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Ring deactivating effect on antimicrobial activities of metal complexes of the schiff base of *p*-nitroaniline and isatin

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ABSTRACT

The Schiff base of "p"-nitroaniline and isatin was prepared by refluxing method [1]. Cobalt (II) and Nickel (II) metal complexes of this ligand were also synthesized. These compounds were analyzed using spectroscopic means, which were ^{1}HNMR, ^{13}C NMR IR and UV spectra. The ligand and the complexes were screened for in vitro antibacterial activity using Mueller-Hinton agar and antifungal activity using Sabouraud dextrose agar media. The antibacterial activity was evaluated against three Gram-positive bacteria (Staphylococcus aureus, Micrococcus luteus and Bacillus subtilis) and three Gram-negative bacteria (Pseudomonas aeruginosa, Escherichia coli and Salmonella typhi). The antifungal activities of the compounds were evaluated against three fungi (Aspergillus niger, Aspergillus flavus and Candida albicans). The Cobalt(II) complex showed activity against Bacillus subtilis and Escherichia coli, with the minimum inhibitory concentration (MIC) value of 5.0 µg/ml. The ligand and the Nickel(II) metal complex showed no activity against all tested bacterial. Also no antifungal activity was displayed by all compounds and this must have been due to the deactivation of the ring system by the electron withdrawing nitro group at the para position of aniline.

Keywords: Complexes, Schiff base, Isatin, Antimicrobial, and ligand.

INTRODUCTION

Isatin is an endogenous indole with a variety of pharmacological actions, including anticonvulsant, anti-microbial and antiviral activities, inhibition of monoamine oxidase [2, 3] and behavioral effects [4]. It has also found a wide application as a precursor in fine organic synthesis, leading to many different products, such as amine-, ether-, nitrile- and oxazole- derivatives [5, 6, 7, 8, 9], which were also proposed as therapeutic agents for coronary diseases such as ischemic heart disease, cardiac arryhythmia, hypertension and depression and even anticancer agents [9, 10]. The study of the metal complexes of the Schiff base ligands derived from isatin has also received much attention [1, 11, 12, 13]. Isatin-thiosemicarbazone Copper(II) complexes related to the antiviral drug, methisazone, were prepared and characterized using spectroscopic techniques [14]. This type of complexes were found to cause significant inhibition of human leukemic cell proliferation [15] presenting the copper atom in a square pyramidal coordination, as determined by crystallographic analysis [10]. A novel non-nucleoside reverse transcriptase inhibitor (NNRTI) has also been designed [16]. This aminopyridimino isatin lead compound was designed as a broad-spectrum chemotherapeutic agent active against HIV, HCV, *Mycobacterium tuberculosis* and various pathogenic bacteria. Metal complexes of the Schiff base of isatin and sulfanilamide have also been prepared and characterized using spectroscopic means [17]. The ligand was discovered to have acted either in a bidentate manner or in a tridentate manner presenting the different metal atoms in various geometries. Also, some novel Schiff bases of 5-substituted isatin derivatives were synthesized with a comprehensive study of their antimicrobial activities [18]. The study revealed that the Schiff base of 5-substituted isatin and "p"-nitroaniline was the most potent with antimicrobial activity (against four gram-negative and three gram-positive bacteria) and also antifungal activity. All these

prompted our study, which was aimed at synthesizing metal complexes of the Schiff base of isatin and "p"-nitroaniline, with the view to comprehensively study the antimicrobial activities of these compounds. These types of compounds are expected to also be of great biological importance, which is quite distinctive of such metal complexes of isatin derivatives [10]. Our interest was to determine the effect of condensing isatin and aniline possessing an electron withdrawing specie (a deactivating nitro group) at the para position on antimicrobial activities. Perhaps the distinctively strong antimicrobial potency of isatin might be able to overcome this deactivating effect, thus producing another potent antimicrobial ligand and metal complexes.

EXPERIMENTAL SECTION

Chemicals

Isatin, Sulfanilamide, $CoCl_2.6H_2O$ and $NiCl_2.6H_2O$ were obtained from Aldrich. All solvents used were analytical grades.

The ^1H NMR (500 MHz) and ^{13}C NMR (500 MHz) spectra were recorded at room temperature on a Bruker 500 NMR Spectrophotometer. The infrared spectra of complexes were measured as KBr discs on a Nicolet Avatar 330 FTIR spectrophotometer in the range 4000 – 400 cm^{-1}. The solid reflectance spectra of the compounds were recorded on a Genesys 10s V1.2 UV-Visible spectrophotometer. The purity of the compounds were checked by TLC pre-coated SiO_2 gel (HF_{254}, 200 mesh) aluminium plates (E Merk) using diethyl ether: petroleum ether (4:1) and visualized in UV chamber. Melting points were determined using a Gallenkemp Variable heater apparatus.

Preparation of P-nitroaniline Schiff base ($C_{14}H_9N_3O_3$)

Isatin (5.00 g; 33.98 mmol) and p-nitroaniline (4.69 g; 33.98 mmol) were weighted into a flask. Toluene (50 ml) was used as the solvent and 2.00 mg (0.01 mmol) of benzene sulfonic acid was used as the catalyst. The reaction was refluxed with a Dean Stark water separator attached. The reaction was monitored for 3 h and by then, the product spot on the TLC plate was intense, with the disappearance of the reactant spots. It was allowed to cool; the orange precipitate was filtered, washed with methanol and weighed. Recrystalization was done using ethanol: chloroform (3.5:1.5). For T.L.C., diethyl ether: petroleum ether (4:1) was used as the eluent. The isatin Rf value was 0.32, "p"-nitroaniline Rf value was 0.48 and the ligand Rf value was 0.30. The recrystalized Schiff base was stored in a dessicator. The yield obtained was 8.44 g; 31.58 mmol (93 %) and the melting point was determined as 275 -276 ^0C.

Preparation of Metal Complexes

The different complexes were prepared by the addition of 7.48 mmol of $CoCl_2.6H_2O$ (1.78 g) and $NiCl_2.6H_2O$ (1.78 g) separately dissolved in methanol (10 ml) to a stirring 3.74 mmol of the ligand (1 g) in methanol (50 ml) with the addition of 5 ml of concentrated ammonia solution. The color of each reaction mixture changed in few minutes. The mixtures were refluxed for 2 h and the products that precipitated out were filtered (after cooling), washed with methanol, dried, weighed and stored in the dessicator. The red cobalt (II) complex was obtained in 60 % yield (1.41 g; 5.93 mmol; $C_{28}H_{18}N_6O_6ClCo$), while the green nickel (II) complex was obtained in 68 % yield (1.60 g; 6.73 mmol; $C_{28}H_{18}N_6O_6ClNi$) with their melting points above 320 ^0C.

Biological activity

Antimicrobial Activity

The synthesized compounds were screened for *in vitro* antibacterial activity using Mueller-Hinton agar and antifungal activity using Sabouraud dextrose agar media. The antibacterial activity was evaluated against three Gram-positive bacteria (*Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus subtilis*) and three Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi*). The antifungal activities of the compounds were evaluated against three fungi (*Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*). Preliminary identification of the bacteria was carried out following the methods described by Cheesbrough (2002) [19]. Biochemical tests to confirm the identity of the organisms were performed at the Microbiology Laboratory of Bowen University, Iwo as described by Cheesbrough (2002) [19]. Ciprofloxacin (100 µg/disc) was used as a standard drug for the bacteria and Ketoconazole (100 µg/disc) for the fungi, while DMF was used as control.

Antibacterial test

The standardization of culture was carried out as described by National Committee for Clinical Standard [20]. An 18 h culture of each test bacteria was suspended in a sterile universal bottle containing nutrient broth. Normal saline was added gradually to it so as to compare the turbidity to that of 0.5 McFarland standard corresponding to approximately 10^8 cells/ml. This was then diluted to produce10^6 cells/ml that was used in the experiments. To carry out the antibacterial susceptibility test, the method described by Emeruwa (1982) [21] was used. One milliliter (1 ml) of test organism (10^6 cells/ml) was inoculated into Petri plates (90 mm diameter), then 19 ml molten sterilized (121 ^oC for 15 min) Mueller Hinton agar (MHA) at 45 ^oC added, and the plates shaken gently for even mixing of the

contents. The agar was allowed to solidify on a flat bench. The agar well diffusion and the disc diffusion methods were used to evaluate the antimicrobial activities of the compounds.

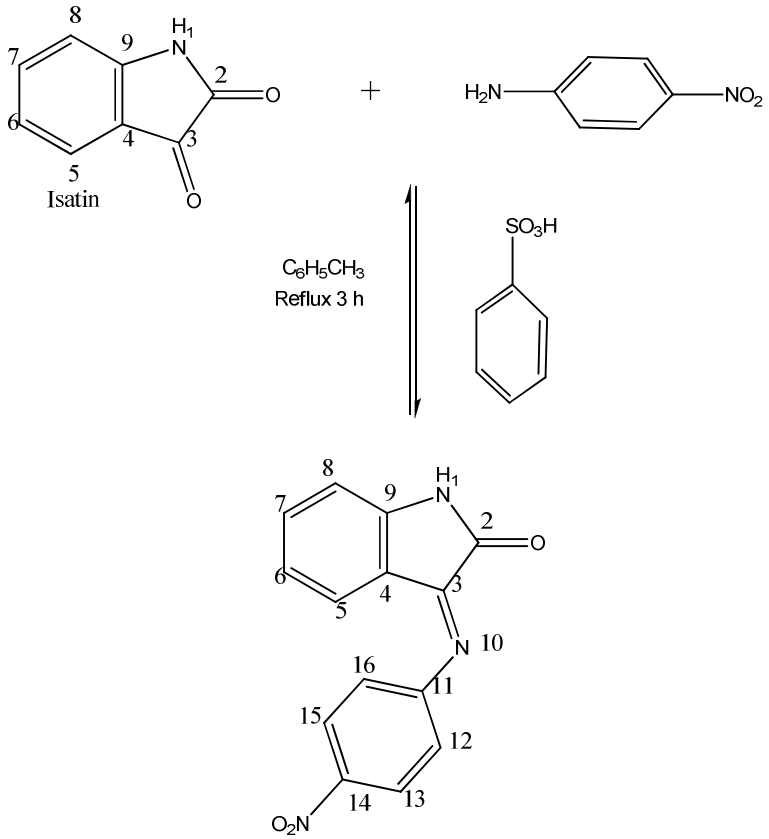
For the agar well diffusion method, wells (5 mm diameter and 4 mm deep) were punched in the agar with the aid of a sterile cork borer. Each of the synthesized compounds was dissolved in dimethylformamide (DMF) at a concentration of 10µg/ml. 0.1 ml of the dissolved compounds was pippetted into the holes. The plates were left on a bench for 1h to diffuse before incubating at 37 ^{o}C for 24 h. For the disc diffusion method, filter paper discs were cut (diameter 5 mm). The discs were sterilized and impregnated with the synthesized compounds (10µg/ml). The discs were picked with sterile forceps and placed on the MHA plates containing the test organisms. Antibacterial activity was evaluated by measuring the diameters of zones of growth inhibition in triplicates and the mean of three results taken.

Minimum inhibitory concentration (MIC)

This was determined by adding 5.0 and 2.5 µg/ml of each complex into test tubes containing sterile nutrient broth. The organisms that showed susceptibility to the complex were then introduced into the broths containing different concentrations of the complex. The tubes were then incubated for 24 h at 37^{o}C. The MIC was taken as the lowest concentration of the extracts that did not permit any visible growth [22, 23].

Antifungal test

The fungal isolates were allowed to grow on a Sabouraud dextrose agar (SDA) (Oxoid) at 25 °C until they sporulated. The fungal spores were harvested after sporulation by pouring a mixture of sterile glycerol and distilled water to the surface of the plate and later scraped the spores with a sterile glass rod. The harvested fungal spores and bacterial isolates were standardized to an OD 600 nm of 0.1 before use. One hundred microliter of the standardized fungal spore suspension was evenly spread on the SDA (Oxoid) using a glass spreader. Wells were then bored into the agar media using a sterile 5 mm cork borer and the wells filled with the synthesized compounds (10µg/ml in DMF) taking care not to allow spillage of the solution to the surface of the agar medium. The plates were allowed to stand on the laboratory bench for 1 h to allow for proper diffusion of the compounds into the media. Plates were incubated at 25 °C for 96 h and observed for zones of inhibition. Activity was evaluated by measuring the diameters of zones of growth inhibition in triplicates and the mean of three results taken [24].



(*E*)-3-(4-nitrophenylimino)indolin-2-one

Figure 1: scheme for the preparation of the ligand

RESULTS AND DISCUSSION

The ligand was prepared as shown in the equation (Figure 1). We have also presented an equation for the preparation of the metal complexes (Figure 2), similar to Adetoye et al, 2009 [17], where the Schiff base of isatin and sulfanilamide (aniline possessing an electron withdrawing sulphonamide group at the para position) with its metal complexes have been prepared. The prepared complexes are insoluble in water and most common organic solvents, but readily soluble in DMSO and DMF. The complexes are amorphous compounds and have not yet been successfully grown into crystals suitable for X-ray crystallographic analysis.

Infra red Spectra

The characteristic vibrational frequencies have been identified by comparing the spectra of the complexes with the free ligand. This is presented in Table 1. The assignments of these infra red bands were made by comparing the spectra of the compounds with reported literature on similar systems [17, 25]. There are four potential donor sites in the ligand. These are the isatin nitrogen, the isatin oxygen, the azomethine nitrogen and one of the oxygens of the nitroaniline.

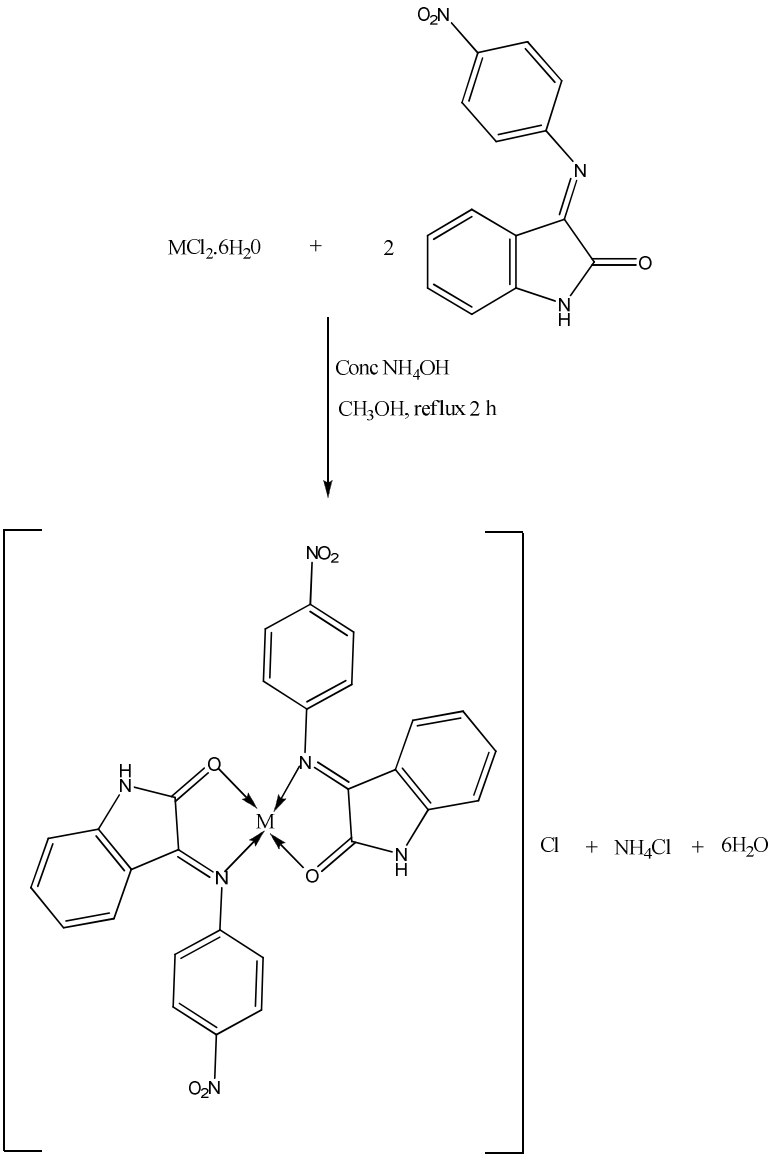


Figure 2: Scheme for the preparation of the metal complexes

The infra red spectrum of the ligand showed a strong band at 3450 cm^{-1} attributed to O-H stretching band. This band appeared strong but at lower frequencies of 3443 cm^{-1} and 3444 cm^{-1} in the Cobalt(II) and Nickel(II) complexes respectively. The medium band appearing at 3280 cm^{-1} in the spectrum of the ligand was attributed to the N-H stretching vibration. This band disappeared in the spectra of the complexes. The strong and medium bands appearing at 1739 cm^{-1} and 1722 cm^{-1} respectively in the spectrum of the ligand have been attributed to ν(C=O) bands. These

bands have been lowered to 1650 cm^{-1} and 1631 cm^{-1} in Cobalt(II) complex, also 1704 cm^{-1} and 1639 cm^{-1} in Nickel(II) complex. The disappearance of the ν(NH) bands and the lowering of the ν(C = O) bands in the complexes suggest the deprotonation of the isatin nitrogen and the involvement of the enol oxygen in chelation [17]. The uncoordinated C = N and C = C stretching vibrations of the ligand occurred as coupled bands expectedly between 1612 cm^{-1} and 1598 cm^{-1} [1], which eventually underwent a shift to a frequency between 1614 - 1578 cm^{-1} on coordination to the metal. The appearance of new bands at 608 – 514 cm^{-1} and 497 – 438 cm^{-1} assignable to ν(M-N) and ν(M-O) stretching frequencies respectively for the Ni (II) and Co (II) complexes, further confirmed coordination of the metal ions to the ligand [17, 26, 27].

Table 1: Relevant Infrared spectra data of isatin, ligand and complexes

COMPOUND	ν(OH) (cm^{-1})	ν (NH) (cm^{-1})	N (C=O) (cm^{-1})	ν (C=N+C=C) (cm^{-1})	ν (C-H) bond (cm^{-1})	υ (N=O) (cm^{-1})	N (C-N+C-O) (cm^{-1})	N (M-N) (cm^{-1})	ν (M-O) (cm^{-1})
Ligand ($C_{14}H_9N_3O_3$)	3450b,m	3280m	1739s 1722s	1612s 1598s	1483m 1462m	1513s 1342s	1096m	-	-
Cobalt(II) Complex	3443s	-	1650m 1631m	1589s, 1578s	1484w 1448m 1411m	1518m 1341s	1022w	514m	494m 480m
Nickel(II) Complex	3444s	-	1704s 1639s	1614s 1587s	1484s 1461s	1518s 1343s	1098m	608m 593m 516m	497m 472m 438m

Note: s – strong, m – medium, b – broad and w – weak

Table 2: Electronic spectra data analyses

Compound	Band position (nm)	Band assignment
Ligand	221; 323; 344;356	$\pi - \pi^*$ $n - \pi^*$
Co (II) complex	233 335; 368 640;820	$\pi - \pi^*$ $n - \pi^*$ d - d
Ni (II) complex	212 338; 350; 386 520; 709	$\pi - \pi^*$ $n - \pi^*$ d – d

Table 3: Antimicrobial activities of synthesized compounds

	Zone of inhibition (mm[a])				
	Ligand	Co (II) Complex	Ni (II) Complex	Ciprofloxacin	Ketoconazole
BACTERIA					
P. aeruginosa	-	-	-	-	NT
S. typhi	-	-	-	18	NT
E. coli	-	8		20	NT
B. subtilis	-	10	-	22	NT
M. luteus	-	-	-	20	NT
S. aureus	-	-	-	22	NT
FUNGI					
A. niger	-	-	-	NT	15
A. flavus	-	-	-	NT	30
C. albicans	-	-	-	NT	30

Note: - Number refers to zone of inhibition; NT - Not tested; [a] - all diameters recorded are the means of triplicate readings

Table 4: Minimum Inhibitory Concentration (MIC) of Cobalt (II) Complexes

Concentration	*E. coli* (min)	*B. subtilis* (min)
10.0 µg/ml	8.0	10.0
5.0 µg/ml	4.0	4.5
2.5 µg/ml	-	-

Note: - Number refers to zone of inhibition in mm

Electronic Spectra

The electronic spectra data for the solid reflectance [27, 17] of the ligand and complexes are presented in Table 2. The ultraviolet spectrum of the ligand showed an absorption band at 221 nm, which has been assigned to $\pi - \pi^*$

transition. The absorption bands at 323 nm, 344 nm and 356 nm were attributed to n - π^* transitions. The interpretations of ultraviolet spectra of metal complexes of this type of isatin-derived Schiff bases revealed that charge transfer bands occur in the same region with π - π^* transitions [17].

The solid reflectance spectra of the Cobalt (II) complex showed an absorption band at 233 nm, which was assigned as π - π^* transition. Also, the absorption bands at 335 nm and 368 nm have been assigned as d - d transitions.

In the spectra of the Nickel(II) complex, the band at 212 nm has been assigned as π - π^* transitions. The bands at 338 nm, 350 nm and 386 nm have been assigned as n - π^* transitions and the absorption bands at 520 nm and 709 nm have been assigned as d - d transitions.

^{1}H and ^{13}C NMR spectra (500 MHz; DMSO-d_6) (ppm)

The numbering systems of the atoms of isatin and the ligand have been presented in Figure 1. Isatin itself showed the following signals in the ^{1}H-NMR spectrum. 1H singlet at δ 11.0 is attributable to CONH. 1H triplet at δ 7.51 (H-6); 1H doublet at δ 7.43 (H-5); 1H triplet at δ 7.00 (H-7) and 1H doublet at δ 6.85 (H-8) are assigned to the protons of the 6-membered ring. The observed chemical shifts for the "p"-nitroaniline Schiff base have also been analyzed. According to literature the comparison of the ^{1}H-NMR of the starting ligand with that of the metal complex of these types of ligands provides the evidence of ligand deprotonation during metal chelation [17].

The ligand spectrum shows two resonance signals due to indole-NH at δ 10.95 and δ 11.02. The proportion of the E : Z isomers were found to be in ratio 67 : 33. The ^{1}H-NMR signals of the two isomers have also been analyzed. The major stereoisomer showed the following signals (δ, ppm): 8.35 (2H, d, J = 8.6 Hz, H-13 and H-15); 7.24 (2H, d, J = 8.6 Hz, H-12 and H-16) and for the protons on indole side of the ligand, the observed values are 7.39 (1H, t, J = 7.7 Hz, H-6); 6.92 (1 H, d, J = 7.6 Hz, H-5); 6.75 (1H, t, J = 7.6 Hz, H-7) and 6.36 (1H, d, J = 7.6 Hz, H-8). The minor isomer showed the following signals (δ, ppm): 8.20 (2H, d, J = 8.4 Hz, H-13 and H-15); 7.18 (2H, d, J = 8.4 Hz, H-12 and H-16); 7.64 (1H, d, J = 7.2 Hz, H-5); 7.51 (1H, t, J = 7.5 Hz, H-6); 7.10 (1H, t, J = 7.4 Hz, H-7) and 6.92 (1H, t, J = 7.6 Hz, H-8). In the ^{13}C NMR spectrum of the "p"-nitroaniline ligand, 12 signals were observed as follows (δ ppm): 157.0 (C=O), 147.9 (C=N), 144.8 (Cq), 135.7 (Cq), 126.5 (Cq), 126.2 (Cq), 124.9 (Cq), 122.6 (Cq), 119.5 (CH), 118.7 (CH), 115.9 (CH) and 112.2 (CH). Thus the four quaternary carbons (C-4, C-9, C-11, C-12, C-13, C-14, C-15, C-14, C-15 and C-16) were observed from 122.6 – 135.7 ppm. These correspond with expectations: 6 CH signals and 6 C signals. Since C-13 and C-15 are in the same environment, likewise C-12 and C-16, their signals are expected to overlap.

Antimicrobial study

The results of antibacterial activity of the metal complexes, ligand and cyprofloxacin (a reference clinical antibiotic used at 100 μg/disc) against the various bacteria has been summarized in Table 3. Also summarized in Table 3 is the result of the antifungal activity of these synthesized compounds and Ketoconazole (100 μg/disc, used as a standard drug). The antibacterial activity was evaluated against three Gram-positive bacteria (*Staphylococcus aureus, Micrococcus luteus* and *Bacillus subtilis*) and three Gram-negative bacteria (*Pseudomonas aeruginosa, Escherichia coli* and *Salmonella typhi*). The Cobalt(II) complex showed activity against *Bacillus subtilis* and *Escherichia coli*. The minimum inhibitory concentration (MIC) was determined as 5.0 μg/ml and the results have also been presented in Table 4. The ligand and the Nickel (II) metal complex showed no activity against all tested bacterial. The antifungal activities of the compounds were evaluated against three fungi (*Aspergillus niger, Aspergillus flavus* and *Candida albicans*). No antifungal activity was displayed by these compounds.

CONCLUSION

Generally, metal(II) complexes have been shown to be, in most cases, more effective than the free ligands. Tweedy's chelation theory [28] predicts that chelation reduces the polarity of the metal atom mainly because of partial sharing of its positive charge with donor groups and possible – electron delocalization over the whole ring. This consequently is expected to increase the lipophilic character of the chelates, favouring its permeation through lipid layers of the bacterial membrane. Thus these synthesized complexes were also expected to possess good antimicrobial activities beyond that of the ligand, but the results of this study have shown otherwise. This could possibly be due to the electron withdrawing effect of the nitro group present on the ligand, otherwise referred to as a strong deactivating effect. The nitro group must have shifted the direction of these delocalized electrons within the ring system towards itself, thus yielding an isatin derivative and its nickel (II) complex with no antimicrobial effect. However in the Cobalt (II) complex, the positive chelation effect of the metal ion must have been slightly stronger than the negative deactivating effect of the nitro group. This must have resulted in the low activity against two gram-negative bacteria, which is not quite comparable with ciprofloxacin, the tested standard drug. Also contrary to the literature of isatin and its derivatives, none of these compounds displayed antifungal activity against the tested

microorganisms. Similarly as already stated for the antimicrobial activity, the possible explanation for these anomalous behaviors could be the strong deactivating effect of the nitro group at the para position of the aniline condensed with isatin to give the ligand and its metal complexes.

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