SUPPLEMENTARY MATERIAL

***In vitro* evaluation of the antioxidant and antimicrobial activity of DIMBOA [2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one]**

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The aim of this study was to evaluate the *in vitro* antioxidant and antimicrobial properties of the natural cyclic hydroxamic acid: 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA). Antioxidant activity of the isolated DIMBOA was examined using DPPH, FRAP and ABTS tests. It was found that DIMBOA exhibits a potent free-radical scavenging activity and a weaker iron (III) ions reducing activity. Antimicrobial activity against selected G(+), G(–) bacterial strains and against yeasts-like reference strains of fungi was investigated using Disk-Diffusion method. It has been shown that DIMBOA possess growth inhibitory properties against many strains of studied bacteria and fungi, like *Staphylococcus aureus*, *Escherichia coli* as well as against *Saccharomyces cerevisiae*.

Keywords: *Zea mays*; DIMBOA; antioxidant; antimicrobial; FRAP; ABTS; DPPH

1. Experimental

1.1. Plant material

In order to obtain the plant material needed for isolation of DIMBOA, three maize (*Zea mays*) varieties were used: KB1903 (1 kg), Kosmo (1 kg) and Kadryl (1 kg). The grains were obtained in February 2014 from Małopolska Hodowla Roślin in Kobierzyce, Poland. They were sown in 0.5 kg portions in metal boxes (48×38×5 cm) with two layers of cellulose towel inside. Maize cultivation was performed in the dark, room temperature (20-25 °C) and watered as needed (approx. 1 L of water overall). After 7 days from germination, the white-yellow aboveground plant parts (9-13 cm) were harvested, weighed and frozen (-16 °C) until the start of extraction.

1.2. Extraction and isolation of DIMBOA

Pure DIMBOA was isolated for further testing from 7-days-old maize seedlings by using a combination of two methods described by Larsen and Christensen (2000) and by Li et al. (2013), yielding respective isolates. The purest sample among them was obtained from 123.84 g seedlings of Kadryl ($m = 43.4$ mg).

1.3. Identification of DIMBOA

The obtained DIMBOA isolate was analyzed by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). The TLC analysis was carried out on RP-18W_F plates (LiChrospher[®], Merck, Germany) and 40% methanol as mobile phase. The HPLC analysis was performed using chromatograph Knauer Smartline series (Germany). Separation was achieved on a YMC (Japan) ODS-AQ chromatographic column (4.6×150 mm) of 3 μ m particle size. The isocratic solvent system consisted of aqueous 0.1% trifluoroacetic acid (TFA) and methanol (55:45, v/v). The flow rate was set at 0.4 mL min⁻¹ with UV/Vis/NIR detection at 190-1020 nm. The chromatograms were recorded at 280 nm. Mass spectrometry and nuclear magnetic resonance, were performed using Apex Ultra Apparatus (Bruker Daltonics, Germany) and Bruker BioSpin 300 MHz Apparatus respectively.

1.4. Free-radical scavenging and reducing activity of DIMBOA

All measurements were performed in triplicate using Spectrophotometer CE 3021 (CECIL instruments, CITY Great Britain). TROLOX[™] (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a positive standard in all tests.

1.4.1. ABTS assay (measuring DIMBOA ability to scavenge ABTS^{•+})

Chemicals: ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) and potassium persulfate (dipotassium peroxydisulfate) were obtained from Sigma-Aldrich, Poland. TROLOX was prepared in HPLC grade methanol (POCh, Poland) to concentration 2.5 mmol L⁻¹, then diluted with deionised water to form known dilutions in range 0-20 mmol L⁻¹. Previously obtained DIMBOA sample was dissolved in methanol to form concentrations 1 mg mL⁻¹, and then was diluted five times with deionised water.

Assay: The assay was performed using method (Re et al. 1999) with modifications. The 7 mmol L⁻¹ ABTS salt and 2.45 mmol L⁻¹ K₂S₂O₈ solutions in deionised water were mixed in

volumetric ratio 1:1. The mixture was in the dark and room temperature for 16 hours in order to produce ABTS radical (ABTS^{•+}). Next, just before the start of the measurements, it was diluted with deionised water to absorbance of 0.7 at 734 nm. 20 µL of sample or TROLOX standard were added to tubes. Next 2 mL of ABTS^{•+} was added, the mixtures were vortexed and absorbance were taken after 6 min incubation at room temperature at 734 nm. Deionised water was used as blank. Measurements were taken in triplicates. The results of the ABTS test are expressed as µmol Trolox per mL units, which were obtained by substituting the calculated percentage of inhibition of sample to the linear regression equation of TroloxTM standard curve (expressed as dependence of percentage of inhibition from the concentration of µmol TroloxTM mL⁻¹) **42.37x + y = 0.0966 (R² = 0.9993)**. Additionally TEAC value was calculated by dividing the IC50 of Trolox for the IC50 of the DIMBOA. Linear regression equation of DIMBOA curve (expressed as dependence of percentage of inhibition from the concentration of µmol DIMBOA mL⁻¹) was **y = 29.803x + 8.660 (R² = 0.9914)**.

1.4.2. FRAP assay

Chemicals: 0.3 mol L⁻¹ acetate buffer (pH = 3.6) was prepared. 0.01 mol L⁻¹ TPTZ (= 2,4,6-tripyridyl-s-triazine; Sigma-Aldrich, Poland) in 0.04 mol L⁻¹ HCl (POCH, Poland) mixture was made. A 0.02 mol L⁻¹ solution of FeCl₃×6H₂O (= iron (III) chloride hexahydrate; Chempur, Poland) in deionised water was made just before the start of the measurements. FeSO₄×7H₂O (= iron (II) sulfate heptahydrate; Chempur, Poland) was used for calibration curve. The compound was dissolved in methanol, and then diluted with deionised water to obtain concentrations: 0.05; 0.1; 0.15; 0.2; 0.3; 0.4 and 0.5 mg mL⁻¹. From the DIMBOA isolate, the 1 mg mL⁻¹ methanolic solution was prepared, which then was diluted with deionised water to finally obtain seven concentrations respectively: 0.1; 0.08; 0.06; 0.04; 0.03; 0.02; 0.01 mg mL⁻¹.

Assay: The assay was performed according to Benzie & Strain (1996) with small modifications. FRAP working solution was prepared just before the start of the analysis: 0.3M acetate buffer (pH = 3.6), 0.01M TPTZ in 0.04M HCl and 0.02M FeCl₃×6H₂O were mixed in volumetric proportion 10:1:1 and kept away from light. Next, 75 µL of the DIMBOA solutions or FeSO₄×7H₂O solutions were mixed with 2.25 mL FRAP working solution and 225 µL deionised water. The obtained mixtures were incubated in 37 °C for 30 min and their absorbance was measured at λ = 593 nm. Deionized water with FRAP working solution was used as a blank. All determinations were carried out in triplicate. Linear regression equation

of the standard curve for iron sulfate (II) ($\text{FeSO}_4 \times 7\text{H}_2\text{O}$) was applied for calculation: $y = 0.6267x - 0.0102$ ($R^2 = 0.998$)

1.4.3. DPPH assay

Chemicals: 8.0 mg DPPH (= 1,1-diphenyl-2-picrylhydrazyl ; Sigma- Aldrich, Poland) was dissolved in pure methanol (20 mL) using the ultrasonic bath SONOREX DIGITAL 10P (Bandelin, Germany). The mixture was then stored in the dark for 2 hours. Next, the 1 mg mL^{-1} methanolic solution of isolated compound was prepared.

Assay: Measurements were performed according to Brand-Williams et al. (1995). At six test-tubes, 0; 20; 40; 60; 80; 100 μL of DIMBOA were mixed with 100; 80; 60; 40; 20; 0 μL of methanol. Next, 2 mL of methanol and 0.25 mL of the prepared solution of DPPH were added to the each tube. Obtained mixtures were vortexed and allowed to stand for 20 min in a dark place at room temperature (25 °C). After this, absorbance was measured at 517 nm. Methanol was used as blank sample. Results are the average of 3 measurements. The scavenging activity of DPPH radicals (%) was calculated with the equation: $(\Delta A_{517} \text{ blank} - \Delta A_{517} \text{ sample}) / \Delta A_{517} \text{ blank} \times 100\%$.

1.5. Antibacterial activity tests

Determination of the antimicrobial activity by Disk Diffusion method: To determine the antimicrobial activity of hydroxamic acid (DIMBOA) sample a filter paper disk method was used as described (Chen et al. 2008), precisely. The microorganisms used in this study, including *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* K326 (MRSA clinical strain), *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Escherichia coli* 295 (ESBL-positive clinical strain), *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030 and *Saccharomyces cerevisiae* BCMM 3963, were obtained from the laboratory of microbiology, Department of Microbiology, Wroclaw Medical University, Poland. Mueller-Hinton agar and Mueller Hinton agar supplemented with 2% dextrose and 0.5 mg L^{-1} methylene blue were used as the basic media for the bacterial and yeast strains, respectively. The bacterial and fungal suspensions of the strains tested, with a turbidity equivalent to 0.5 McFarland standard, were spread on agar plates using sterile swabs. The standard paper disks (6 mm diameter) were impregnated with DMSO-dissolved DIMBOA sample (2 mg per disk) and placed aseptically on the agar plates. Standard discs of

gentamicin (10 µg, Oxoid Ltd., UK) were used as positive controls for bacteria while of amphotericin B (10 µg Abtek Biologicals Ltd., UK) for fungi. The plates thus prepared were incubated at 37 °C for 24 or 48 hours for the bacterial and fungal strains, respectively. After incubation, the inhibition zones around the disks were measured in millimetres. All assays were performed in triplicate, and the results were expressed as means ± standard deviation (SD) of three independent experiments.

1.5.1. Minimal inhibitory concentration (MIC) determination: MIC values of DIMBOA for *Staphylococcus aureus* ATCC 25923 and 22 methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates were determined the method previously described (Erdemoglu et al., 2007). DIMBOA sample was serially diluted into concentrations ranging from 2.0 to 0.0625 mg mL⁻¹. The MIC was defined as the lowest concentration of DIMBOA that inhibits bacterial growth after 18h of incubation at 35°C. All experiments were performed in triplicate.

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Table S1. The antioxidant activity of DIMBOA measured in DPPH, ABTS and FRAP methods.

	ABTS method	DPPH method	FRAP method
DIMBOA [$\mu\text{mol TROLOX mL}^{-1}$]	3.54	1.56	0.33

Figure S1. TLC chromatograms of the resulting DIMBOA sample: A – analysed under UV light of 254 nm (prior to visualization), B – visualized with 3% FeCl₃ in 2% HCl solution, analysed in daylight, C – visualized with DPPH methanolic solution, analysed in daylight.

Figure S2. HPLC chromatogram of the DIMBOA sample, recorded at a wavelength of $\lambda = 280$ nm.

Figure S3. The HSQC correlation of the resulting DIMBOA sample.

Figure S4. Graph plotting DPPH radical-scavenging activity (%) versus the concentration of DIMBOA sample.

Figure S5. The antioxidant activity (FRAP) of the isolated DIMBOA sample.

Figure S6. In vitro susceptibilities of: *Staphylococcus aureus* ATCC 25923 (A), *Staphylococcus aureus* MRSA K326 (B), *Candida albicans* ATCC 90028 (C) and *Pseudomonas aeruginosa* ATCC 27853 (D) to DIMBOA (left disc) vs. control (right disc).