Research Article

Imidazole and Azo-Based Schiff Bases Ligands as Highly Active Antifungal and Antioxidant Components

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We describe, herein, the synthesis, full characterization, and optical properties of four different ligands L1-L4 which associate an azo group, an imidazole unit, and a Schiff base fragment. The UV-visible absorption bands are characteristic of $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions with an additional charge transfer between the azobenzene moiety and the imino group. Finally the determination of MIC$\textsubscript{80}$ values against pathogenic fungi such as $S$. apiospermum, $A$. fumigatus, and $C$. albicans revealed that these ligands have effective antifungal properties with highest activities (MIC$\textsubscript{80}$) on $C$. albicans for the azole based ligands L1-L3. DPPH radical scavenging of the studied ligands was also tested.

1. Introduction

Imidazole derivatives constitute an important class of heterocycles being the core fragment of different natural products and biological systems. They occupy a unique place in the field of medicinal chemistry owing to their potent biological activity [1]. They are well known to possess many pharmacological properties and to play a very important role in diverse biochemical processes [2, 3]. Many substituted imidazoles display wide range of biological applications such as antiprotozoal, antifungal, and antihypertensive agents [4–6]. In addition, imidazoles constitute an entire or partial part of the binding sites of various transition metal ions such as Ni$^{2+}$, Cu$^{2+}$, or Zn$^{2+}$ in a large number of metalloproteins and have thus been utilized as effective ligands to chelate these transition metals. For example, imidazole based ligands have been used to afford tetranuclear copper(II) and nickel(II) metal complexes, [7] copper(II) or zinc(II) coordination polymers, [8] 1D coordination chains, [9] tripod metal complexes, [10] and many other complexes used for treatment of Wilson and Menkes diseases [11]. On the other hand, azobenzene [12, 13] and its numerous derivatives are currently used as dyes and represent 60–70 % of the world production of “absorbing molecules” [14]. They are known to possess a unique photoisomerization process which leads to two stable cis and trans geometries. The trans-to-cis photoisomerization occurs by UV light irradiation while the reverse cis-to-trans takes place under blue light irradiation [15, 16]. This process is the key principle of various applications such as chemosensors, [17] optical storage media, [18] optical switches, [19, 20] nonlinear optics, [21] as well as trigger for protein folding [22]. Furthermore, azo compounds have also a variety of biological activities including antibacterial, [23] antifungal, [24] pesticidal, [25] antiviral, and anti-inflammatory activities [26]. Schiff bases ligands with chelating abilities have been recognized as privileged ligands to form stable complexes with a large variety of transition metals [27]. They have been used, for example, in metallo-ligand self-assembly to generate discrete supramolecular assemblies such as metallo-supramolecular helicates, [28–30] cages [31], and capsules, [32] and have also been used with a variety of transition metals in catalysis [33, 34]. In addition, Schiff base derivatives
showed a variety of biological and pharmacological activities as antimicrobial, [35] antidepressant, [36] cytotoxic, [37] analgesic, [38] anti-HIV, [39] antileishmanial, [40] anticonvulsant, [41] fungicides, [42] anti-inflammatory [43], and anticancer [44]. In this present investigation work, we are interested by the combination of the azo group, the imidazole unit and the Schiff base fragment. Thus, we report herein on the synthesis, characterization, and optical properties of four different Schiff bases ligands L1-L4. We also report the possible use of such systems in biological applications, in particular due to their antifungal properties and antioxidant activities. Note that the X-ray crystal structure of ligand L2 has been recently described by us [45].

2. Results and Discussions

2.1. Synthesis. The synthesis of the Schiff bases was started by the preparation of the azo-based salicylaldehyde (1a-c) [46] by a coupling reaction between 2-hydroxybenzaldehyde and the substituted anilines as it was previously reported in the literature (Scheme 1). The resulting salicylaldehydes (1a-c) were subjected to condensation reaction with one equivalent of N-(3-Aminopropyl)imidazole or one equivalent of propylamine to afford in good yields the Schiff bases L1-L4.

2.2. UV-Visible Absorption Spectroscopy. Figure 1 shows the normalized UV-visible absorption spectra of the Schiff bases ligands L1-L4 that were recorded in dichloromethane solution (~2·10⁻⁵M) at room temperature. Ligands L1-L3 exhibit two strong electronic absorption bands at around λ = 275 nm and 350 nm while the two absorption bands of L4 are blue shifted to λ = 262 nm and 335 nm. These absorption bands are assigned to π → π* and n → π* transitions in the azobenzene moiety and the imidazole rings for L1-L3 and in the azobenzene fragment for L4. In the visible region the four ligands show and additional broad absorption band which might be assigned to a charge transfer band between the azobenzene moiety and the imino group.
than the one of miconazole. Once again, B (8 μg/mL) exerted significant antifungal activities (1.5 and 3 μg/mL) for the three ligands L1, L2, and L3 whose inhibition zone diameters of 21, 19, and 16.5 mm, respectively, were superior to the one of the reference antifungal amphotericin B. For A. fumigatus, a so-called nsolid medium, we noticed certain discordance for few results between the solid and liquid media. For example, it was the case for L4 against A. fumigatus and S. apiospermum. But we can notice that L4 has a structural formula very different from the other three ligands L1-L3. In fact, the first three ligands contain an imidazole ring which is absent in L4. As the disk diffusion method consists of test compounds in solid agar medium, we may hypothesize that steric hindrance and/or lipophilic character of the molecules alter their diffusion in such medium. All these effects favor the fungal growth. These results strengthen the interest of the broth microdilution method for a large scale evaluation of compounds, disk diffusion method being suitable for a rapid antifungal screening.

The weaker antifungal activities of L4 observed against C. albicans may be explained by the lack of azole group in its formula. This group found in antifungals used in therapy is known to interact with the lanosterol 14α-demethylase (or CYP51A1) which is implicated in the biosynthesis of the ergosterol, important component of the fungal plasmatic membrane. Curiously in liquid medium, L4 exerted a good activity against the other fungi S. apiospermum and A. fumigatus suggesting antifungal targets different from the ergosterol synthesis. As related in previous paper concerning

### Table 1: Antifungal activities determined on solid medium and expressed by the diameter of the growth inhibition zone (mm).

| Compounds tested | S. apiospermum | Fungi | A. fumigatus | C. albicans |
|------------------|----------------|-------|--------------|-------------|
| L1               | 32             | 28    | 17           |
| L2               | 19             | 13    | 14.5         |
| L3               | 21             | 33    | 14.5         |
| L4               | 16.5           | 12    | 12.5         |
| Miconazole       | 28.5           | 18.5  | 18.5         |

### Table 2: Antifungal activities determined in liquid medium and expressed by "MIC<sub>80</sub> in μg/mL".

| Compounds tested | S. apiospermum | Fungi | A. fumigatus | C. albicans |
|------------------|----------------|-------|--------------|-------------|
| L1               | 4              | 1.5   | 0.8          |
| L2               | 4              | 12    | 0.1          |
| L3               | 4              | 3     | 0.2          |
| L4               | 4              | 1.5   | 3            |
| Amphotericin B   | 4              | 8     | 1            |

a: MIC<sub>80</sub> corresponds to the minimum inhibitory concentration of the compound that inhibits 80% of the fungal growth.

2.3. Biological Activities

2.3.1. Antifungal Activity. Antifungal activities of the ligands L1 to L4 were evaluated on three important fungal species pathogenic for humans: Candida albicans, Aspergillus fumigatus, and Scedosporium apiospermum, especially implicated in patients with cystic fibrosis [47]. First, for a rapid antifungal screening, we have used a disc diffusion method on solid medium. The results reported in Table 1 indicate that, concerning C. albicans, we notice that the ligands L1, L2, L3, and L4 exhibit an antifungal activity which is lower than the one of the reference antifungal miconazole, while ligand L1 shows an antifungal activity with the same order of magnitude to the one of the reference miconazole. Against S. apiospermum all the ligands were active; three of them showed activities according to this ascending order L4 < L2 < L3 but lower than the one of miconazole. Once again, L1 had a growth inhibition zone diameter (32 mm) superior to the one of miconazole (28.5 mm). The best antifungal activities against A. fumigatus were found for two ligands L1 and L3 whose inhibition zone diameters of 28 and 33 mm, respectively, were much superior to the one of miconazole (16.5 mm). A weak activity on the growth of A. fumigatus was observed with L2 and L3. In all the tested fungi, it appears clearly that ligand L1 presents the best antifungal results, which clearly indicate that any additional substituent on the phenyl ring is not beneficial for the antifungal activity.

In liquid medium, the antifungal activities measured in triplicate were confirmed (Table 2). Against S. apiospermum, the four ligands had the same MIC<sub>80</sub> (4 μg/mL) which was equal to the one of the reference antifungal amphotericin B. For A. fumigatus, as observed on solid medium, L1 and L3 exerted significant antifungal activities (1.5 and 3 μg/mL, respectively) which were superior to the one of amphotericin B (8 μg/mL). It was also the case for L4 (1.5 μg/mL) in contrary to the result obtained on solid medium. However, in correlation with the solid medium, L2 had a weaker effect on the growth of A. fumigatus in liquid medium. High antifungal activities were detected against C. albicans for three ligands in opposite ascending order of the MIC<sub>80</sub> values: L1 (0.8 μg/mL) < L3 (0.2 μg/mL) < L2 (0.1 μg/mL). All of these values were lower than the MIC<sub>80</sub> value (1 μg/mL) of amphotericin B. L4 had a weaker antifungal effect with a MIC<sub>80</sub> value three times more the value of amphotericin B. As for solid media L1 had an excellent antifungal activity, although it is less than L2 and L3. The fact that L4 shows a weak effect clearly indicates that the presence of the imidazole unit is of great importance for such biological activities.

As previously described for thiosemicarbazone ligands, [48] we noticed certain discordance for few results between the solid and liquid media. For example, it was the case for L4 against A. fumigatus and S. apiospermum. But we can notice that L4 has a structural formula very different from the other three ligands L1-L3. In fact, the first three ligands contain an imidazole ring which is absent in ligand L4. As the disk diffusion method consists of test compounds in solid agar medium, we may hypothesize that steric hindrance and/or lipophilic character of the molecules alter their diffusion in such medium. All these effects favor the fungal growth. These results strengthen the interest of the broth microdilution method for a large scale evaluation of compounds, disk diffusion method being suitable for a rapid antifungal screening.

The weaker antifungal activities of L4 observed against C. albicans may be explained by the lack of azole group in its formula. This group found in antifungals used in therapy is known to interact with the lanosterol 14α-demethylase (or CYP51A1) which is implicated in the biosynthesis of the ergosterol, important component of the fungal plasmatic membrane. Curiously in liquid medium, L4 exerted a good activity against the other fungi S. apiospermum and A. fumigatus suggesting antifungal targets different from the ergosterol synthesis. As related in previous paper concerning
thiosemicarbazones, [48] there is the possibility that the activity may be linked to the relative lipophilicity of the molecule that makes the penetration in the fungal cell through the cell membrane and finally its destabilization easier. More generally for all the molecules tested, we may hypothesize that the capacity of these heterocyclic ligands to chelate metal ions which are essentials in numerous biological processes may impact the physiology of the fungi [49, 50].

Considering the need for new azole antifungals able to overcome the increase of life-threatening fungal infections and the emergence of resistant fungal isolates, our newazole ligands may be worthy of interest to develop new therapeutic agents.

2.3.2. Antioxidant Activity. Antioxidants are important bio-active species since they are capable of capturing free radicals responsible for many diseases such as cancer [51]. Due to the mobility of a proton (OH, NH, . . .) in their structure, they act by direct scavenging of reactive oxygen species (ROS).

DPPH (2,2 Di phenyl-1-picyryl hydrazyl) radical scavenging capacities of the Schiff bases L1-L4 were evaluated using UV-visible spectroscopy at different concentrations and compared to that of the well-known antioxidant ascorbic acid. Data summarized in Table 3 show that all the tested compounds exhibit an antioxidant activity although it is less than the ascorbic acid. This promising activity is probably due to the hydroxyl group that is commonly known to be responsible for many diseases such as cancer [51]. Due to the mobility of a proton (OH, NH, . . .) in their structure, they act by direct scavenging of reactive oxygen species (ROS).

3. Experimental

3.1. General Methods and Materials. All organic solvents were commercially available, distilled, and dried by appropriate methods.

1H and 13C NMR spectra were obtained from a Bruker Avance DRX 300 spectrometer. Chemical shifts are expressed in parts per million (ppm) downfield from external TMS. Perkin Elmer spectrophotometer was used to record the UV-visible absorption spectra. Mass spectra were measured on Bruker Biflex-III TM. IR spectra were measured on a Bruker vertex 70. A Thermo-Scientific Flash 2000 Analyzer was used to obtain (C, H and N) elemental analyses.

3.2. Synthesis of the Precursors and the Ligands

3.2.1. General Procedure for the Synthesis of 2-Hydroxy-5-((aryl diaz enyl)benzaldehyde. A diazonium solution was prepared by dissolving 0.01 mol of amine in 8 mL of water and 5 mL of concentrated hydrochloric acid was cooled to 0°C, treated with 15 mL of aqueous 1.0 M sodium nitrate dropwise, and stirred for 15 min. The resulting solution was added dropwise to a solution of salicylaldehyde (0.01 mol) dissolved in 50 mL of 10% aqueous sodium hydroxide. After the resulting mixture had been stirred for an hour at 0-5°C, the precipitate was filtered and the products were obtained by recrystallized from ethanol.

3.2.2. 2-Hydroxy-5-(phenyl diaz enyl)benzaldehyde (1a). Yield 84%, mp: 128°C. 1H NMR (300 MHz, DMSO) δ/ppm: 11.6 (s,1H), 10.4 (s,1H), 8.22 (d, 1H, J = 3), 8.12 (q, 1H, J = 3), 7.85 (dd,2H, J = 6, f = 3), 7.70 (m, 2H), 7.62 (m, 1H), 7.22 (d, 1H, J = 9). 13C NMR (75 MHz, DMSO) δ/ppm: 190.6, 163.3, 151.8, 144.8, 131.1, 129.6, 129.4, 123.8, 122.58, 122.3, 118.4. MALDI TOF MS calcd: m/z = 226.07 Da. Found m/z = 227.10 [M+1]+. Selected IR bands (cm−1) 1385.84, 1960, 1570, 1446, 1301, 1281, 1154, 1019, 952, 905, 843, 809, 759, 735, 708, 682, 641.

3.2.3. 2-Hydroxy-5-(o-tolyldiazenyl)benzaldehyde (1b). Yield 88%, mp: 130°C. 1H NMR (300 MHz, DMSO) δ/ppm: 11.55 (s, 1H), 10.40 (s,1H), 8.21 (d, 1H, J = 3), 8.12 (dd,1H, J = 6, f = 3), 7.59-7.45 (m, 3H), 7.34 (m, 1H), 7.24 (d, 1H, J = 9), 2.68 (s, 3H). 13C NMR (75 MHz, DMSO) δ/ppm: 190.6, 163.1, 149.8, 145.3, 137.2, 132.0, 129.4, 124.6, 124.1, 118.4, 115.1, 171. MALDI TOF MS calcd: m/z = 240.09 Da. Found m/z = 241.10 [M+1]+. Selected IR bands (cm−1) 3185.84, 1960, 1570, 1446, 1301, 1281, 1154, 1019, 952, 905, 843, 809, 759, 735, 708, 682, 641.

Table 3: The antioxidant activity of L1-L4 in DPPH.

| Compound | Concentration (µg/ml) |
|----------|-----------------------|
| L1       | 10 50 100 200 400 600 |
| L2       | - - - - - -          |
| L3       | - - - - - -          |
| L4       | - - - - - -          |
| Ascorbic Acid | 49.18 52.07 55.31 65.04 82.34 98.73 |
| Blank    | - - - - - -          |

We observed also that the tested ligands L1-L3, which structures differ only by the presence of a methyl group, gave similar inhibitory concentrations and that they are more active than the ligand L4 whose formula lacks the imidazole ring. This result led us to conclude that the presence of the imidazole ring significantly contributes to the bioactivity of the studied compounds.
3.2.4. 2-Hydroxy-5-(p-tolildiazenyl)benzaldehyde (Ie). Yield 89%, mp: 154°C. $^1$H NMR (300 MHz, DMSO) $\delta$/ppm: 11.53 (s, 1H), 10.39 (s,1H), 8.19 (d, 1H, J = 3), 8.10 (dd, 1H, J = 6, J = 3), 7.80 (d, 2H, J = 6), 7.42 (dd, 2H, J = 6, J = 3), 7.22 (d, 1H, J = 9), 2.42 (s, 3H). $^{13}$C NMR (75 MHz, DMSO) $\delta$/ppm: 190.6, 163.1, 149.9, 144.8, 141.3, 129.9, 129.6, 123.5, 122.6, 122.4, 118.3, 210.0. MALDI TOF MS calcld: $m/z = 240.09$ Da. Found $m/z = 241.10$. 

3.2.5. General Procedure for the Synthesis of the Schiff Bases Based on Imidazole: L1-L3. N-($\alpha$-Aminophenyl)imidazole (4mmol) was added to a methanol solution (30 mL) of 2-Hydroxy-5-($\alpha$-aryldiazenyl)benzaldehyde (4 mmol). The mixture was refluxed for 2 h and cooled to room temperature. The solvent was removed on a rotary evaporator and the orange products were rinsed and recrystallized with mixture of methanol and ether. The products were obtained as orange crystals.

3.2.6. Schiff Base Ligand L1. Yield 84 %, m.p. 110°C. $^1$H NMR (300 MHz, DMSO) $\delta$/ppm: 8.65 (s, 1H), 8.03 (d, 1H, J = 2.4), 7.91 (dd, 1H, J = 6.6, J = 2.4), 7.78 (dd, 1H, J = 7.2, J = 1.5), 7.65 (s, 1H), 7.55-7.5 (m, 2H), 7.46-7.48 (m, 1H), 7.2 (s, 1H), 6.92-6.89 (m, 2H), 4.04 (t, 2H, $J = 6.9$), 3.56 (t, 2H, $J = 6.9$), 2.12 (qd, 2H, $J = 6.9$). $^{13}$C NMR (75 MHz, DMSO) $\delta$/ppm: 31.4, 44.3, 55.7, 118.0, 118.2, 118.7, 122.6, 127.1, 127.2, 131.0, 130.0, 137.1, 145.4, 152.6, 164.1, 165.9. MALDI TOF MS calcld: $m/z = 333.16$ Da. Found $m/z = 334.4$. 

3.2.7. Schiff Base Ligand L2. Yield 79 %, m.p. 82°C. 1H MR (300 MHz, DMSO) $\delta$/ppm: 8.65 (s, 1H), 8.01 (d, 1H, J = 2.4), 7.91 (dd, 1H, J = 6.6, J = 2.4), 7.65 (s, 1H), 7.52 (dd, 1H, J = 7.8, J = 1.5), 7.35-7.34 (m, 1H), 7.33 (dd, 1H, J = 7.6, J = 1.5), 7.28-7.25 (m, 1H), 7.20 (s, 1H), 6.93-6.90 (m, 2H), 4.01 (t, 2H, $J = 7.2$), 3.55 (t, 2H, $J = 6.9$), 2.61 (s, 3H), 2.10 (qd, 2H, $J = 6.9$). $^{13}$C NMR (75 MHz, DMSO) $\delta$/ppm: 176.6, 31.7, 44.2, 53.7, 115.4, 115.7, 119.8, 120.1, 126.5, 127.0, 130.4, 131.7, 137.2, 137.8, 144.1, 150.4, 166.8, 168.6. MALDI TOF MS calcld: $m/z = 347.17$. Found $m/z = 348.4$. 

3.2.8. Schiff Base Ligand L3. Yield 82%, m.p. 95°C. 1H MR (300 MHz, DMSO) $\delta$/ppm: 8.63 (s,1H), 7.99 (d,dH,J = 2.7), 7.88 (dd, 1H, J = 6.6, J = 2.4), 7.71 (d, 2H, J = 8.1), 7.65 (s, 1H), 7.32 (d, 2H, J = 8.1), 7.19 (s, 1H), 6.90-7.00 (m, 2H), 4.04 (t, 2H, J = 7.2), 3.55 (t, 2H, J = 6.6), 2.34 (qd, 2H, $J = 6.9$). $^{13}$C NMR (75 MHz, DMSO) $\delta$/ppm: 21.5, 31.8, 44.3, 55.7, 117.9, 118.2, 118.7, 122.6, 126.9, 1271, 129.8, 129.9, 137.1, 141.1, 145.5, 150.7, 163.8, 166.0. MALDI TOF MS calcld: $m/z = 347.17$. 

3.2.9. Schiff Base Ligand L4. Propylamine (4 mmol) was added to a methanol solution (30 mL) of 2-Hydroxy-5-($\alpha$-tolildiazenyl)benzaldehyde (4 mmol). The mixture was refluxed for 2h and cooled to room temperature. The solvent was removed on a rotatory evaporator and the orange product was recrystallized with mixture of methanol and ether. Product was obtained as orange crystals. Yield 90 %. $^1$H NMR(300MHz,DMSO)$\delta$/ppm: 1H NMR(300MHz,DMSO)$\delta$/ppm: 14.42 (s, 1H), 8.45 (s, 1H), 8.02 (dd, 1H, J = 2.4, J = 9), 7.92 (d, 1H, J = 2.4), 7.65-7.63 (m, 1H), 7.37-7.33 (m, 2H), 7.32-7.27 (m, 1H), 7.09 (d, 1H, J = 9), 3.65 (t, 2H, J = 6), 2.74 (s, 3H), 1.8 (m, 2H), 1.06 (t, 3H, J = 9). $^{13}$CNMR (75 MHz, DMSO) $\delta$/ppm: 11.2, 17.1, 23.2, 56.5, 115.04, 116.4, 120.6, 120.6, 125.0, 130.5, 131.1, 131.2, 136.6, 142.9, 150.0, 165.8, 170.7. MALDI TOF MS calcld: $m/z = 281.15$ Da. Found $m/z = 282.4$. 

3.2.10. Microorganisms. Antifungal activity was assayed on human pathogenic fungi, including Candida albicans (ATCC 1066), Aspergillus fumigatus (CBS 1132), and Scedosporium apiospermum (IHEM 15115). The yeast was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the opportunistic molds were furnished for A. fumigatus by the Centraalbureau voor Schimmelcultures (CBS, Delft, Netherlands) and for S. apiospermum by the Institute of Hygiene and Epidemiology-Myco section, Institute of Public Health (IHEM, Brussels, Belgium). All the strains were maintained on yeast extract-peptone-dextrose agar (YPD) plates which contained 0.05% chloramphenicol at 37°C during an incubation time of 48 h for yeast, 72 h for A. fumigatus, and 7 days for S. apiospermum.

3.2.11. Determination of Antifungal Activity. Disk Diffusion Method on Solid Medium. Antifungal activities of the different ligands were evaluated using a disk diffusion method adapted from routinely antifungal tests [55]. Casitone agar plates of 90 mm diameter were used for the experiments. Antifungal inocula were prepared for yeast by suspending one colony in 10 mL of sterile distilled water. For the filamentous fungi, the mycelium was recovered by scraping the YPD plates with 10 mL of sterile distilled water. Conidia were then harvested from the suspension after a centrifugation at 1500 g for 5 min. The supernatant was then adjusted by spectrophotometry at 630 nm to an absorbance of 0.1. Casitone Petri dishes were flooded with 10 mL of the spore suspensions. Excess of the
suspension was eliminated and the Petri dishes were dried 10 min at 37°C.

Compounds were dissolved in DMSO at a final concentration of 10 mg/mL and 25 μL aliquots were applied on 12 mm diameter paper disks (ref 06234304, Prolabo 33173 Gradignan, France). Then, disks were deposited in the center of the casitone agar plates previously inoculated by fungal suspensions.

After an incubation time of 48 h for C. albicans, 72 h for A. fumigatus, and 7 days for S. apiospermum at 37°C, diameters of the growth inhibition zones were measured. Growth control was performed using filter paper disks soaked with an equal volume of, respectively, drug-free solvent (DMSO), and positive control was made with miconazole (Neosensitabs tablets, Rosco Diagnostic, Denmark).

**Microdilutions Method in Liquid Medium.** Antifungal tests were performed by following the guidelines of the Clinical Laboratory Standards Institute (CLSI) corresponding for yeasts to the reference method M27-A3 [56] and for filamentous fungi to the M38-A2 reference method [57]. Briefly, the fungal suspensions were prepared in RPMI-1640 culture medium (Sigma) added with 2 mM of L-glutamine, buffered with 0.165 M of morpholine propane-sulfonic acid (MOPS), and adjusted spectrophotometrically at 630 nm to final inocula concentrations of 0.5 to 2.5 x 10⁸ colony-forming units (CFU) per mL for C. albicans and 0.5 to 5.0 x 10⁴ CFU per mL for A. fumigatus and S. apiospermum. Broth microdilution tests were performed using sterile 96-wells flat-shaped microtitre plates. Each well was inoculated with 195 μL of the corresponding fungal working suspension.

Serial twofold dilutions of ligand were made in DMSO, 40 times the strength of the final drug concentration (10-0.0025 mg.mL⁻¹), and were dispensed in triplicate at a volume of 5 μL per well. Final concentrations for the drugs were from 250.00 to 0.061 μg.mL⁻¹. According to this procedure, the final concentration of DMSO solvent was lower than 2.5 % which did not affect significantly the growth of any fungus. After a 48 h incubation time for the yeast, 72 h for A. fumigatus, and 7 days for S. apiospermum at 37°C, the spectrophotometric reading of each well was performed at 630 nm with a Dynatech Laboratories MRX TC automatic plate spectrophotometric reader. The minimum inhibitory concentration of the compound that inhibits 80% (MIC⁸₀) of the fungal growth was calculated from the turbidimetric data compared to that of the DMSO-free control as the lowest compound concentration giving rise to an inhibition of growth equal or greater than 80 % of the compound-free control.

### 3.2.12. Antioxidant Activity.

DPPH free radical scavenging assays were performed according to the procedure described by Blois et al. [58]. A DPPH solution was prepared by dissolving 2 mg DPPH in 100 mL of methanol and then 250 μL of this solution was mixed with 50 μL of different concentrations of compounds L1-L4 dissolved in methanol. After 30 min incubation in the dark and at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical (DPPH) in percentage (I%) was calculated as follows:

$$I\% = \left(\frac{A_0 - A_i}{A_0}\right) \times 100$$  \hspace{1cm} (2)

where A0 is the absorbance of the control (containing all reagents except the test compound), and Ai is the absorbance of the tested sample.

IC₅₀ values were also defined as the concentration of the sample that causes 50 % of DPPH radicals inhibition. These values were calculated from the plot of inhibition percentages against sample concentration. Ascorbic acid was used as a positive control. Each measurement was performed in triplicate.

### 4. Conclusion

In this paper, we describe the synthesis, full characterization, and optical properties of four different Schiff bases ligands L1-L4 which associate an azo group, an imidazole unit, and a Schiff base fragment. The antifungal activities of the prepared ligands were evaluated and the results indicate two main tendency: (a) the presence of an azole group in this ligands is of great importance for the biological activities since L1-L3 exhibit higher growth inhibition zone diameters against the studied S. apiospermum, A. fumigatus, and C. albicans fungi and lower MIC⁸₀ values for C. albicans as compared with the azole free ligand L4; (b) the presence of an additional substituent on the phenyl ring of the azo group lowers the MIC⁸₀ activity of the ligands. Note that L1-L3 azole based ligands show excellent MIC⁸₀ values against C. albicans with concentrations as low as 0.1 μg/mL which is ten times lower than the reference concentration (1 μg/mL). In addition the four ligands show a significant antioxidant activity. All these findings clearly indicate the great potential of these new azole ligands as valuable candidates for developing new therapeutic agents. The complexation ability of these novel Schiff bases ligands towards transition metal cations such as Cu(II), Fe(II), Co(II),... as well as their corresponding biological activities is in progress.

### Data Availability

The experimental data used to support the findings of this study are included within the article.

### Conflicts of Interest

The authors declare that they have no conflicts of interest.

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