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Newbouldiaquinone A: A naphthoquinone–anthraquinone ether coupled pigment, as a potential antimicrobial and antimalarial agent from *Newbouldia laevis*

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Abstract

The study of the chemical constituents of the roots of *Newbouldia laevis* (Bignoniaceae) has resulted in the isolation and characterization of a naphthoquinone–anthraquinone coupled pigment named newbouldiaquinone A (**1**) together with 14 known compounds: apigenin, chrysoeriol, newbouldiaquinone, lapachol, 2-methylanthraquinone, 2-acetylfuro-1,4-naphthoquinone, 2,3-dimethoxy-1,4-benzoquinone, oleanolic acid, canthic acid, 2-(4-hydroxyphenyl)ethyl triacontanoate, newbouldiamide, 5,7-dihydroxydehydroiso-α-lapachone, β-sitosterol, and β-sitosterol glucopyranoside. The structure elucidation of the isolated compounds was established based on spectroscopic studies, notably of the 2D NMR spectra. The antimalarial activity of compound (**1**) against *Plasmodium falciparum* in vitro shows moderate chemo suppression of parasitic growth. Its antimicrobial activity against a wide range of microorganisms was 13- and 24-fold more active against *Candida gabrata* and *Enterobacter aerogens* than the reference antibiotics nystatin and gentamycin.

Keywords: *Newbouldia laevis*; Bignoniaceae; Ether-coupled naphthoquinone–anthraquinone; Antimalarial and antibacterial activity

1. Introduction

Newbouldia laevis SEEM. or "Boundary Tree" is a medium sized angiosperm in the Bignoniaceae family. It is native to tropical Africa, and grows to a height of about 10 m (Okeka, 2003). The species *N. laevis* is widely used in African folk medicine for the treatment of several diseases such as an astringent in diarrhea and dysentery. It is also employed in the treatment against worms, malaria, sexually transmitted disease, and in the reduction of dental caries (Eyong et al., 2005). However, little is known of its antimalarial properties despite the fact that this species is widely used by local healers to treat malaria. In an earlier study, we reported the isolation and structure elucidation of newbouldiaquinone, a naphthoquinone-anthraquinone C–C coupled pigment (Eyong et al., 2005). To obtain the minor compounds, the roots, seeds and leaves of *Newbouldia laevis* were again collected and extracted with CH_2Cl_2:MeOH (1:1). The root fraction was subjected to different chromatographic procedures resulting in the isolation and structure elucidation of newbouldiaquinone A (**1**), another naphthoquinone–anthraquinone pigment from *N. laevis*, but coupled via an ether bridge rather than a C–C bond (Eyong et al., 2005). We now report on the structure elucidation and antibacterial potency of **1** against a wide

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range of microorganisms and on antimalarial screening of this compound together with other quinones and derivatives from this plant.

2. Results and discussion

The MeOH:CH_2Cl_2 (1:1) extract of the leaves, stem and root of *N. laevis* was fractionated by silica gel column chromatography to give several fractions, which were further chromatographed on silica gel to give a new naphthoquinone–anthraquinone dimeric quinone, newbouldiaquinone A (**1**) together with 14 known compounds. The latter group of compounds were identified by comparison of their spectroscopic data with the literature data as apigenin (Hiermann and Kartnig, 1978), chrysoeriol (Harrison and Kulshreshtha, 1984), newbouldiaquinone (Eyong et al., 2005), lapachol (Khan and Mlungwana, 1999), 2-methylanthraquinone (Boivert and Brussard, 1988), 2-acetylfuro-1,4-naphthoquinone (Lopes et al., 1984), 2,3-dimethoxy-1,4-benzoquinone (Matsumoto and Kobayashi, 1985), oleanolic acid (Ikuta and Hokawa, 1988), canthic acid (Chatterjee et al., 1979), 2-(4-hydroxyphenyl)ethyl triacontanoate (Ali and Houghton, 1999), newbouldiamide (Eyong et al., 2005), 5,7-dihydroxydehydroiso-α-lapachone (Gafner et al., 1996), β-sitosterol (Schuhr et al., 2003), and β-sitosterol glucopyranoside (Seo et al., 1978). The α-lapachone and β-lapachone were synthesized from lapachol using the Hooker procedure (Hooker, 1936).

Newbouldiaquinone A (**1**) was obtained as a yellow powder with m.p. 260 °C. The UV spectrum of **1** exhibited absorption maxima at 270, 308 and 388 nm, suggesting a naphthoquinone derivative (Gorman et al., 2003). This was supported by IR bands at 1647 cm^{-1} for carbonyl absorption, a broad signal at 3404 cm^{-1} for a non-chelated hydroxyl group and also by a sharp signal at 1236 and 1036 cm^{-1} for an ether function. Analysis of the chemical ionisation mass spectrum (CI-MS) gave a molecular ion at m/z 411.1 $[M - H]^+$, corresponding to the molecular formula $C_{25}H_{14}O_6$, supported by the ^1H NMR, ^{13}C NMR and DEPT analysis. The mass spectrum also showed peaks at m/z 394.1 $[M - O]^+$, 348 $[M - O - CO - H_2O]^+$ and 322 $[M - H_2O - 2CO - CH_2]^+$, exhibiting loss of methyl, water and multiple loss of carbon monoxide, suggesting the presence of hydroxyl, methyl, and carbonyl groups. This fragmentation pattern is typical for hydroxyanthraquinone and/or napthoquinone (Aguinaldo et al., 1993; Singh and Singh, 1986).

The ^1H NMR in $CDCl_3$ (see Section 3) of newbouldiaquinone A (**1**) had one singlet at δ 2.51 of three protons, characteristic of a methyl group attached to an aromatic system (Aguinaldo et al., 1993). It also had 10 strongly deshielded protons, 6 of which appear above δ 8.10, characteristic of protons peri to carbonyl groups, suggesting the presence of an anthraquinnone–anthraquinone, anthraquinone–naphthoquinone or naphthoquinone–naphthoquinone ring system. From the proton–proton correlation spectroscopy (COSY), two pairs of four protons were coupling to one another while two protons did not have any coupling suggesting the presence of two pairs of AA′BB′ spin system of four aromatic protons each. The first AA′BB′4spin system of four aromatic protons at δ 8.37–8.32 (2H, *m*, H-5, H-8), 7.95 (2H, *td*, $J = 8.5$, 1.5 Hz, H-6, H-7) along with 8.27 (1H, *s*, H-4) and 8.18 (1H, *s*, H-1) indicated that compound **1** contains an anthraquinone, possessing an unsubstituted ring A and a di-substituted ring C at positions 2 and 3 (Chart 1, partial structure A). A second AA′BB′ spin system of four aromatic protons at 8.25 (1H, *dd*, $J = 8.5$, 1.5 Hz, H-8′), 8.22 (1H, *dd*, $J = 8.5$, 1.5 Hz, H-5′), 7.90 (2H, *td*, $J = 8.5$, 1.5 Hz, H-6′, H-7′) is attributed to a second partial structure B, i.e., a naphthoquinone, possessing an unsubstituted ring A.

The structures of the two fragments were similar to those in newbouldiaquinone (Eyong et al., 2005) and were also confirmed by the analysis of the ^{13}C NMR spectral data (see Section 3).

The acetylation of compound **1** gave a monoacetate **2** with M^+ at m/z 452 with the addition of one acetyl unit suggesting that it contains only one free hydroxyl group, probably at position 2′ of the naphthoquinone moiety from biogenetic consideration as well as from HMBC interactions (Fig. 1). Moreover, the signals at δ 2.30 in ^1H

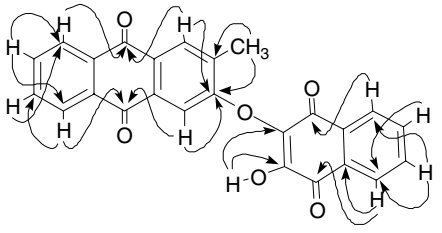

Fig. 1. Important HMBC data for compound **1**.

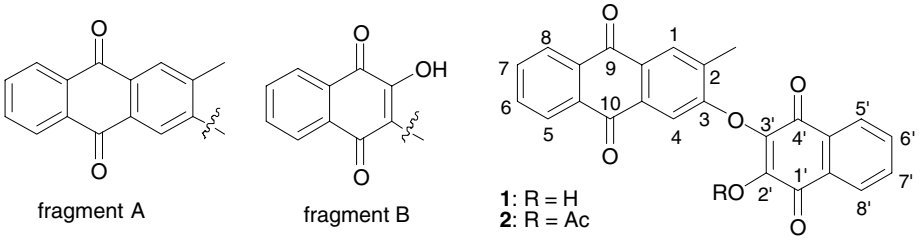

Chart 1. Structures of fragments A and B and newbouldiaquinone A (**1**) and its acetate **2**.

Table 1
In vitro activity (parasitemia %) of lapachol, newbouldiaquinone A (**1**), β-lapachone, and α-lapachone against *Plasmodium falciparum*

Days	Control	Lapachol	Newbouldiaquinone A	β-Lapachone	α-Lapachone
0	0.55	0.55	0.55	0.55	0.55
1	0.79	0.52	0.41	0.37	0.5
2	1	0.937	0.78	0.66	0.5
3	1.65	1.3	0.9	0.84	0.86
4	2.17	1.97	1.7	1	0.957

NMR and at δ 21.5 and 170.1 in ^{13}C NMR further confirm the monoacetylation of **1**. Thus, the remaining oxygen must be incorporated in an ether linkage. The absence of any quinoidal protons for H-2′ or H-3′ that usually occurs at ca. δ 6.87 (Hassanean et al., 2000), for naphthoquinones and/or benzoquinones confirms our partial structures. The ether, therefore, links the position C-3′ of fragment B and position 2 or 3 of fragment A since positions 1 and 4 show signals for peri-hydrogens in the ^{1}H NMR spectrum. The methyl group is thus attached at C-2 of ring C in the anthraquinone due to strong HMBC correlations with carbon C-1, suggesting a coupled anthraquinone–naphthoquinone skeleton at C-3′ and C-3 by ether linkage. Consequently, the structure of compound **1** was established as 3-(2-hydroxyl-naphthoquinon-3-*O*-yl)-2-methyl-anthracen-9,10-dione, named newbouldiaquinone A (**1**).

Newbouldiaquinone A (**1**), lapachol, α-lapachone and β-lapachone were tested against *Plasmodium falciparum* in vitro. All of them showed moderate suppression of parasitic growth (Table 1).

Table 2
Antimicrobial activity of newbouldiaquinone A (**1**) and of reference antibiotics

Microbial strains	(**1**) μg/ml (μM)[a]	RA[b] μg/ml (μM)[a]
Gram-negative bacteria		
Entrobacter freundii	0.31 (0.75)	4.88 (9.0)
Enterobacter aerogens	0.31 (0.75)	9.76 (18)
Enterobacter cloacae	0.31 (0.75)	4.88 (9.0)
Escherichia coli	0.31 (0.75)	1.22 (2.25)
Klebsiella pneumoniae	0.31 (0.75)	2.44 (4.5)
Morganella morgani	0.61 (1.49)	2.44 (4.5)
Proteus mirabilis	0.31 (0.75)	2.44 (4.5)
Proteus vulgaris	4.88 (11.9)	1.22 (2.25)
Pseudomonas aeruginosa	9.76 (23.8)	4.88 (9.0)
Shigella dysenteriae	4.88 (11.9)	2.44 (4.5)
Shigella flexneri	1.22 (2.97)	2.44 (4.5)
Salmonella typhi	4.88 (11.9)	2.44 (4.5)
Gram-positive bacteria		
Bacillus cereus	9.76 (23.8)	2.44 (4.5)
Bacillus megaterium	9.76 (23.8)	4.88 (9.0)
Bacillus stearothermophilus	9.76 (23.8)	4.88 (9.0)
Bacillus subtilis	4.88 (11.9)	2.44 (4.5)
Staphylococcus aureus	9.76 (23.8)	4.88 (9.0)
Streptococcus faecalis	9.76 (23.8)	4.88 (9.0)
Yeasts		
Candida albicans	4.88 (11.9)	4.88 (5.21)
Candida krusei	4.88 (11.9)	4.88 (5.21)
Candida gabrata	0.31 (0.75)	9.76 (10.4)

[a] MIC: minimal inhibition concentration or the lowest concentration that prevents the growth of the tested pathogens.
[b] RA: reference antibiotics (gentamycin for bacteria, nystatin for yeast).

Newbouldiaquinone A (**1**) is a powerful antimicrobial agent (Table 2). Very pronounced activities were observed against Gram-negative bacteria with the minimal inhibition concentration (MIC) varying from 0.31 to 9.76 μg/ml (0.75–23.8 μM). The inhibition effect observed was greater than that of the reference antibiotics (RA). However, it appeared to be less active against Gram-positive bacteria. Its antifungal activity was also important with *Candida gabrata* being the most sensitive yeast. Newbouldiaquinone A (**1**) is 13- and 24-fold more active against *Candida gabrata* and *Enterobacter aerogens* than nystatin and gentamycin, respectively.

3. Experimental

3.1. General experimental procedures

^{1}H, 2D ^{1}H–^{1}H COSY, ^{13}C, 2D HMQC and HMBC spectra were recorded with a Bruker Avance 500 MHz spectrometer. Chemical shifts are referenced to internal TMS ($\delta = 0$) and coupling constants J are reported in Hz. Optical spectra were recorded with a NICOLET 510P FT-IR spectrometer, a UV-2101PC spectrometer, and Perkin–Elmer 241 polarimeter.

3.2. Plant material

The plant *Newbouldia laevis* SEEM. (Bignoniaceae) was collected at Mamfe, South West province of the Republic of Cameroon in December 2004, earlier identified by Mr. Ndive Elias (Plant taxonomist), Botanical Garden, Limbe Cameroon. A voucher specimen (No. 1754/SRFK) has been deposited at the National Herbarium, Yaounde, Cameroon.

3.3. Extraction and isolation

Dried and powdered leaves (1 kg), seeds (1 kg), root bark (2.5 kg), and stem bark (3 kg) of *N. laevis* were separately extracted with a mixture of MeOH:CH_2Cl_2 (1:1) at room temperature for 24 h. The suspensions were filtered and each filtrate was concentrated under vacuum to give 300, 80, 80 and 90 g of crude residue, respectively. 50 g of the 300 g crude extract of the leaves was separated using sepadex LH-50 to give yellow eluents that were regrouped based on their TLC pattern and purified by column chromatography on silica gel eluting with hexane:EtOAc

(7.5:2.5) to afford Apigenin (40 mg). The crude extract from the seeds (80 g) was subjected to column chromatography (silica gel, hexane, hexane–EtOAc and EtOAc, in order of increasing polarity) yielding 205 fractions ($F_{1–205}$). Fractions $F_{35–40}$, from elution with a mixture of hexane–EtOAc (9:1) yielded β-sitosterol (200 mg), and fractions $F_{107–112}$, which were eluted with hexane:EtOAc (5.5:4.5) and subjected to a second CC, afforded newbouldiamide (80 mg). Column fractions $F_{151–162}$ [hexane:EtOAc (5.5:4.5)], $F_{170–172}$ [hexane:EtOAc (4:6)] that was similarly subjected to a second CC yielded chrysoeriol (40 mg) and fractions $F_{190–200}$ hexane–EtOAc (3.0:7.0) afforded β-sitosterol glucopyranoside (500 mg). Similarly, the crude extract of the root bark (80 g) was also chromatographed on a silica gel column and eluted with gradient mixtures of hexane:EtOAc yielding 200 fractions ($F_{1–200}$). Fractions $F_{20–F_{30}}$ were eluted with hexane:EtOAc (9.5:0.5) and afforded 2-acetylfuro-1,4-naphthoquinone (6.5 mg) and 2-methylanthraquinone (80 mg). Fractions $F_{31–35}$, eluted with a mixture of hexane:EtOAc (9:1), gave lapachol (200 mg); fractions $F_{96–106}$ (hexane:EtOAc 8.5:1.5) gave newbouldiaquinone (30 mg). Fractions $F_{136–138}$ on CC using hexane:EtOAc (8:2), gave canthic acid (50 mg), $F_{145–150}$ hexane:EtOAc (7.5:2.5) afforded a 5,7-dihydroxydehydro-iso-α-lapachone while $F_{165\text{-}170}$ eluted with hexane:EtOAc (7:3) afforded newbouldiaquinone A (**1**). Finally, the crude extract of stem bark (90 g) was subjected to CC using hexane–EtOAc yielding 151 fractions ($F_{1–151}$). Fractions $F_{17–20}$ eluted with hexane afforded 2,3-dimethoxy-1,4-benzoquinone (8 mg) and fractions $F_{48–50}$ gave 2-(4-hydroxyphenyl)ethyl triacontanoate on subjecting to CC using hexane:EtOAc (9.5:0.5) while $F_{90–100}$ (Hex:EtOAc 8.0:2.0) gave oleanolic acid (80 mg).

3.3.1. Newbouldiaquinone A (*1*)

Yellow powder, m.p. 260 °C; UV λ_{max}, nm (log ε): 270 (2.9), 308 (4.80), 388 (4.50); IR ν_{max} $CHCl_3$ cm^{-1}: 3404, 1647, 1236, 1036;. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.37–8.32 (2H, *m*, H-5, H-8), 8.27 (1H, *s*, H-4), 8.25 (1H, *dd*, $J = 8.5$, 1.5 Hz, H-8′), 8.22 (1H, *dd*, $J = 8.5$, 1.5 Hz, H-5′), 8.18 (1H, *s*, H-1), 7.95 (2H, *td*, $J = 8.5$, 1.5 Hz, H-6, H-7) and 7.90 (2H, *td*, $J = 8.5$, 1.5 Hz, H-6′, H-7′), 2.51 (3H, *s*, CH_3-2); ^{13}C NMR (125 MHz, $CDCl_3$): δ 183.5 (C-10), 182.9 (C-1′), 182.7 (C-9), 181.6 (C-4′), 145.4(C-3), 140.1 (C-3′), 139.9 (C-2′), 135.2 (C-6′), 134.9 (C-7′), 134.8 (C-6), 133.9 (C-7), 133.6 (C-8a), 132.7(C-10a), 132.6 (C-9a), 132.5 (C-4a), 130.9 (C-4a′), 130.8 (C-8a′), 129.8 (C-1), 128.2 (C-4), 127.2 (C-5), 127.1 (C-8), 126.4 (C-8′), 126.3 (C-5′), 121.3 (C-2), 20.4 (CH_3); CI–MS (CH_4): *m/z* 411.1 $[M + 1]$; EIMS *m/z* (rel. int.): 410.1 $[M]^+$ (41), 394.1 $[M - O]^+$ (100), 348.1 $[M - O - CO - H_2O]^+$ (30), 322.1 $[M - H_2O - 2CO - CH_2]^+$(20), 252.1 (20), 176.1 (14), 126.1 (8), 105.0 (16), 76.0 (21), 50.0 (9).

3.3.2. Acetylation

A solution of dry pyridine (0.5 ml) and Ac_2O (0.5 ml) were added to compound **1** (5 mg), and left overnight. After usual hydrolytic (HCl) workup, compound **2** was isolated and purified by filtration over a short batch of silical gel (CH_2Cl_2) (3 mg); m.p.: 205 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.37–8.33 (2H, m, H-5, H-8), 8.27 (1H, *s*, H-4), 8.24 (1H, *dd*, $J = 8.5$, 1.5 Hz, H-8′), 8.21 (1H, *dd*, $J = 8.5$, 1.5 Hz, H-5′), 8.15 (1H, *s*, H-1), 7.88–7.83 (4H, m, H-6, H-7, H-6′, H-7′), 2.50 (3H, s, CH_3-2) and 2.30 (3H, s, CH_3COO); ^{13}C NMR (125 MHz, $CDCl_3$):δ 183.3 (C-10), 183.0 (C-1′), 182.7 (C-9), 181.6 (C-4′), 170.1 (CH_3COO)145.3(C-3), 141.9 (C-3′), 140.8 (C-2′), 135.5 (C-6′), 134.2 (C-7′), 134.8 (C-6), 133.9 (C-7), 133.6 (C-8a), 132.7(C-10a), 132.5 (C-9a), 132.4 (C-4a), 130.8 (C-4a′), 130.7 (C-8a′), 129.6 (C-1), 128.1 (C-4), 127.4 (C-5), 127.0 (C-8), 126.7 (C-8′), 126.5 (C-5′), 121.4 (C-2), 21.5 (CH_3COO), 20.4 (CH_3-2); EI–MS *m/z* (rel. int.): 452.1 $[M]^+$ (30), 392.1 $[M - HOAc]^+$ (25), 410.1 $[M - CH_3CO + H]^+$ (30), 176.1 (12), 126.1 (11), 105.1 (16), 76.2 (29), 50.1 (12).

3.4. Antimalarial test

Newbouldiaquinone A (**1**), lapachol, α-lapachone and β-lapachone were dissolved in water + DMSO 0.02% v/v (Andrade-Neto et al., 2004). The compounds were administered over a period of four days to the culture and the number of parasites was determined daily. An untreated culture of plasmodia served as a control (for results see Table 1).

3.4.1. Culturing of *P. falciparum* NF54 strain

P. falciparum isolate NF54 and R strain were maintained in small Petri dishes (5 cm) according to a protocol from Moloney (Moloney et al., 1990) and Trager (Trager and Williams, 1992) in a gaseous phase of 90% N_2, 5% CO_2 and 5% O_2. Parasites were cultured in human erythrocytes (blood group A^+) in RPM1640 medium (Sigma) supplemented with 25 μM HEPES, 20 mM sodium bicarbonate, and 10% heat inactivated human A+ plasma at 10% (v/v) hematocrit. The parasitemia of infected erythrocytes was determined by light microscopy and estimated by Giemsa-stained smears. Parasitemia detected in the cultures were scored visually with a 100-fold oil immersion objective, counting at least 1000 infected erythrocytes to determine the parasitemia.

3.4.2. Inhibitor experiments by monitoring multiplication and growth of plasmodia

Cultures were adjusted to a parasitemia of 0.5%. Aliquots were diluted 1:10-fold in RPMI-medium, dispensed into 12-well microculture trays and incubated at 37 °C in a candle jar. Thereafter, growth medium was changed once a day for four days and inhibitors were added to the media in concentration of 20 μM as indicated. Each substance was analyzed in four independent wells of the microculture tray. Parasitemia was estimated as triplicates daily in each of the four independent wells from Giemsa-stained smears by counting 1000 erythrocytes (for results see Table 1).

3.5. Antimicrobial test

3.5.1. Microbial strains

A total of 21 microbial cultures belonging to six Gram positive bacterial species (*Bacillus cereus*, *Bacillus megaterium*, *Bacillus substilis*, *Bacillus stearothermophilus*, *Staphylococcus aureus*, *Streptococcus faecalis*), 12 Gram negative bacteria (*Escherichia coli*, *Shigella dysenteriae*, *Proteus vulgaris*, *Proteus mirabilis*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Morganella morgani*, *Enterobacter aerogens*, *Citrobacter freundii*, *Enterobacter cloacae*), and three yeasts from Candida species (*Candida albicans*, *Candida krusei* and *Candida gabrata*) were used in this study. Three of the four Bacillus species were provided by "l'institut Appert de Paris" while, *Bacillus cereus* was provided by the A.F.R.C Reading Laboratory of Great Britain. The other strains were clinically isolated from patients in the Centre Pasteur de Yaounde-Cameroon (health institution). They were then maintained on agar slant at 4 °C in the Laboratory of the Applied Microbiology and Molecular Pharmacology (Faculty of Science, University of Yaounde I) where the antimicrobial tests were performed. The strains were activated at 37 °C for 24 h on nutrient agar (NA) (bacteria) or Sabouraud glucose agar (yeasts). The nutrient broth (NB) was used to determine the minimal inhibition concentration of compound (**1**) against the tested pathogens.

3.5.2. Antimicrobial assays

MICs of compound (**1**) were evaluated against the pathogens. The inocula of micro organisms were prepared from 12 h broth culture and the suspensions were adjusted to 0.5 Mc Farland turbidity. Compound (**1**) was first dissolved in dimethyl sulfoxide (DMSO) 10% v/v to the highest dilution (39.06 μg/ml), and serial twofold dilutions were made in a concentration ranged from 0.031 to 39.06 μg/ml in the 96 wells microplate containing NB. MIC values of the tested compounds against pathogens were determined based on the microdilution method, as the lowest concentration at which there was 100% growth inhibition of the tested pathogens (Zgoda and Porter, 2001). Gentamycin (bacteria) and nystatin (yeasts) diluted prior in water were also used as reference antibiotics. Negative control was made with DMSO 10% v/v.

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