Finally, phase 2/step 2 tests include assays mimicking conditions of actual use.

Organometallic compounds, which are defined as metal–organic complexes containing at least one direct covalent metal–carbon bond, offer interesting opportunities for the design of novel classes of biocides.⁷ These metal–carbon bonds can exert major electronic and steric effects and thus offer a novel approach to designing medicinal compounds, based on mechanisms differing substantially from those of purely organic molecules.⁷ For example, the ferrocenyl derivative of chloroquine, a ferroquine antimalarial candidate, has been found ten times more active than the parent compound against chloroquine-resistant *Plasmodium falciparum*.⁸ Similarly, we have synthesized previously a series of ferrocenyl analogues of tamoxifen with anti-proliferative and antimicrobial effects.^{9, 10} Broad-spectrum activity combined with low hemolytic activity was observed for 1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-ferrocenyl-but-1-ene citrate (compound **1**).¹¹ In the present work, we report the development of a new biocide based on this compound and our assessment of its antiseptic efficacy according to European standards. A liquid

formulation containing the new molecule was compared to a commercial biocide in terms of effectiveness against various healthcare-associated microorganisms.

2. Materials and methods

2.1. Chemical compounds and antiseptic formulation

The compound 1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-ferrocenyl-but-1-ene citrate (**1**) was synthesized as described previously.¹⁰ Its structure is represented in Figure 1. The commercial antiseptic solution Cytéal[®], which contains hexamidine diisethionate (**2**), chlorhexidine digluconate (**3**) and chlorocresol (**4**), was used for comparison (Table 1). Our disinfectant formulation contained compound (**1**) plus the same concentrations of cocamidopropyl betaine, edetic acid and coprah diethanolamide as in the commercial product.

2.2. Microbial strains and culture conditions

Escherichia coli ATCC 10536, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 6538, *Enterococcus hirae* ATCC 10541, *Candida albicans* ATCC 10231 and *Acinetobacter baumannii* (clinical isolate) were grown aerobically in LB broth (Lennox, Difco Laboratories, Sparks, MD, USA) at 37°C. The microbial strains were sub-cultured at least three times in this medium before use.

2.3. Antimicrobial activity

Compound (**1**) was dissolved in water, doxycycline was dissolved in DMSO, and inhibitory concentrations thereof were assessed using polystyrene micro-assay plates (96-well Microtest, Becton Dickinson Labware, Sparks, MD, USA) as described by Hammami et al.¹² Briefly, microplates loaded with two-fold serial dilutions of each sample (starting at 254 µM) were seeded with log-phase culture of target strain diluted in LB. The microplates were then incubated at 37°C for 24 h and absorbance at 620 nm was measured using an Infinite[®] F200 PRO

spectrophotometer (Tecan US Inc., Durham, NC, USA). The inhibitory activity of the tested compounds was calculated using the formula:

$$\text{IA (\%)} = 100 \times \left(1 - \frac{ODx - ODi}{ODc - ODi} \right)$$

Where *i* is the value of the negative control, *c* is the value of microbial culture without inhibitor, and *x* is the value of microbial culture containing the test compound.

The antimicrobial activity of compound (1) alone and in formulation was compared at a concentration of 300 µg mL⁻¹ to that of Cytéal® (a commercial analogue) using *Staphylococcus aureus* ATCC 6538 in agar well diffusion tests as described by Hammami et al.¹²

2.4. Antiseptic assay

The antiseptic and disinfectant effects of the compound (1) formulation were measured in accordance with European standards EN 1040, EN 13727 and EN 1499.¹³⁻¹⁵ For compliance with EN 1040 and EN 13727, we prepared the microbial suspensions in contact with the tested disinfectant, then filtered and washed the filters to remove the active substance for incubation (37°C for 24 h) before reading. Each strain was tested at antiseptic strengths of 10%, 80% and 90% with contact times of 5 min, 30 min and 24 h. The efficacy of the compound (1) formulation *in situ* was evaluated using the hand-washing test with *Escherichia coli* ATCC 10536 with and without friction, according to standard EN 1499. After washing with soap, fingertips were immersed in the microbial suspension for 5 seconds, allowed to dry for 5 minutes, then rubbed with 1 mL of antiseptic for 60 seconds and dipped in LB, which was serially diluted immediately. One mL of each dilution was filtered and the filter was washed with sterile distilled water to remove active substances, followed by incubation of the filter (37°C for 24 h) and then reading.

The cocamidopropyl betaine, edetic acid and coprah diethanolamide formulation devoid of compound (1) was also tested in place of the mild soap required by EN 1499.

2.5. Statistical analyses

The distribution of the percentages of bacteria did not significantly deviate from normality. Data were subjected to ANOVA using the GLM procedure of SAS (SAS Institute, 2003) and compared with the 2-tailed Student's t test. P-values < 0.05 were considered significant. Error bars denote the SD, unless otherwise stated.

3. Results and discussion

3.1. Antimicrobial activity of compound (1)

Compound (1) is a synthetic ferrocene analogue of tamoxifen with a broad spectrum of action (Table 1).^{10, 11} It was found much more potent than doxycycline against *Candida albicans* ATCC 10231, inhibiting the fungus completely at 3.97 μM versus 254 μM (Figure 2). The nominal IC_{50} values for these compounds are respectively 5 μM and 200 μM . Doxycycline is a tetracycline derivative with improved pharmacokinetic properties that prevents docking of acylated tRNA to the ribosome, thereby inhibiting translation.¹⁶ Compound (1) has been shown to alter bacterial membrane structure, causing significant efflux of K^+ and Na^+ and ultimately cell death.¹¹ This mode of action, combined with low hemolytic activity, makes this substituted diaryl butane attractive for development as an antiseptic. The efficacy of compound (1) at different concentrations was also evaluated in the absence or presence of bovine serum albumin (3 g L^{-1}) mimicking unclean surfaces (Table 2). All tested microorganisms were inhibited by 5, 30 min or 24 h of contact at strengths of 80% and 90%, regardless of the presence of interfering substances. No significant differences found between microbial counts in absence or presence of interfering substances at strengths of 80 and 90%. Nevertheless, at lower strength of compound (1) (10%)

required time for efficient action was delayed in presence of interfering substances. For example, 30 min of contact time were necessary at strength of 10% for effective killing of *Pseudomonas aeruginosa* in presence of BSA, while five minutes were sufficient in clean conditions. Compound (1) at 80% strength exhibited antiseptic properties meeting the requirements of standard EN 13727 (5 log reduction in microbial count) in the absence of interfering substances and was only slightly less effective in its presence.

3.2. Efficacy of the antiseptic formulation

The compound (1) formulation contained the same surface-active compound (cocamidopropyl betaine), complexing agent (edetic acid) and foam-stabilizing agent (diethanolamide of coprah) as the commercial product Cytéal[®]. Figure 3 shows the potency of the formulation [concentration of (1) = 300 µg mL⁻¹] against *Staphylococcus aureus* ATCC 6538 compared to compound (1) alone and to the commercial product. The formulation appeared as inhibitory as Cytéal[®], and more inhibitory than compound (1) alone. Table 3 shows the reductions of the counts of the five microbes exposed to different strengths of our formulation or Cytéal[®] in the absence or presence of interfering substances. In general, compound (1) formulation at strength of 90% was efficient as Cytéal[®] at 80%. For example, *Escherichia coli* reduction of about 7 log cycles after contact for 30 min with compound (1) formulation and Cytéal[®] were obtained with strengths of 90% and 80%, respectively. Nevertheless, the two products appeared equally effective as disinfectants, regardless of possible interference from the protein, even though our formulation contained much less active ingredient (300 mg L⁻¹). The active ingredients in Cytéal[®] (Table 1) are hexamine diisethionate (2,500 mg L⁻¹), chlorhexidine digluconate (12,882.5 mg L⁻¹) and p-chlorocresol (7,500 mg L⁻¹).

The antiseptic effect of our formulation was then assessed against a clinical isolate of *Acinetobacter baumannii* in the presence of interfering substance (Table 4). Although 24 h of incubation were required to kill this resistant germ, this is nevertheless interesting in view of the known resistance of *A. baumannii* to antibiotics and antiseptics.¹⁷ In phase 2/step 2, our formulation was tested using the hand-washing test with *Escherichia coli* ATCC 10536 as a measure of efficacy *in situ* (standard EN 1499). As shown in Figure 4, 60 seconds of contact were sufficient to reduce significantly *E. coli* counts by 4 log cycles, which meets the standard. The hands of healthcare personnel are common vehicles of HAI transmission, and hygiene is the first line of defense against the risk of propagating infectious microbes.¹⁸ On the basis of these results, our formulation has potential for application as a disinfectant/antiseptic.

4. Conclusions

A novel derivative of tamoxifen (**1**) was found to be a potent inhibitor of pathogens known to cause HAIs, in accordance with European standards EN 1040 and EN 13727. The formulation met the phase 1 criterion for antiseptics of reducing microbial contaminant counts by 5 log cycles. It also met the phase 2/step 1 criterion of efficacy in the presence of interfering substances, using these microorganisms and a clinical isolate of *Acinetobacter baumannii* highly resistant to antibiotics and antiseptics. Phase 2/step 2 tests confirmed the capacity of the formulation to reduce *E. coli* ATCC 10536 counts by 4 log cycles in 60 seconds on human hands. Our formulation containing the novel ferrocenyl diaryl butane citrate compound is therefore a potent biocide, and the present demonstration could lead to the development of a new commercial antiseptic.

Acknowledgments

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Figure legends

Figure 1. Molecular structure of compound (1) (1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-ferrocenyl-but-1-ene citrate)

Figure 2. Inhibition of the growth of *Candida albicans* ATCC 10231 in the presence of increasing concentrations of compound (1) (circle) and doxycycline (square)

Figure 3. Inhibition of the growth of *Staphylococcus aureus* ATCC 6538 by compound (1) alone (a), Cytéal[®] (b), and the compound (1) formulation (c), all at an active ingredient concentration of 300 µg mL⁻¹

Figure 4. Assessment of the antiseptic efficacy of the compound (1) formulation using the hand-washing test (standard EN 1499) with *Escherichia coli* ATCC 10536 (phase 2/step 2)

Table 1. Composition of the active ingredients in our formulation and commercial antiseptic product Cytéal®

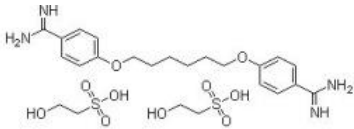
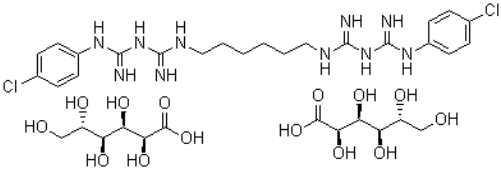
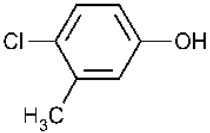
Products	Active ingredients	Concentration (mg L ⁻¹)	Total active ingredient (mg L ⁻¹)
Our formulation	Compound (1)	300.0	300.0
	 <p>(2) Hexamidine diisethionate</p>	2,500.0	
Cytéal®	 <p>(3) Chlorhexidine digluconate</p>	12,882.5	22 882.5
	 <p>(4) p-Chlorocresol</p>	7,500.0	

Table 2: Antiseptic efficacy of compound (1) against HAI-associated microbes in the presence or absence of interfering substances

Strains	Inoculum	[C] Time	With interfering substances									Without interfering substances								
			90%			80%			10%			90%			80%			10%		
			5 min	30 min	24 h	5 min	30 min	24 h	5 min	30 min	24 h	5 min	30 min	24 h	5 min	30 min	24 h	5 min	30 min	24 h
<i>Escherichia coli</i> ATCC 10536	N _i :1.75 10 ⁸	N _a	58	0	0	>3 10 ²	200	150	>3 10 ²	>3 10 ²	>3 10 ²	4	3	0	46	9	0	>3 10 ²	>3 10 ²	0
		R	3 10 ⁶	1.75 10 ⁸	1.75 10 ⁸	-	8.7 10 ⁴	1.16 10 ⁵	-	-	-	4.4 10 ⁶	5.8 10 ⁶	1.75 10 ⁸	3.8 10 ⁵	1.9 10 ⁶	1.75 10 ⁸	-	-	1.75 10 ⁸
<i>Pseudomonas aeruginosa</i> ATCC 15442	N _i :1.25 10 ⁷	N _a	0	0	0	0	0	0	>3 10 ²	0	0	0	0	0	0	0	0	0	0	0
		R	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	-	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷	1.25 10 ⁷
<i>Staphylococcus aureus</i> ATCC 6538	N _i :1.4 10 ⁸	N _a	10	2	0	>3 10 ²	180	80	>3 10 ²	>3 10 ²	>3 10 ²	2	1	0	65	3	0	>3 10 ²	>3 10 ²	>3 10 ²
		R	1.4 10 ⁶	7 10 ⁷	1.4 10 ⁸	-	7 10 ⁴	1.7 10 ⁵	-	-	-	7 10 ⁶	1.4 10 ⁷	1.4 10 ⁸	2 10 ⁵	4.6 10 ⁶	1.4 10 ⁸	-	-	-
<i>Enterococcus hirae</i> ATCC 10541	N _i :5.6 10 ⁷	N _a	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0
		R	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	2.8 10 ⁵	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷	5.6 10 ⁷
<i>Candida albicans</i> ATCC 10231	N _i :7 10 ⁷	N _a	0	0	0	1.8 10 ²	0	0	>3 10 ²	>3 10 ²	>3 10 ²	0	1	1	>3 10 ²	100	0	>3 10 ²	>3 10 ²	1
		R	7 10 ⁶	7 10 ⁶	7 10 ⁶	3.8 10 ⁴	7 10 ⁶	7 10 ⁶	-	-	-	7 10 ⁶	7 10 ⁶	7 10 ⁶	23 10 ¹	7 10 ⁴	7 10 ⁶	-	-	7 10 ⁶

N_i: Initial contamination; N_a: Contamination after treatment; R: Reduction

Table 3: Antiseptic efficacy of the compound (**1**) formulation and Cytéal® in the presence of interfering substances

Strains	Inoculum	[C] Time	Compound (1) formulation									Cytéal®								
			90%			80%			10%			90%			80%			10%		
			5 min	30 min	24 h	5 min	30 min	24 h	5 min	30 min	24 h	5 min	30 min	24 h	5 min	30 min	24 h	5 min	30 min	24 h
<i>Escherichia coli</i> ATCC 10536	N _i :1.75 10 ¹⁰	N _a	>3 10 ²	240	0	>3 10 ²	>3 10 ²	>3 10 ²	>3 10 ²	>3 10 ²	>3 10 ²	>3 10 ²	200	0	>3 10 ²	167	0	>3 10 ²	>3 10 ²	>3 10 ²
		R	-	7.3 10 ⁶	1.75 10 ¹⁰	-	-	-	-	-	-	-	8.7 10 ⁶	1.75 10 ¹⁰	-	1.04 10 ⁷	1.75 10 ¹⁰	-	-	-
<i>Pseudomonas aeruginosa</i> ATCC 15442	N _i :1.25 10 ⁷	N _a	3	0	0	51	2	0	>3 10 ²	79	56	-	-	-	-	-	-	-	-	-
		R	4.1 10 ⁵	1.25 10 ⁷	1.25 10 ⁷	2.5 10 ⁴	6.2 10 ⁵	1.25 10 ⁷	-	1.6 10 ⁴	2.2 10 ⁴	-	-	-	-	-	-	-	-	-
<i>Staphylococcus aureus</i> ATCC 6538	N _i :1.4 10 ¹⁰	N _a	250	240	0	>3 10 ²	>3 10 ²	>3 10 ²	>3 10 ²	>3 10 ²	>3 10 ²	69	0	0	112	140	2	>3 10 ²	>3 10 ²	40
		R	5.6 10 ⁶	5.8 10 ⁶	1.4 10 ¹⁰	-	-	-	-	-	-	2 10 ⁷	1.4 10 ¹⁰	1.4 10 ¹⁰	1.25 10 ⁷	1 10 ⁷	7 10 ⁸	-	-	3.5 10 ⁷
<i>Enterococcus hirae</i> ATCC 10541	N _i :5.6 10 ⁷	N _a	1	2	0	>3 10 ²	20	4	>3 10 ²	16	11	-	-	-	-	-	-	-	-	-
		R	5.6 10 ⁶	2.8 10 ⁶	5.6 10 ⁷	-	2.8 10 ⁵	1.4 10 ⁶	-	3.5 10 ⁵	5.1 10 ⁵	-	-	-	-	-	-	-	-	-
<i>Candida albicans</i> ATCC 10231	N _i :7 10 ¹⁰	N _a	25	29	0	97	50	0	>3 10 ²	200	110	80	0	0	98	2	0	200	53	29
		R	2.8 10 ⁸	2.41 10 ⁸	7 10 ¹⁰	7.2 10 ⁷	1.4 10 ⁸	7 10 ¹⁰	-	3.5 10 ⁷	6.36 10 ⁷	-	8.7 10 ⁷	7 10 ¹⁰	7 10 ¹⁰	7.1 10 ⁷	3.5 10 ⁹	7 10 ¹⁰	3.5 10 ⁷	1.32 10 ⁷

N_i: Initial contamination; N_a: Contamination after treatment; R: Reduction

Table 4: Antiseptic effect of compound (1) against a clinical isolate of *Acinetobacter baumannii* in the presence of interfering substances

Strain and initial count	Time	Formulation strength								
		90%			80%			10%		
		5 min	30 min	24 h	5 min	30 min	24 h	5 min	30 min	24 h
<i>Acinetobacter baumannii</i> N _i :6.39 10 ⁸	N _a	>3 10 ²	>3 10 ²	24	>3 10 ²	>3 10 ²	>3 10 ²	>3 10 ²	>3 10 ²	>3 10 ²
	R	–	–	2.66 10 ⁶	–	–	–	–	–	–

N_i: Initial contamination; N_a: Contamination after treatment; R: Reduction

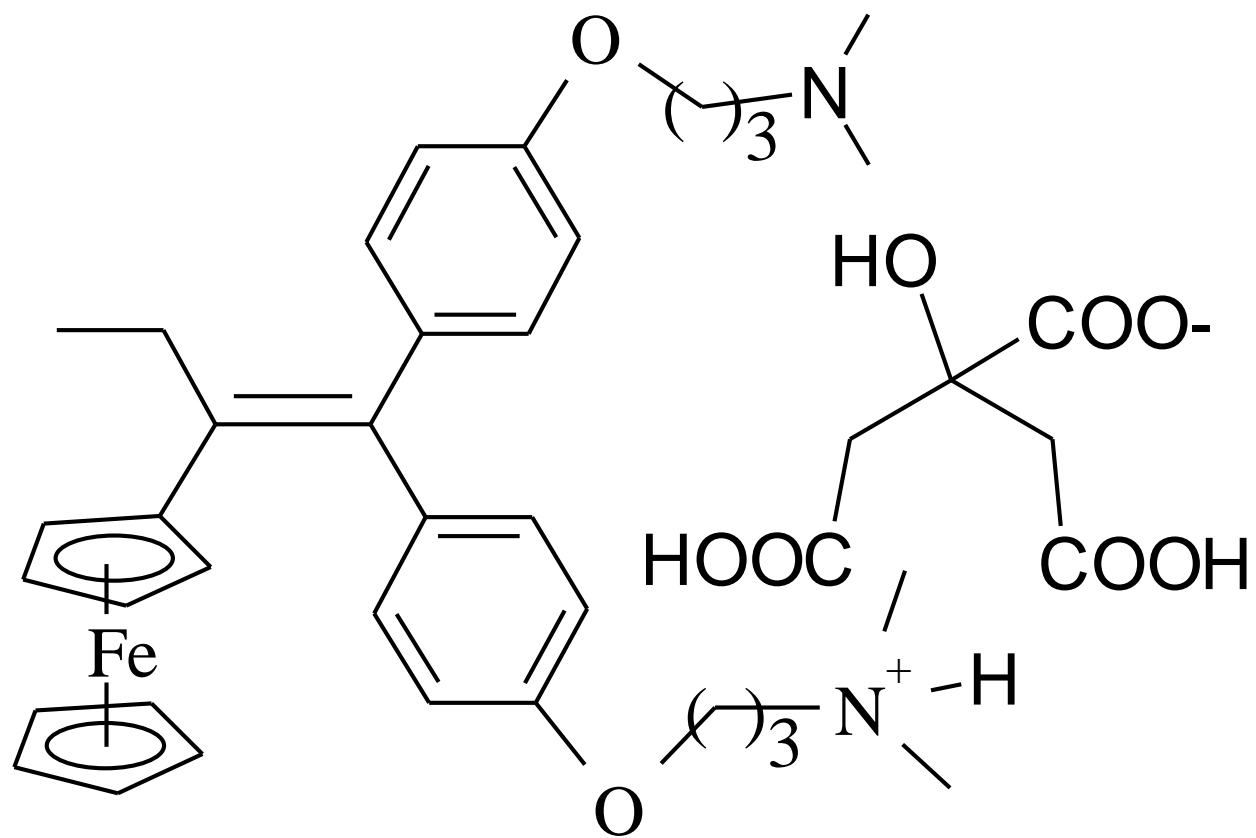


Figure 1.

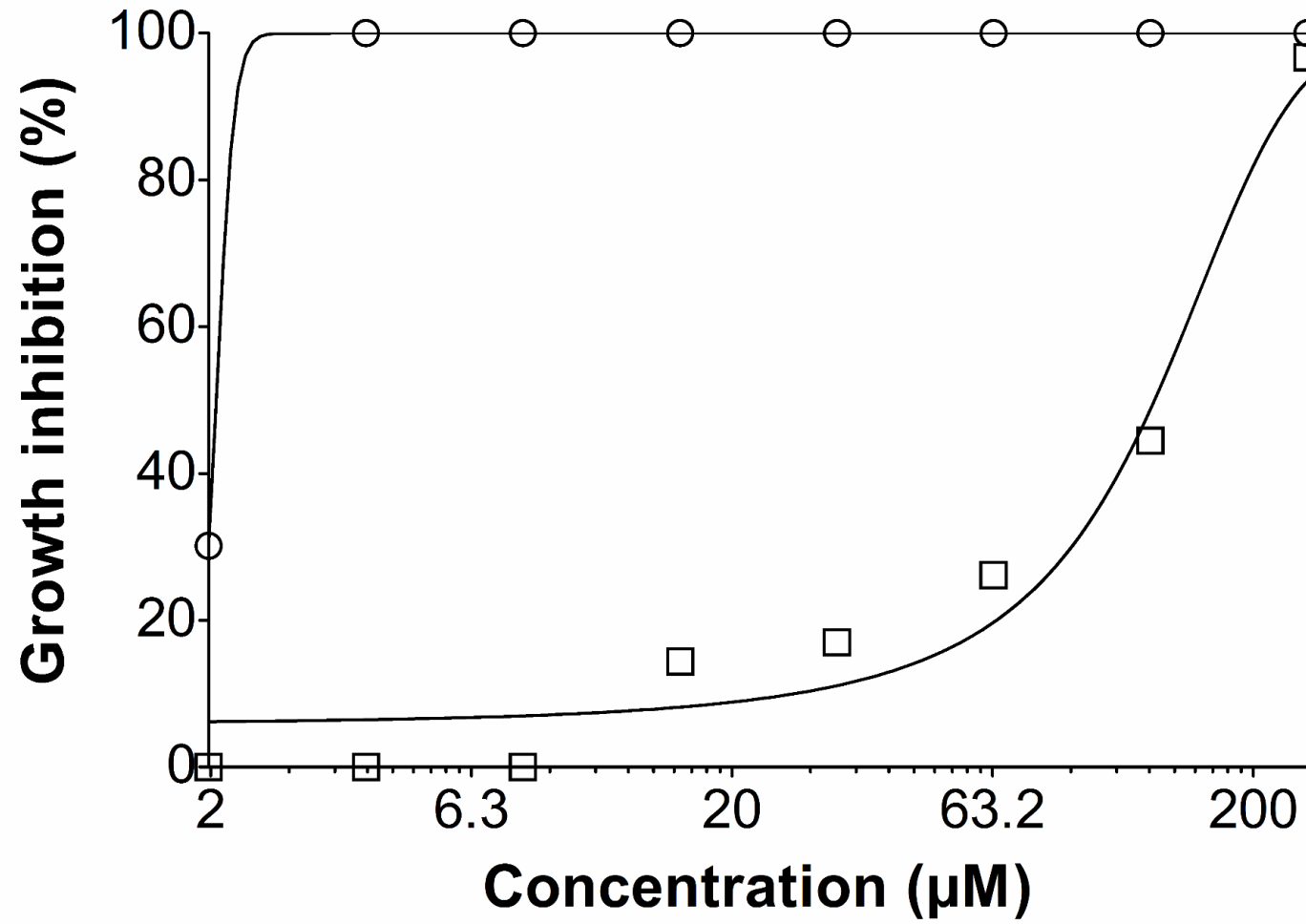


Figure 2.

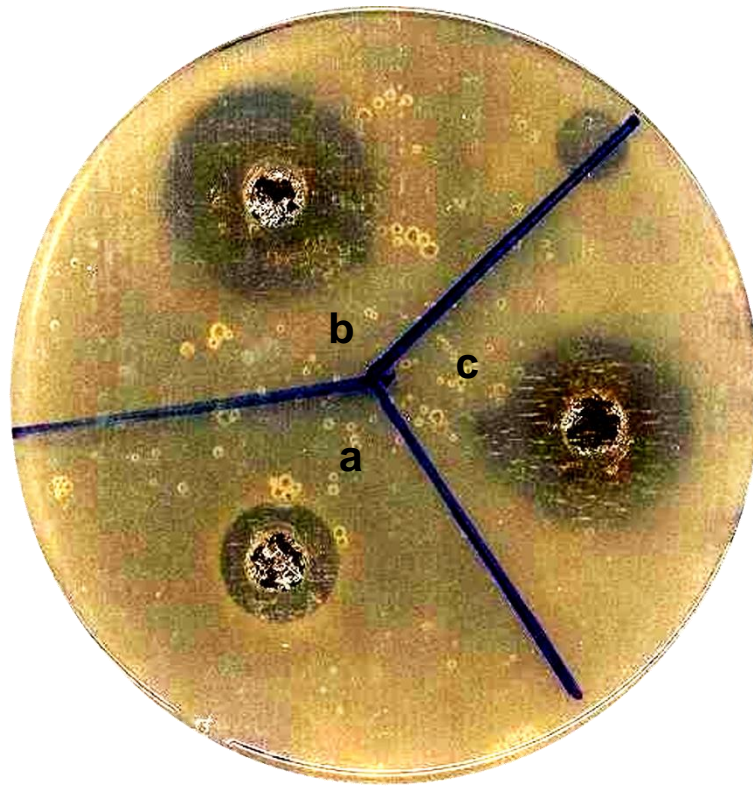


Figure 3.

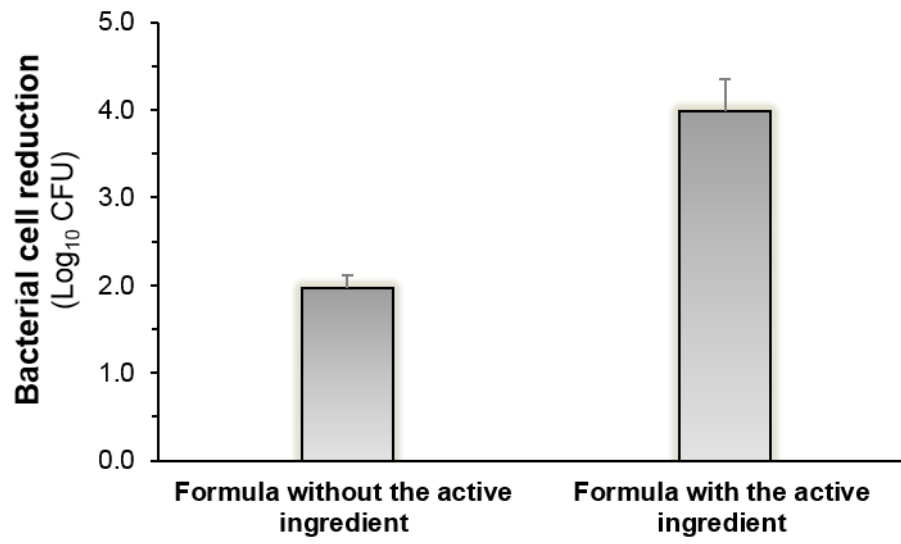


Figure 4.