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A new Schiazolyl Schiff base: Antibacterial and antifungal effects and modulation of oxidative stress in vitro on human endothelial cells

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ABSTRACT: Schiff bases (SBs) are chemical compounds displaying a significant pharmacological potential. They are able to modulate the activity of many enzymes involved in metabolism and are found among antibacterial, antifungal, anti-inflammatory, antioxidant, and antiproliferative drugs. A new thiazolyl-triazole SB was obtained and characterized by elemental and spectral analysis. The antibacterial and antifungal ability of the SB was evaluated against Gram-positive and Gram-negative bacteria and against three Candida strains. SB showed good antibacterial activity against L. monocytogenes and P. aeruginosa; it was two times more active than ciprofloxacin. Anti-Candida activity was twofold higher compared with that of fluconazole. The effect of the SB on cell viability was evaluated by colorimetric measurement on cell cultures exposed to various SB concentrations. The ability of the SB to modulate oxidative stress was assessed by measuring MDA, TNF-α, SOD1, COX2, and NOS2 levels in vitro, using human endothelial cell cultures exposed to a glucose-enriched medium. SB did not change the morphology of the cells. Experimental findings indicate that the newly synthetized Schiff base has antibacterial activity, especially on the Gram-negative P. aeruginosa, and antifungal activity. SB also showed antioxidant and anti-inflammatory activities.

Keywords**:** *Schiazolyl Schiff, Antibacterial, modulation, oxidative stress, endothelial cells*

INTRODUCTION

Aerobic organisms have antioxidant defense systems against reactive oxygen species- (ROS-) induced damage produced in various stress conditions. ROS are also involved in the innate immune system and have an important role in the inflammatory response; they attract cells, by chemotaxis, to the inflammation site. Nitric oxide (NO) is another important intracellular and intercellular signaling molecule involved in the regulation of multiple physiological and pathophysiological mechanisms. It acts as a biological modulator. NO is able to regulate vascular tone and can function as a host defense effector. Also, it can act as a cytotoxic agent in inflammatory disorders. NO synthase (NOS) enzyme family catalyzes NO production. Inhibition of inducible NOS (iNOS) might be beneficial in the course of treatment of certain inflammatory diseases [1]. The reactions between NO and ROS, such as superoxide radicals ($O_2^{\cdot-}$), lead to the production of a potent prooxidant radical (peroxynitrite), thus inducing endothelial and mitochondrial dysfunction. The major cellular defense against peroxide and peroxynitrite radicals are the superoxide dismutases (SODs) that catalyzes the transformation of peroxide radicals into hydrogen peroxide (H_2O_2), which is further transformed by catalase into water and molecular oxygen. Also, SODs play an

important role in preventing peroxynitrite formation [2]. All isoforms have in their catalytic site a transition metal, such as copper and manganese [3].

Schiff bases (SBs) are chemical structures that have a significant pharmacological potential. SBs contain an azomethine group obtained through the condensation of primary amines with carbonyl compounds [10]. The pharmacophore potential of this group is due to their ability to form complex compounds with bivalent and trivalent metals located in the active center of numerous enzymes involved in metabolic reactions. The relationship between a chemical structure and biological activity (SAR) underlines the importance of the azomethine group for the synthesis of new compounds with antibacterial, antifungal, and even antitumor activities [11–13].

Multiple studies showed the ability of SBs to act as antiproliferative and antitumoral agents [14–16]. The azomethine pharmacophore is used in developing new bioactive molecules [17]. The discovery of selective cytotoxic drugs influenced oncological therapy. However, completely satisfactory answers for metastasis onset have not yet been found. Due to the increased prevalence of neoplasia and to the existence of various cellular tumor lines resistant to cytotoxic therapy, the research of new active agents is justified [18, 19].

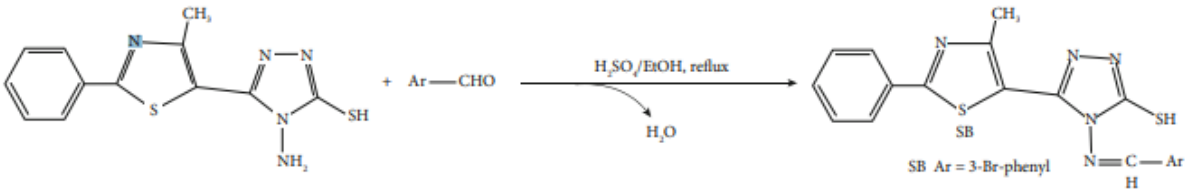
The current study is aimed at testing a newly synthetized heterocyclic SB in terms of antimicrobial activity against Gram-positive and Gram-negative bacteria and antifungal effects against *Candida* strains [20, 21], as well as to evaluate the biocompatibility of the SB *in vitro* on human endothelial cells and the ability of this SB to modulate oxidative stress, by assessing enzymes involved in cellular antioxidant defense.

2. Materials and Methods

2.1. Synthesis of the Schiff Base

All reagents and solvents used were purchased from Sigma-Aldrich and were used without further purification. The starting compound was previously reported and was synthesized by us according to methodologies described in the literature [21].

The synthesis of Schiff base (SB) 4-(3-bromobenzylideneamino)-5-(4-methyl-2-phenylthiazol-5-yl)-4H-1,2,4-triazole-3-thiol was made using a general procedure (Scheme 1) [21]. 2 mmol (0.578 g) of 4-amino-5-(4-methyl-2-phenylthiazol-5-yl)-4H-1,2,4-triazole-3-thiol was suspended in 10 mL of absolute ethanol. The resulting suspension was added with an alcoholic solution of 2 mmol of 3-bromobenzaldehyde in 5 mL of absolute ethanol and 2-3 drops of concentrated H_2SO_4, as a catalyst. The reaction mixture was refluxed for 6 h. The obtained precipitate was filtered hot and washed with absolute ethanol, and then, it was dried and recrystallized from dimethyl sulfoxide (DMSO).



Scheme 1: Synthesis of the Schiff base (SB).

2.2. In Vitro Antibacterial and Antifungal Screening

2.2.1. Preparation of Sample Solution

SB was dissolved in DMSO, at a final concentration of 100 μg/mL. Sample solution was stored at 4°C [22, 23].

2.2.2. Inhibition Zone Diameter Measurements

Antimicrobial activity was tested *in vitro* using the agar disk diffusion method through the measurement of the inhibition zone diameters. Agar plates were inoculated with a standardized inoculum of the test microorganisms: two Gram-negative bacterial strains—*Salmonella enteritidis* ATCC 14028 and *Escherichia coli* ATCC 25922, two Gram-positive bacterial strains—*Listeria monocytogenes* ATCC 19115 and *Staphylococcus aureus* ATCC 49444, and a fungal strain—*Candida albicans* ATCC 10231. Petri plates with Mueller Hinton Agar (20.0 mL) were used for all bacterial tests. Mueller-Hinton medium supplemented with 2% glucose (providing adequate growth of yeasts) and 0.5 g/L methylene blue (providing a better definition of the inhibition zone diameter) was used for antifungal testing. Each paper disk was impregnated with 10 μL of solution (100 μg compound/disk). The filter paper disks were placed on Petri dishes previously seeded "in layer" with the tested bacterial strain inoculums. Then, Petri dishes were maintained at room temperature to ensure the equal diffusion of the compound in the medium, and afterwards, the dishes were incubated at 37°C for 24 hours. Inhibition zones were measured after 24 hours of incubation. Assessment of the antimicrobial effect was realized by measuring the diameter of the growth inhibition zone. Ciprofloxacin (10 μg/well) and fluconazole (25 μg/well) were used as *standard* antibacterial and antifungal *drugs.* DMSO was used for comparison, as a negative control, for all experiments, and it did not inhibit the growth of microorganisms (diameter = 6 mm). The clear halos with a diameter larger than 10 mm were considered positive results [22, 23]. Tests were performed in triplicate, and values are presented as the average value ± standard deviation.

3.Result

3.1. Chemical Characterization of the SB

The SB structure was confirmed by elemental analysis and on the basis of its mass spectrum (MS), infrared spectrum (IR), and nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra [21].

4-(3-Bromobenzylideneamino)-5-(4-methyl-2-phenylthiazol-5-yl)-4H-1,2,4-triazole-3-thiol. Yield 80.3% (0.366 g); m.p. 268-270°C; light yellow powder; Anal. Calcd for $C_{19}H_{14}BrN_5S_2$ (456.38): C, 49.89; H, 3.06; N, 15.33; S, 14.02; Found: C, 50.1; H, 3.07; N, 15.33; S, 14.07; IR (ATR, cm^{-1}): 3104 (*v* $NH_{triazole}$), 1618 (*v* -N=CH-), 1274 (*v* C=S); 1055 (*v* C-Br); ^{1}H NMR (500 MHz, DMSO-d_6, *δ*/ppm): 14.18 (s, 1H, NH), 9.52 (s, 1H, -N=CH-), 7.97–8.06 (d, 2H, ArH), 7.92 (s, 1H, ArH), 7.77 (d, 1H, ArH), 7.59 (d, 1H, ArH), 7.47-7.54 (m, 4H, ArH), 2.41 (s, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6, *δ*/ppm): 170.12 (C=S), 159.15 (C), 157.66 (CH=N), 153.81 (C), 151.07 (C), 143.96 (C), 135.16 (C), 134.51 (C), 131.21 (CH), 130.93 (2CH), 130.29 (CH), 129.29 (2CH), 128.94 (CH), 128.68 (C), 127.36 (CH), 127.14 (CH), 15.92 (CH_3); MS (EI, 70 eV) *m*/*z* (%): 457 (M + 1).

3.2. Antimicrobial Activity

Results obtained by measuring the diameters of growth inhibition zones of the tested microorganisms, compared to ciprofloxacin and fluconazole, used as standard reference drugs, are presented in Table 1.

Table 1: Inhibition zone diameters on tested microorganisms

Samples	Diameter of the inhibition zone (mm)				
	SA	LM	EC	ST	CA
Schiff base	14	14	14	18	18
Ciprofloxacin	28	18	27	22	—
Fluconazole	—	—	—	—	25

SA: Staphylococcus aureus; LM: Listeria monocytogenes; EC: Escherichia coli; ST: Salmonella typhimurium; CA: Candida albicans

MIC, MBC, and MFC values of the new compound are presented in Tables 2 and 3. The results showed that MIC values ranged from 1.95 (*Listeria monocytogenes*) to 62.5 µg/mL, MBC values were between 3.9 and 125 µg/mL, and MFC scores ranged between 62.5 and 125 µg/mL.

Table 2: Minimum inhibitory concentrations (MIC)

Samples	Minimum inhibitory concentration (MIC (µg/mL))					
	SA	LM	PA	ST	CA (ATCC 10231)	CA (ATCC 18804)
Schiff base	31.25	1.95	1.95	62.5	62.5	31.25
Ciprofloxacin	1.95	3.9	3.9	0.97	—	—
Fluconazole	—	—	—	—	62.5	62.5

SA: *Staphylococcus aureus*; LM: *Listeria monocytogenes*; PA: *Pseudomonas aeruginosa*; ST: *Salmonella typhimurium*; CA: *Candida albicans*.

Table 3: Minimum bactericidal (MBC) and minimum fungicidal concentrations (MFC).

Samples	MBC (μg/mL)				MFC (μg/mL)		
	SA	LM	PA	ST	CA (ATCC 10231)	CA (ATCC 18804)	CK (ATCC 6258)
Schiff base	62.5	3.9	3.9	125	125	62.5	62.5
Ciprofloxacin	3.9	7.8	7.8	1.95	—	—	—
Fluconazole	—	—	—	—	125	125	125

SA: *Staphylococcus aureus*; LM: *Listeria monocytogenes*; PA: *Pseudomonas aeruginosa*; ST: *Salmonella typhimurium*; CA: *Candida albicans*; CK: *Candida krusei.*

3.3. In Vitro Antioxidant Capacity

The antioxidant capacity of the SB was determined by the DPPH bleaching method, and BHT and trolox were used as positive controls. The results are displayed in Table 4. The new compound showed a very low IC_{50} value (16.10 μg/mL), similar to that of BHT (16.39 μg/mL).

Table 4: Antioxidant capacity using the DPPH method.

Samples	IC50 (μg/mL)
Schiff base	16.10 ± 1.2
BHT	16.39 ± 0.9
Trolox	11.98 ± 0.4

3.4. Cell Viability

SB did not lead to significant changes in HUVEC viability for doses lower than 0.1 μg/mL (Figure 1). Higher concentrations led to a dose-dependent viability decrease, compared with control.

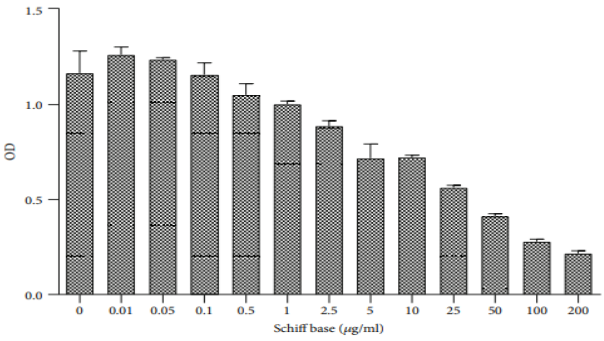


Figure 1: Cell viability testing. Schiff base (SB) was tested for multiple concentrations (0.01-200 μg/mL). Cell viability is presented as OD 540 nm (mean values ± standard deviation at 540 nm, n = 3).

3.5. Assessment of the Ability of the SB to Modulate Inflammatory Response and Oxidative Stress on HUVECs

Lipid peroxidation level (MDA), the ability to modulate inflammatory response (TNF-*α*, COX2), and the activity of enzymes involved in the prooxidant/antioxidant equilibrium (SOD1, NOS2) were appreciated. The ability of the SB to modulate oxidative stress was tested *in vitro* on HUVECs, using a glucose-enriched medium [36–38]. A SB concentration of 0.001 *μ*g/mL was used for all experiments.

The effect of the newly synthetized compound on lipid peroxidation (MDA level) was assessed. SB administration decreased the MDA level compared with both control and glucose-enriched medium, thus reducing the lipid peroxidation in endothelial cells (Figure 2).

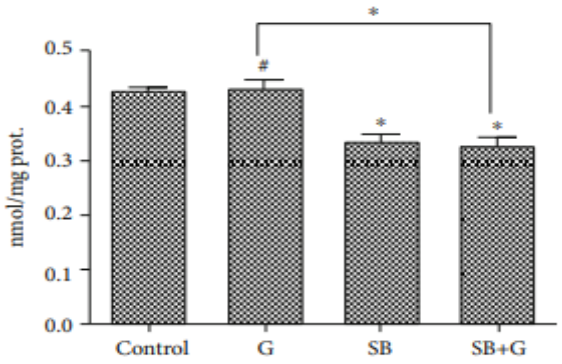


Figure 2: Lipid peroxidation levels (MDA) in endothelial cells exposed to medium (control), glucose (G), Schiff base (SB), and combination treatment (SB+G). Each bar represents the mean ± standard deviation (n = 3). # Not significant. ∗p < 0:05.

The TNF-*α* level was quantified through ELISA for the same SB concentration (Figure 3). Glucose-enriched medium slightly increased the TNF-*α* level. SB also increased the TNF-*α* level both alone and in combination with glucose.

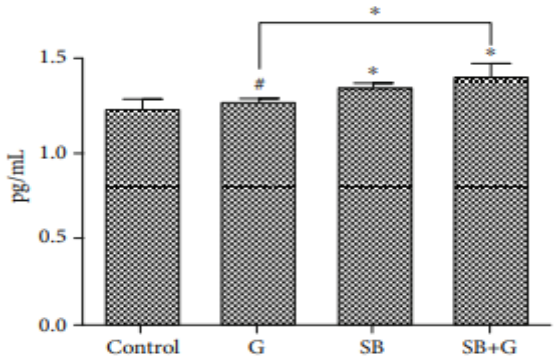


Figure 3: TNF-α levels in endothelial cells exposed to medium (control), glucose (G), Schiff base (SB), and combination treatment (SB+G). Each bar represents the mean ± standard deviation (n = 3). # Not significant. ∗p < 0:05.

The same SB concentration (0.001 *μ*g/mL) was used to further test its effect on the protein level of the enzymes involved in the oxidant/antioxidant equilibrium and in the inflammatory response (SOD1, NOS2, and COX2).
An inflammatory marker (COX2) and antioxidant enzyme (constitutive SOD1 and inducible NOS2) expression was quantified by Western Blot (Figure 4).

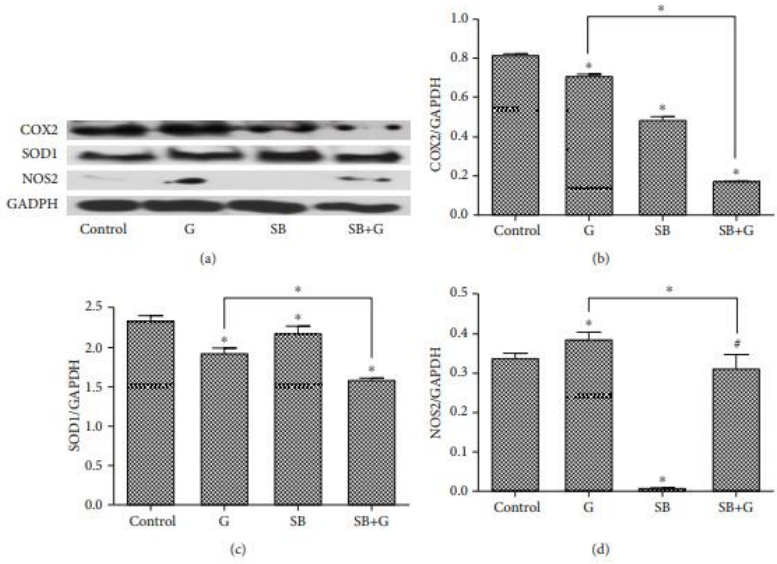


Figure 4: Protein levels of COX2, SOD1, and NOS2 in endothelial cells exposed to medium (control), glucose (G), Schiff base (SB), and combination treatment (SB+G). Comparative Western blot images showing expressions of COX2, SOD1, and NOS2 in HUVECs (b, c, d). Image analysis of Western blot bands (a) was performed by densitometry; results were normalized to GAPDH. Each bar represents the mean ± standard deviation (n = 3). # Not significant. ∗p < 0:05.

COX2, an inflammatory marker, significantly decreased after both glucose and SB treatments, compared to control. Combined exposure (SB+G) strongly decreased the protein level of COX2 (Figure 4(b)). This finding is consistent with MDA levels and may be due to the antioxidant effect of the SB in this experimental setting. Interestingly, it is not consistent with TNF-*α*, a fact which might be explained by a different mechanism than oxidative stress that triggers an increase of TNF-*α*. Exposure to glucose-enriched medium significantly decreased SOD1. SB slightly decreased SOD1 activity compared with the control group, but SOD1 activity was maintained at a significantly higher level, compared with glucose e ($p < 0:05$). Combination (SB+G) treatment significantly decreased SOD1 compared with both glucose and control (Figure 4(c)). Exposure to glucose increased significantly NOS2. The SB drastically decreased the NOS2 level compared with both the control and glucose groups (Figure 4(d)).

Correlation analysis, using Spearman's coefficient for rank correlation (Table 5), revealed statistically significant positive correlations between MDA and enzyme (COX2, SOD1, and NOS2) levels. On the other hand, the TNF-*α* level negatively correlates with both MDA and all enzymes measured.

Table 5: Spearman's coefficient of rank correlation (rho) between the oxidative stress and inflammation markers in HUVECs.

	MDA	TNF-α	COX2	SOD1	NOS2
MDA	1.00	-0.776∗∗	0.699∗	0.462	0.595∗
TNF-α	-	1.00	-0.818∗∗	-0.566	-0.455
COX2	-	-	1.00	0.755∗∗	0.431
SOD1	-		-	1.00	-0.144
NOS2	-	-	-	-	1.00

∗p < 0:05, ∗∗p < 0:01

Cell morphology does not seem to be affected by exposure to the Schiff base compared to control. When exposed to high-glucose concentration, cells had a tendency to conglomerate and to form multilayered spherical bodies, with alteration of the actin filament disposition. The aspect of the cells receiving combination treatment was similar to those of controls (Figure 5).

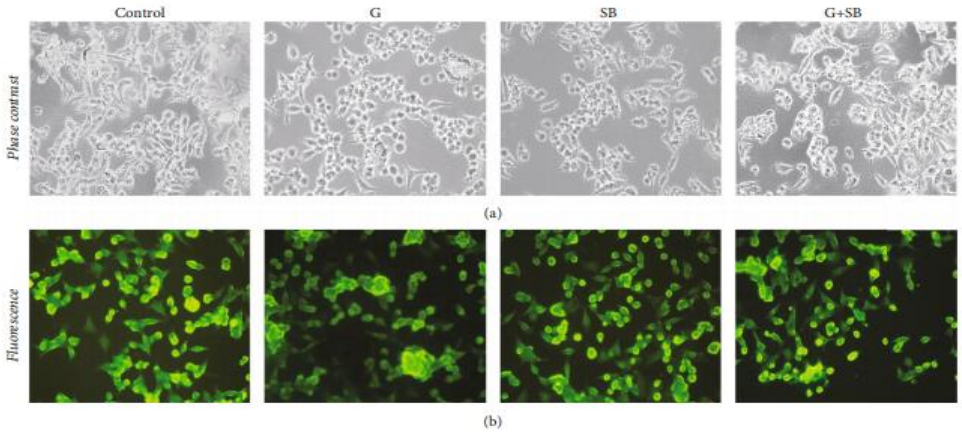


Figure 5: Images of HUVECs treated with medium (control), glucose (G), Schiff base (SB), and combination treatment (SB+G), stained with phalloidin-FITC; the same microscopic field is presented as phase contrast images (a) and fluorescence images (b) for comparison (pictures taken through an Olympus BX inverted microscope, original magnification 20x).

4. Discussions

The structure of the Schiff base was established by elemental analysis and on the basis of its mass spectrum (MS), infrared spectrum (IR), and nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra. The results of the C, H, N, S quantitative elemental analysis were in agreement with the calculated values, within ±0.4% of the theoretical values. The spectral data confirmed the formation of the SB. The recorded mass spectrum revealed the correct molecular ion peak (M + 1), as suggested by the molecular formula. The absence of the NH_2 asymmetric and symmetric stretching vibrations at 3281 cm^{-1} and 3186 cm^{-1}, and the presence of N=CH stretch absorption bands at 1618 cm^{-1} in the IR spectrum of the final compound provided strong evidences for the formation of the SB. The ^{1}H-NMR spectrum of the starting compound was recorded a signal characteristic for the amino protons, as a

singlet, at 5.73 ppm. The absence of this signal from the ^{1}H-NMR spectrum of the newly synthesized compound and the presence of a singlet characteristic to the N=CH proton at 9.52 ppm further confirmed the condensation between the 4-amino-5-(4-methyl-2-phenylthiazol-5-yl)-4*H*-1,2,4-triazole-3-thiol and the 3-bromo-phenyl-carbaldehyde. The ^{13}C-NMR spectrum of the newly synthesized compound was consistent with the proposed structure.

The aim of the present study was to evaluate the antibacterial and antifungal activity of a new SB as well as its ability to modulate oxidative stress.

The new thiazolyl SB exerted moderate to good antibacterial activity against tested strains (Tables 1–3). The inhibition of bacterial growth was more pronounced in Gram-negative bacteria, especially in *Pseudomonas aeruginosa* strain, where the SB showed better activity compared with ciprofloxacin, used as the reference drug. Regarding antifungal activity, the compound showed a better anti-*Candida* effect than fluconazole, used as the reference drug. Previous studies showed that SBs have the ability to modulate oxidative stress [17, 39]. This ability can be exploited in order to use them as antibacterial drugs and/or as potential oxidative stress modulators in medicine. The SB was tested on endothelial cells exposed to a glucose-enriched environment.

High-carbohydrate intake, impaired glucose tolerance, and diabetes mellitus lead to hyperglycemia and chronic inflammatory status. Endothelial lesions are often involved in the pathology of these conditions [40]. During inflammatory episodes, such as response to injury, nitric oxide (NO) is released in order to modulate vascular tone. Since glycocalyx plays an important role in transducing the fluid stress to the cytoskeleton of the endothelial cells, vasodilator substance production is stimulated [40–42]. High-glucose concentration increases oxidative stress and influences the structure of the cytoskeleton. Exposure to high-glucose hyperosmolar medium induces, using an AQP1-dependent mechanism, remodeling of the F-actin and cytoskeleton [43]. Our results are consistent with these findings (Figure 5). A high-glucose level led to mitochondrial dysfunction and increased production of ROS [44, 45].

A glucose-enriched environment also triggers the release of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-*α*), by the cells involved in immune reactions [46, 47], along with other proinflammatory molecules, such as CRP, interleukin 6, intercellular adhesion molecule 1, and VCAM-1. In diabetic patients, TNF-*α* was related with an atherogenic profile and with vascular complications [48]. A similar effect was obtained in our study, where higher levels of TNF-*α* were observed after hyperglycemia exposure. This effect was also seen after SB treatment and was augmented by the combined SB and high-glucose concentration. However, TNF-*α* production was negatively correlated with MDA and antioxidant enzymes (Table 5). This suggests that the increased TNF-*α* was not produced through enhanced oxidative stress, but through a different mechanism. Its clarification requires further studies. Since TNF-*α* acts as a promoter of leucocyte adhesion to the endothelium, the SB might be beneficial as antimicrobial, local immune response, and oxidative status modulator in the treatment of infectious diseases.

The results obtained by the DPPH study showed that the SB exhibited antioxidant activity. The low IC_{50} value, similar to the positive control (BHT), reflects a strong antioxidant activity *in vitro*. The new compound showed radical scavenging activity according to the DPPH method, the presence of the -SH group being probably responsible of the radical scavenging activity [49–51]. The effect of the SB on the oxidative stress was also tested *in vitro* on cell cultures (HUVECs), by assessing the MDA level, a marker of lipid peroxidation and the expression of two enzymes involved in the oxidative equilibrium (SOD1 and NOS2). The results showed that, at the tested concentration (0.001 *μ*g/mL), SB decreased lipid peroxidation (MDA) and the protein level of certain enzymes involved in the modulation of oxidative stress and inflammatory response (COX2 and NOS2). These changes are consistent with the DPPH result and suggest an anti-inflammatory effect of the tested SB, mostly by interfering with the prooxidant mediators.

The ability of the SB, in low concentrations, to decrease lipid peroxidation, might be explained by its capacity to form complexes with the bivalent and trivalent metal ions located in the active center of the enzymes involved in the onset of the oxidative stress or in the scavenging of the prooxidant molecules [52–57]. The antioxidant effect on the human cells (Figures 2 and 4) is also consistent with the absence of morphological changes of the cells observed in the present study (Figure 5).

Considering antibacterial activity, especially against *Pseudomonas aeruginosa*, the decrease of the NOS2 protein level in HUVECs after SB exposure, it might be possible that the synthesis of NO by bacteria could also be reduced. One of the many proposed roles of NO in bacteria is to help protect the bacteria from host cell antibiotic-induced oxidative stress; therefore, the inhibition of bacterial nitric oxide synthase has been identified as a promising antibacterial strategy, especially for resistant bacteria [58].

Nitric oxide synthase (NOS) inhibitor NO-donating drugs were reported to inhibit IL-1β production, modulate PGE_2 production, and protect against apoptosis in human endothelial cells and human monocytes [59]. In type 2 diabetes, hyperglycemia stimulates endothelial cell migration in the retina, leading to retina neoangiogenesis and visual impairment by CXC receptor-4 stimulation and activation of the PI3K/Akt/eNOS signaling pathway. Therefore, SB modulation of the NOS2 might be beneficial for the endothelial dysfunction in hyperglycemia [60, 61].

Recent studies showed that the antibacterial and antifungal activity in general and antibiofilm activity of some newly identified classes seem to correlate with their ability to induce ROS synthesis [5]. The SB showed an anti-*Candida* effect, with a twofold increased activity compared with the consecrated antifungal fluconazole (Tables 2 and 3). Also, the results showed that SB reduced the SOD1 level and increased the activity of the proinflammatory cytokine (TNF-α). The antifungal effect could also be explained by the ability of the tested SB to form complexes between the azomethine group and the metal from the active center of the enzymes and also by its capacity to induce ROS production, similar with some antifungal azoles (e.g., miconazole) [5].

Additional studies are needed in order to clarify the effect of such compounds as SB and their role as adjuvant antioxidant, antimicrobial, and local immune response modulators (TNF-α) in the treatment of infectious diseases.

5. Conculsions

The new Schiff base exhibited antibacterial effects on both Gram-positive and Gram-negative bacteria, as well as antifungal activity against *Candida albicans*. The results of the present study show that the new SB plays a role in the prooxidant/antioxidant equilibrium. In the tested dose, SB does not change endothelial cell morphology, has an antioxidant effect, as demonstrated by the DPPH test, decreased lipid peroxidation (MDA), and decreased the inducible NOS2 level. Therefore, it can be considered a potential candidate with promising antioxidant properties that may be used as an adjuvant therapy in diseases caused by excessive free radical production. The decrease in COX2 and NOS2 levels also might suggest an anti-inflammatory action. A possible mechanism for the antibacterial activity on Gram-negative bacilli could include the decrease of the bacterial NOS level and the formation of complexes with metals located in the active center of certain bacterial enzymes. Also, the SB might potentially act as an antifungal agent, through ROS production in fungal biofilm cells. Its clarification requires further studies.

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