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A N   I N T E R N A T I O N A L   J O U R N A L

New Aminoporphyrins Bearing Urea Derivative Substituents: Synthesis, Characterization, Antibacterial and Antifungal Activity

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ABSTRACT

This work studied the synthesis of 5,10,15-tris(4-aminophenyl)-20-(N,N-dialkyl/diaryl-N-phenylurea) porphyrins (P_1-P_4 with alkyl or aryl groups of Ph, iPr, Et and Me, respectively) and also the preparation of their manganese (III) and cobalt (II) complexes (MnP and CoP). The P_1-P_4 ligands were characterized by different spectroscopic techniques (^1H NMR, FTIR, UV-Vis) and elemental analysis, and metalated with Mn and Co acetate salts. The antibacterial and antifungal activities of these compounds in vitro were investigated by agar-disc diffusion method against Escherichia coli *(-),* Pseudomonas aeruginosa *(-),* Staphylococcus aureus *(+),* Bacillus subtilis *(+) and* Aspergillus oryzae *and* Candida albicans. *Results showed that antibacterial and antifungal activity of the test samples increased with increase of their concentrations and the highest activity was obtained when the concentration of porphyrin compounds was 100 µg/mL. The activity for the porphyrin ligands depended on the nature of the urea derivative substituents and increased in the order $P_1 > P_2 > P_3 > P_4$, which was consistent with the order of their liposolubility. MnP and CoP complexes exhibited much higher antibacterial and antifungal activity than P_1-P_4 ligands. Further, the growth inhibitory effects of these compounds was generally in the order CoP complexes > MnP complexes > P_1-P_4 ligands. Among these porphyrin compounds, CoP_1 displayed the highest antibacterial and antifungal activity, especially with a concentration of 100 µg/mL, against all the four tested bacteria and two fungi, and therefore it could be potential to be used as drug.*

Key words: Porphyrin, Aminoporphyrins, Antibacterial/Antifungal Activity, Synthesis, Carbamoyl Chloride

INTRODUCTION

Antibiotic resistance is one of the most severe public health problems globally that is of serious concern. It affects children and adults who have common infections, once easily treatable with antibiotics. It is important to find alternate treatments for microbial infections. Porphyrins and their metallo-derivatives are significant biomimetic compounds that are used in the studies in the areas of chemistry, biology and biotechnology (Banfi et al. 2006; Orlandi et al. 2012, 2013; Dosselli et al. 2013; Katsunori et al.

2013). In the recent years, porphyrins have been studied as flexible model compounds. It is possible to obtain useful biologically active materials by changing the peripheral functional groups in porphyrin skeleton and central metal atom in porphyrin core (Li et al. 1997; Beirão et al. 2014; Prasanth et al. 2014; Meng et al. 2015; Zoltan et al. 2015). Their properties can be tuned for specific application by metalation and/or by introducing substituents selectively at their *β* or *meso*-position (Goodrich et al. 2013; Nowak-Król and Gryko 2013). Porphyrins with only one, two, or more substituents present a compact

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architecture that suites a wide variety of applications, or further synthetic elaboration. To synthesize porphyrins bearing a molecular recognition site, porphyrin synthons that have functional groups at the *p*-positions of the *meso*-phenyl groups are usually employed, since these functional groups might be further modified by chemical treatments to enhance selectivity in the porphyrin-mediated reactions.

Following interest in development of porphyrins as selective electrodes (Karimipour et al. 2012) and as biomimetic oxidation catalysts (Mohajer et al. 2004; Karimipour et al. 2007, 2013), this work aimed to study the synthesis and characterization of new conjugates of aminoporphyrins in which one urea derivatives joins porphyrin by using carbamoyl chlorides. Also, the corresponding cobalt (II) and manganese (III) porphyrin derivatives were prepared. Finally, the biological activity of the newly synthesized porphyrin ligands and their complexes was tested against antibiotic-resistant fungi (*Aspergillus oryzae* and *Candida albicans*) and some Gram (+) and Gram (-) bacteria (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus subtilis*).

MATERIAL AND METHODS

All chemicals and solvents used for this work were obtained from Merck and Fluka Companies. All solvents and pyrrole were used after distillation. The melting points of the synthesized compounds were determined in open-glass capillaries on A-KRUSS-KSP1D melting point apparatus. IR absorption spectra were recorded on JASCO-680 using KBr pellets. UV-Vis spectroscopy was performed using JASCO-570 spectrometer, and ^{1}H NMR spectra were recorded on a BRUKER spectrometer operating at 400MHz. The ^{1}H NMR chemical shifts are reported as parts per million (ppm) downfield from TMS (Me$_4$Si) used as an internal standard. The elemental analysis was performed using an ELEMENTAR series elemental analyzer equipped with a vario EL software (vario EL cube) supplement based on absorption technique with TCD detector.

Synthesis of *N,N*-diphenyl and diisopropylcarbamoyl chloride

In a 50 mL two-necked flask, containing triphosgene (3.6 g, 12 mmol) in benzene/xylene (20 mL, 1:1, v/v), diphenylamine/diisopropylamine (39 mmol) was added slowly. The reaction was carried out in the presence of dry solid sodium hydroxide as mild catalyst (600 mg, 15 mmol) for 30 min under refluxing condition. The reaction mixture was cooled at room temperature and filtered. The filtrate was evaporated *in vacuo* to dryness and the residue was washed with hot hexane to remove unreacted triphosgene; the mixture was filtered and the resulting residue was crystallized from hexane to give corresponding carbamoyl chloride (Scheme 2; **a** and **b**) (Zare et al. 2012).

Diphenylcarbamoyl chloride (grey-green crystalline powder, yield: 73%), mp: 84 ^{o}C, IR (KBr, v, cm^{-1}): 760 (C-Cl), 1350 (C-N), 1650 (C=O), 3050 (C-H aromatic) and diisopropylcarbamoyl chloride (white to yellow solid, yield: 68%), mp: 58 ^{o}C, IR (KBr, v, cm^{-1}): 760 (C-Cl), 1400 (C-N), 1680 (C=O), 3050 (C-H sp^3).

Synthesis of *N,N*-dimethyl and diethylcarbamoyl chloride

In a 50 mL two-necked flask, containing triphosgene (4.5 g, 15 mmol) in benzene/xylene (15 mL, 1:1, v/v), 40% aqueous dimethylamine solution/diethylamine (49 mmol) was added dropwise. The reaction was stirred and refluxed in the presence of dry solid sodium hydroxide as mild catalyst (750 mg, 19 mmol) for 30 min to give corresponding carbamoyl chloride (Scheme 2, **c** and **d**) (Zare et al. 2012).

Dimethylcarbamoyl chloride (yellow liquid with a pungent odor, yield: 56%), bp: 167 ^{o}C, IR (KBr, v, cm^{-1}): 680 (C-Cl), 1400 (C-N), 1500 (C=O), 3000 (C-H sp^3). Diethylcarbamoyl chloride (clear to slightly grayish liquid, yield: 61%), bp: 122 ^{o}C, IR (KBr, v, cm^{-1}): 675 (C-Cl), 1400 (C-N), 1500 (C=O), 3050 (C-H sp^3).

Synthesis of 5,10,15,20-tetrakis(4-nitrophenylporphyrin); H$_2$T(4-NO$_2$PP)

H$_2$T(4-NO$_2$PP) was prepared according to a previously reported method (Adler et al. 1967). A mixture of 4-nitrobenzaldehyde (11 g, 73 mmol) and acetic anhydride (12 mL, 127 mmol) in distilled propionic acid (300 mL) was refluxed for 5 min. Freshly distilled pyrrole (5 mL, 72 mmol) was then added and progress of the reaction was monitored by TLC and UV-Vis spectroscopy. After completion of the reaction (12 h), the solution was cooled and the black heavy

precipitate was collected by filtration, washed by deionized water and dried. The crude material was taken into pyridine (80 mL), and the reaction mixture was then refluxed for 2 h. The mixture was kept in refrigerator at 0°C for 24 h, and then filtered and dried under ambient condition. $H_2T(4-NO_2PP)$ (~20%) was characterized by spectroscopic techniques. UV-Vis (λ_{max} nm in CH_2Cl_2 at 298 °K), [log ε($10^3 M^{-1}cm^{-1}$]: (Soret) 425 [208], (Q-IV) 517 [15], (Q-III) 552 [8], (Q-II) 591 [6], (Q-I) 657 [4]. IR (KBr, ν, cm^{-1}), 3318 (pyrrole NH), 1591 (aromatic C=C), 1513 (aromatic NO_2), 1336 (aromatic NO_2), 1102 (aromatic C-H in-plane bend). ^1H NMR ($CDCl_3$): −2.95 (s, 2H, pyrrole NH), 8.82 (s, 8H, β-pyrrole), 8.66 (d, 8H, J = 8.6 Hz, 2,6-(4-nitrophenyl)), 8.40 (d, 8H, J = 8.4 Hz, 3,5-(4-nitrophenyl)).

Synthesis of 5,10,15,20-tetrakis(4-aminophenylporphyrin); $H_2T(4-NH_2PP)$

$H_2T(4-NH_2PP)$ was prepared by the reduction of $H_2T(4-NO_2PP)$ with $SnCl_2.2H_2O$ and concentrated hydrochloric acid (Chen and Hsieh 1997). In a 100 mL three-necked flask, $H_2T(4-NO_2PP)$ (1.7 g, 2.1 mmol) was dissolved in concentrated hydrochloric acid (20 mL, 37%) and the resulting green mixture was vigorously stirred at room temperature for 1 h. Simultaneously, in a two-necked 100 mL flask, a solution containing stannous chloride dihydrate (1.1 g, 4.88 mmol) and concentrated hydrochloric acid (30 mL, 37%) was prepared with stirring by performing argon atmosphere for 1 h. Then, this solution was quickly added to the mixture containing $H_2T(4-NO_2PP)$, by pipette and the reaction was refluxed at 75-80 °C for 2 h. After completion of the reaction, ammonium hydroxide (25%) was added dropwise until the neutral pH. Then, it was kept under chemical hood for 24 h, filtered and dried. The dark green product was dissolved in 100 mL NaOH solution (5%) and stirred for 30 min. Again the solution was filtered and washed several times with distilled water and dried. The powder residue placed in a cellulose extraction thimble and final purification was performed by use of Soxhlet extraction apparatus in the presence of chloroform (250 mL) for 72 h. After the purification was completed, the solvent was evaporated and the purple residue was pulverized to a fine powder (scheme 3). $H_2T(4-NH_2PP)$ (~80%) was characterized by spectroscopic techniques. UV-Vis (λ_{max} nm in CH_2Cl_2 at 298 °K), [log ε($10^3 M^{-1}cm^{-1}$]: (Soret) 428 [257], (Q-IV) 522 [12], (Q-III) 562 [12], (Q-II)

596 [6], (Q-I) 654 [5]. IR (KBr, ν, cm^{-1}), 3322 (pyrrole and amine NH), 1598 (aromatic C=C), 3348 (asy NH_2), 3360 (sy NH_2), 1180 (aromatic C-H in-plane bend), 741 (aromatic C-H out-of-plane bend). ^1H NMR ($CDCl_3$): δ -2.73 (s, 2H, pyrrole NH), 4.06 (s, 8H, amine NH_2), 8.90 (s, 8H, β-pyrrole), 7.99 (d, 8H, J = 8.1 Hz, 2,6-(4-aminophenyl)), 7.07 (d, 8H, J = 8.1 Hz, 3,5-(4-aminophenyl)).

Synthesis of 5,10,15-tris(4-aminophenyl)-20-(N,N-dialkyl/diaryl-N-phenylurea) porphyrins

$H_2T(4-NH_2PP)$ (679 mg, 1 mmol) was added to dry THF (150 mL) and the reaction flask was transferred into an ice-water bath. After dissolving the porphyrin, triethylamine (300 mg, 2.99 mmol) was added as HCl scavenger to the reaction mixture. Then, dialkyl/diarylcarbamoyl chloride (1 mmol) solution in dry THF (25 mL) was added dropwise over 30 min and the reaction mixture was vigorously stirred in ice-water bath for 3 h. Finally, the precipitate was filtered and washed thoroughly with ethanol and hot water to extract $Et_3HN^+Cl^-$ salt and obtain compounds P_1-P_4 (Scheme 4).

5,10,15-tris(4-aminophenyl)-20-(N,N-diphenyl-N-phenylurea) porphyrin; P_1

(Yield: 79%, mp > 300 °C). ^1H NMR ($CDCl_3$): −2.67 (s, 2H, NH), 4.10 (s, 6H, NH_2), 7.31 (s, 1H, NH; amide), 8.95 (s, $8H_β$, pyrrole), 8.27 (m, $6H_m$, 4-aminophenyl), 8.30 (m, $6H_o$, 4-aminophenyl), 8.03 (m, $2H_m$, 4-N,N-phenyl-N-phenylurea), 8.12 (m, $2H_o$, 4-N,N-phenyl-N-phenylurea). UV-Vis (λ_{max} nm in CH_2Cl_2): 419 (Soret), 521, 572, and 677. IR (KBr, ν, cm^{-1}): 1650 (C=O), 3350 (NH_2 amine), 3300 (NH amide), 1540 (C=C Ph). EA calcd for $C_{57}H_{43}N_9O$: C, 78.69; H, 4.98; N, 14.49; O, 1.84. Found: C, 78.16; H, 5.11; N, 14.35; O, 1.78.

5,10,15-tris(4-aminophenyl)-20-(N,N-diisopropyl-N-phenylurea) porphyrin; P_2

(Yield: 72%, mp > 300 °C). ^1H NMR ($CDCl_3$): -2.73 (s, 2H, NH), 3.35 (m, 6H, NH_2), 7.83 (s, 1H, NH; amide), 8.87 (s, $8H_β$, pyrrole), 6.90 (d, $8H_m$), 7.83 (d, $8H_o$), 3.24-3.52 (m, 12H, CH_3 bonded to CH $_{iPr}$), 2.3-2.6 (m, 2H, CH $_{iPr}$). UV-Vis (λ_{max} nm in CH_2Cl_2 at 298 °K): 423 (Soret), 518, 574, and 678. IR (KBr, ν, cm^{-1}): 1640 (C=O), 3360 (NH_2 amine), 3300 (NH amide), 3050 (aromatic C-H), 2890 (aliphatic C-H), 1200 (C-N). EA calcd for

$C_{51}H_{47}N_9O$: C, 76.38; H, 5.91; N, 15.72, O, 1.99. Found: C, 76.85; H, 5.49; N, 15.98; O, 2.11.

5,10,15-tris(4-aminophenyl)-20-(*N,N*-diethyl-*N*-phenylurea)porphyrin; P₃

(Yield: 77%, mp > 300 °C). ^{1}H NMR (CDCl₃): -2.67 (s, 2H, NH), 4.08 (s, 6H, NH₂), 8.03 (d, 1H, NH; amide), 8.93 (s, 8H$_\beta$, pyrrole), 7.10 (d, 8H$_m$), 7.32 (m, 8H$_o$), 3.78 (t, 4H, CH₂(ethyl)), 1.57 (m, 6H, CH₃(ethyl)). UV-Vis (λ_{max} nm in CH₂Cl₂ at 298 °K): 421 (Soret), 519, 575, and 672. IR (KBr, ν, cm^{-1}): 1670 (C=O), 3340 (NH₂ amine), 3300 (NH amide), 2950 (aromatic C-H), 1200 (C-N). EA calcd for $C_{49}H_{43}N_9O$: C, 76.04; H, 5.60; N, 16.29, O, 2.07. Found: C, 75.74; H, 5.39; N, 16.34; O, 2.12.

5,10,15-tris(4-aminophenyl)-20-(*N,N*-dimethyl-*N*-phenylurea) porphyrin; P₄

(Yield: 74%, mp > 300 °C). ^{1}H NMR (CDCl₃): -2.66 (s, 2H, NH), 4.05 (s, 6H, NH₂), 8.03 (d, 1H, NH; amide), 8.93 (s, 8H$_\beta$, pyrrole), 7.10 (d, 8H$_m$), 7.29 (m, 8H$_o$), 1.89 (m, 6H, methyl). UV-Vis (λ_{max} nm in CH₂Cl₂ at 298 °K): 418 (Soret), 516, 579, and 676. IR (KBr, ν, cm^{-1}): 1670 (C=O), 3365 (NH₂ amine), 3300 (NH amide), 1200(C-N). EA calcd for $C_{47}H_{39}N_9O$: C, 75.68; H, 5.27; N, 16.90, O, 2.15. Found: C, 76.25; H, 5.41; N, 17.13; O, 1.98.

Synthesis of MnP₁-MnP₄

MnP₁-MnP₄ was prepared using the Adler method by mixing of P₁-P₄ (0.7 mmol) and manganese (II) acetate tetrahydrate (490 mg, 2.0 mmol) in *N,N*-dimethylformamide (DMF) (100 mL) (Adler et al. 1970). The reaction mixtures were briefly stirred in reflux condition for 2 h and then cooled at room temperature. The solution was filtered and the precipitate was dissolved in small amount of CH₂Cl₂ and purified by silica gel chromatography with CHCl₃/EtOAc as eluant to obtain MnP₁-MnP₄ as green powder (yield = 47-58%) (Scheme 5) and confirmed by electronic spectra. UV-Vis (λ_{max} nm in CH₂Cl₂ at 298 °K); MnP₁: 466 (Soret), 514, 561, MnP₂: 470 (Soret), 525, 559, MnP₃: 475 (Soret), 520, 559, MnP₄: 468 (Soret), 513, 560. These spectroscopic data were similar to data previously reported manganese porphyrin systems (Michael et al. 1984).

Synthesis of CoP₁-CoP₄

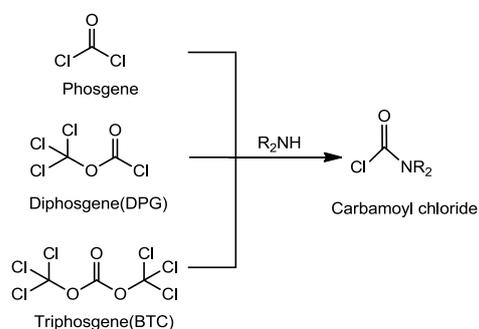Cobalt porphyrins derivatives were synthesized according to Lauher and Ibers (1974). The P₁-P₄ (0.65 mmol) was placed in a 250 mL two-necked round bottom flask and 80 mL of freshly distilled chloroform was added; the mixture was stirred and heated for 10 min to dissolve the porphyrin completely. A solution consisting of cobalt (II) acetate tetrahydrate (2.0 g, 8.0 mmol) in 50 mL methanol was added and the mixture was heated to reflux for 2 h. The solution was cooled at room temperature and the reaction mixture added to a separatory funnel containing of 100 mL of distilled water. The chloroform was placed at the bottom, while the methanol and excess inorganic salt was dissolved in the water. After repeating this operation, most of the inorganic salts and methanol were removed and the chloroform solution of the cobalt porphyrin was washed several times with fresh water. Finally, the chloroform solution was dried over Na₂SO₄, the drying agent was filtered off, and then the filtrate was evaporated to dryness. The CoP₁-CoP₄ was separated as dark-red crystals (yield: 59-83%) (Scheme 5). UV-Vis (λ_{max} nm in CH₂Cl₂ at 298 °K); CoP₁: 412 (Soret), 541, 573, CoP₂: 416 (Soret), 545, 583, CoP₃: 415 (Soret), 539, 580, CoP₄: 414 (Soret), 543, 581 (Chizhova et al. 2013).

RESULTS AND DISCUSSION

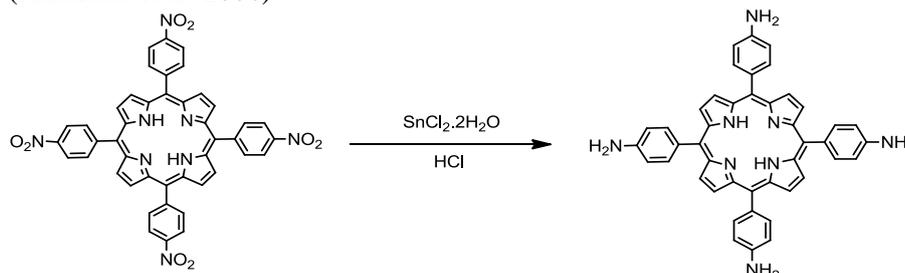### Antibacterial and antifungal activity test

The *in vitro* antibacterial and antifungal activity test of newly synthesized porphyrin compounds were carried out against *E. coli* (ATCC 25922), *S. aureus* (ATCC 6538), *P. aeruginosa* (ATCC 9027), *B. subtilis* (ATCC 6633), *A. oryzae* (ATCC 20423) and *C. albicans* (isolated from a patient). The antibacterial activities were evaluated by the agar-disc diffusion method as per National Committee for Clinical Laboratory Standards guidelines (NCCLS 1997). The filter paper sterilized discs saturated with measured quantity of the sample (25, 50 and 100 μg/mL) were placed on plate containing solid bacterial medium (Muller Hinton agar/broth) and fungal medium (sabouraud dextrose agar). The assay plates were incubated at 25°C for 72 h, at 25°C for 5 days and at 37°C for 24 h for yeast, fungus and bacteria, respectively. After incubations, the diameters of the clear zone of inhibition surrounding the sample were measured in millimeters by digital caliper (Figs. 1-6, *vide infra*).

Several reagents such as phosgene (carbonyl dichloride) (Ryan et al. 1996; Marrs et al. 1996; Goren et al. 1991), diphosgene (trichloromethyl chloroformate) (Hood and Murdock 1919; Kurita et al. 1976; Skorna and Ugi 1977) and triphosgene (bis(trichloromethyl) carbonate) (Ramsperger and Waddington 1933; Babad and Zeiler 1973; Nolan et al. 2012) have been used previously for phosgenation. The synthetic applications and characteristics of triphosgene have been reviewed recently. Compared to phosgene and diphosgene, it exhibits superior properties. Generally, aliphatic and aromatic carbamoyl chlorides are prepared from amines and phosgene group compounds by a simple one-step condensation method (Scheme 1) (Tilley and Sayigh 1963; Johnson 1967).

In this study, carbamoyl chlorides **a-d** were prepared by reacting the secondary amines (R_2NH; R= Ph, i-Pr, Et and Me) with reagent triphosgene, a stable phosgene substitute (Scheme 2) (Zare et al. 2012).

The reactions were clean and only the carbamoyl chlorides were detected by spectroscopic analysis of the crude products. The produced carbamoyl chlorides had a chloride leaving group that allowed for addition-elimination reactions with amines. This directed us to design a series of non-symmetrical aminoporphyrins containing urea derivatives using these carbamoyl chlorides.

Scheme 2 - Synthesis of carbamoyl chloride derivatives.

Synthesis of aminoporphyrins bearing urea derivative substituents and their metal complexes

As reported previously (Adler et al. 1967), 5,10,15,20-tetrakis(4-nitrophenylporphyrin); H_2T(4-NO_2PP) was prepared using freshly distilled 4-nitrobenzaldehyde and pyrrole and identified by UV-Vis spectroscopy. Starting with the prepared H_2T(4-NO_2PP) and reduction of the -NO_2 groups with $SnCl_2$/HCl, it can be obtained 5,10,15,20-tetrakis(4-aminophenylporphyrin); H_2T(4-NH_2PP), as a potent starting porphyrin wherein each p-phenyl positions occupied by amino-groups (Scheme 3) (Chen et al. 1997).

The reaction of H_2T(4-NH_2PP) with carbamoyl chlorides **a-d** in a 1:1 molar ratio gave aminoporphyrins P_1-P_4 (72-79%) in which only one amino group converted into urea derivatives. A typical profile of these reactions is reported in Scheme 4.

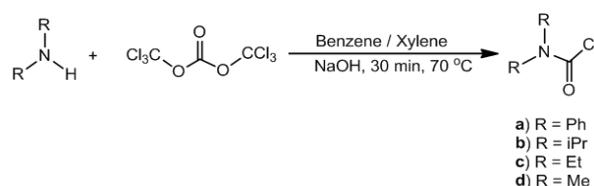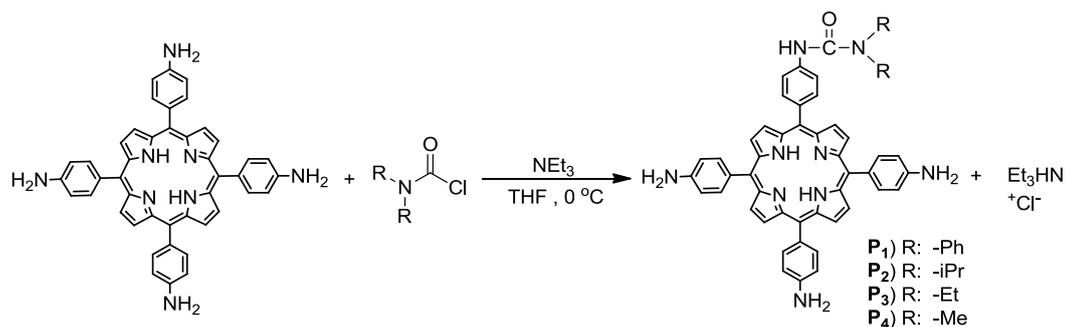**Scheme 1 -** Phosgenation reagents and carbamoyl chloride structures.

In this work triphosgene was used for the synthesis of carbamoyl chloride derivatives instead of phosgene and diphosgene to avoid handling hazardous and high risk materials. Carbamoyl chloride is used in the various reactions such as nucleophilic substitution reactions, Grignard reagents (Marcos et al. 2010), amine reactions (Montel et al. 2008), production of urea derivatives (Fish et al. 2008; Gumaste and Deshmukh 2004; Kamisaki et al. 2009), cycloaddition reactions to generate heterocycles (Grzyb and Batey 2008) and also polymerization reactions to produce polymers such as polyurethanes (Hamdach et al. 2006).

Scheme 3 - Synthesis of 5,10,15,20-tetrakis(4-aminophenylporphyrin).

Scheme 4 - Synthesis of 5,10,15-tris(4-aminophenyl)-20-(*N,N*-dialkyl/diaryl-*N*-phenylurea) porphyrins.

Formation of the P_1-P_4 was confirmed by the IR, ^{1}H NMR and UV-Vis spectroscopy. The IR spectra of the compounds showed a strong absorption at about 1650 cm^{-1} due to the carbonyl stretch. In ^{1}H NMR, the pyrrole and phenyl ring protons appeared in 7.29-8.93 ppm and the methyl groups emerged at 1.57-3.52 ppm. The two inner -NH signals appeared at -2.66- (-2.73) ppm as a weak singlet. The signals at about 4 and 8 ppm indicated the free -NH$_2$ and the -NH of amide substituent, respectively (see Materials and Methods section).

In comparison with H$_2$T(4-NH$_2$PP), the unchanged Soret absorption band of P_1-P_4 showed that *N,N*-dialkyl/diarylcarbamoyl chloride reacted selectively only with a peripheral amino group and the key aromatic 18e structure of the porphyrin skeleton stayed intact. The conversion of H$_2$T(4-NH$_2$PP) to P_1-P_4 was monitored by the IR spectral change. In P_1-P_4 compounds, the -NH amide group stretching vibrations displayed a characteristic band at about 3300 cm^{-1} rang, while this band did not appear in H$_2$T(4-NH$_2$PP). The Mn and Co porphyrin complexes (MnP and CoP) were synthesized by the reaction of P_1-P_4 and the corresponding metal acetate salts (Scheme 5). The formation of porphyrin complexes and bonding modes were inferred from the positions of characteristic bands in UV-Vis spectra (*vide supra*).
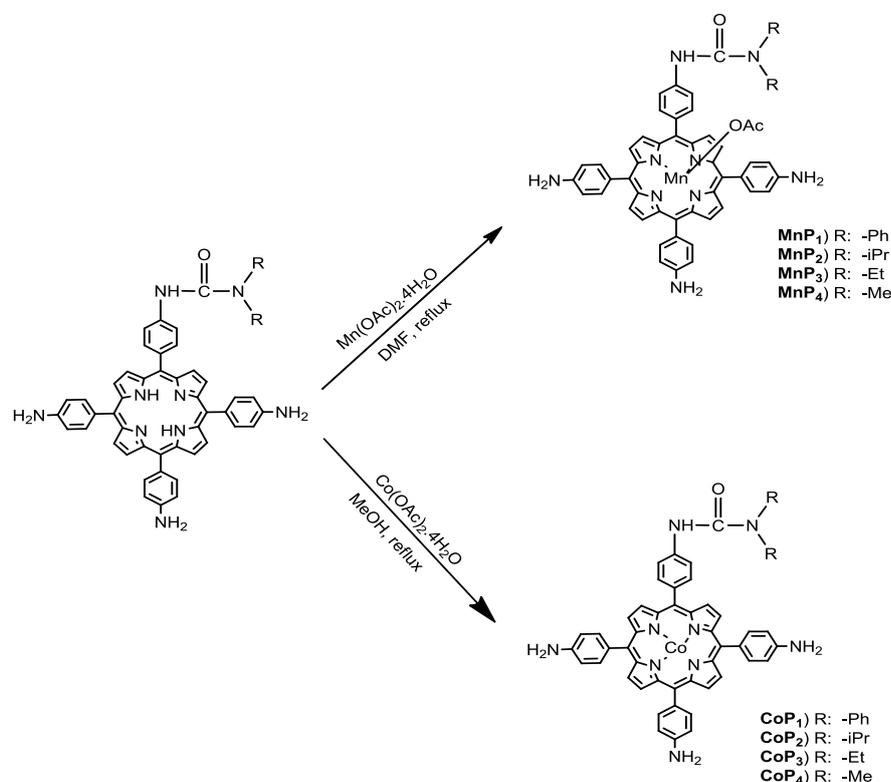
Scheme 5 - Synthesis of manganese and cobalt complexes of P_1-P_4 porphyrin.

Antibacterial and Antifungal bioassay (*in vitro*)
Tetrapyrrolic compounds such as porphyrins and their complexes are considered an important class of organic compounds, which have wide applications in many biological aspects (Boulton et al. 2001; Chiller et al. 2001; Maisch et al. 2004; Meisel and Kocher 2005; Satyasheel and Mahendra 2013; Bajju et al. 2014). The biological activity of the porphyrins and their complexes in inhibition of bacterial growth could be attributed to one of the following mechanisms. The first mechanism could be by the inhibition of the bacterial cell wall synthesis by bounding to the precursor of the cell wall, and second mechanism revealed that some antibodies had similar stereo structure to substrate (D-alanyl D-alanine). Hence, it would act competitive inhibitions for the enzymes (transpeptidase and /or carboxpeptidase), which were the main enzymes catalyzed the end step in the biosynthesis of peptidoglycans of the bacterial cell wall. Other mechanisms could contribute to the results found in the study that included the inhibition of biosynthesis of bacterial proteins by linking to the ribosome by doing so. The ribosome would not be in contact with tRNA (transfer ribonucleic acid), hence, the bacteria would not survive. Other mechanisms postulated that some antibodies inhibited the de *novo* synthesis of bacterial DNA by splitting the DNA in DNA-enzyme complexes by inhibiting DNA ligase (Murray et al. 1991; Baron et al. 1994).

In this study, the antibacterial activity of all new porphyrins and their complexes against the studied bacteria was indicated by the growth free "zone of inhibition" near the respective disc. The sensitive bacteria grew everywhere, except in the areas around the disc containing porphyrin compounds and antibiotic in the medium. The *in vitro* activity tests were carried out using the growth inhibitory zone (well method) (Indu et al. 2006; Tabatabaeian et al. 2013). The potency of components was determined against the two Gram-negative bacteria (*E. coli*, and *P. aeruginosa*), two Gram-positive bacteria (*S. aureus* and *B. subtilis*) and two fungi (*A. oryzae*, and *C. albicans*).

The results reported in Figures 1-3 for bacteria and Figures 4-6 for fungi, and also two samples of the growth inhibition halos are represented in Figure 7. The synthesized compounds showed varying degree of inhibitory effects: low (up to 10 mm), moderate (up to 16 mm) and significant (above 16 mm). The parent $H_2T(4-NH_2PP)$ could not be thoroughly investigated due to its very low solubility in DMSO. However, the urea substituents on the porphyrins P_1-P_4 ligands and the respective metalloporphyrins increased their solubility in DMSO. P_1 possessed a moderate activity against the bacterial strains at 25 μg/mL (Fig. 1) and 50 μg/mL (Fig. 2), but indicated moderate to high activity at 100 μg/mL (Fig. 3). P_2 exhibited low activity against all the strains at 25 μg/mL, but it was improved with increasing the concentration, which indicated moderate activity against *S. aureus* and *B. subtilis* at 50 and 100 μg/mL. Also, P_3 and P_4 exhibited weak to moderate activity against these bacterial strains. Hence, both antibacterial and antifungal activities for the ligands increased in the order $P_1 > P_2 > P_3 > P_4$. The observed difference in activity could attribute to the difference in lipophilicity of the porphyrin ligands (*vide infra*) containing different urea derivative substituents.
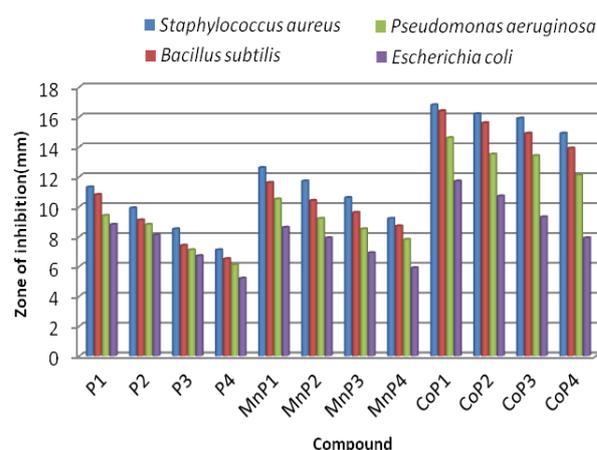

Figure 1 - Antibacterial activity of porphyrins and their complexes (25 μg/mL) by disc-diffusion method.
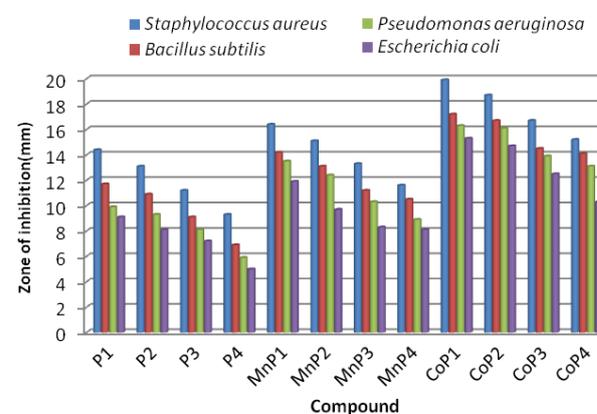

Figure 2 - Antibacterial activity of porphyrins and their complexes (50 μg/mL) by disc-diffusion method.
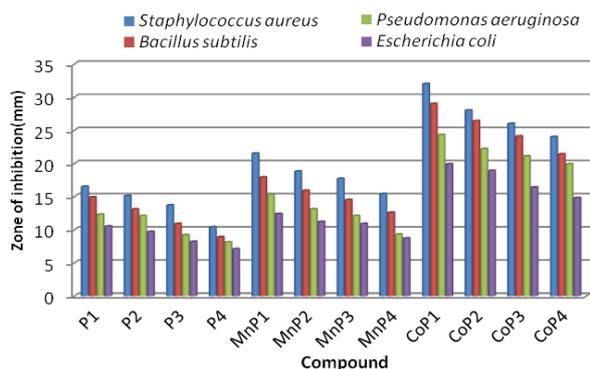
Figure 3 - Antibacterial activity of porphyrins and their complexes (100 µg/mL) by disc-diffusion method.

As the second step, MnP_1-MnP_4 complexes were tested for their inhibitory effects on the growth of bacterial strains. MnP_1 showed moderate activity against *S. aureus*, *B. subtilis* and *P. aeruginosa*, but was weak against *E. coli* at 25 µg/mL concentration, whereas significant activity was observed against *S. aureus* and moderate activity against all other bacterial strains at 50 µg/mL for this compound. At higher concentration (100 µg/mL), MnP_1 exhibited significant activity against the four bacterial strains, except *E. coli*. Similarly, MnP_2 showed moderate activity against *S. aureus* and *B. subtilis* at 25 µg/mL, whereas with increasing the concentration of MnP_2 solution to 100 µg/mL, the inhibition diameter was increased. It showed significant activity against *S. aureus*, *B. subtilis* and moderate against *P. aeruginosa* and *E. coli*. The reduction in halo diameters was observed for MnP_3 and MnP_4 (Figs. 1-3). Hence, antibacterial and antifungal activity for these manganese porphyrins was in the order $MnP_1 >$ $MnP_2 > MnP_3 > MnP_4$.

Finally, a similarly inhibitory effect on the microorganisms as $CoP_1 > CoP_2 > CoP_3 > CoP_4$ was also detected for Co(II) complexes. The inhibitory effect of CoP_1 and CoP_2 complexes was investigated, which showed the most antibacterial activity against these bacterial strains. The CoP_1 exhibited the highest effect and showed significant activity against *S. aureus* and *B. subtilis* and also moderate against *P. aeruginosa* and *E. coli* even at low concentration (25 µg/mL). This metalloporphyrin had maximum antibacterial against all four bacterial strains between P_1-P_4 porphyrins and their synthesized complexes in this study, which showed significant activity against *S. aureus* (32 mm), *B. subtilis* (29 mm), *P. aeruginosa* (24.3 mm) and *E. coli* (19.9 mm) at

100 µg/mL, which were admissible. Similarly, CoP_2 exhibited moderate to significant activity, especially at 50 and 100 µg/mL. CoP_3 showed significant activity against *S. aureus* at all the three concentrations and moderate against the others and also showed significant activity against the four bacterial strains at 100 µg/mL. CoP_4 showed moderate to significant activity against all bacterial strains, except for *E. coli*. The antifungal activities of the porphyrins and their complexes were quantitatively assessed by the presence, or absence of inhibition zones and zone diameters against *C. albicans* and *A. oryzae* according to Zahid et al. (2010) (Figs. 4-6). These compounds showed remarkable inhibition zones at 100 µg/mL. The porphyrins P_1-P_2 showed weak antifungal activity but their complexes, especially cobalt derivatives exhibited moderate to significant activity against *C. albicans* and *A. oryzae*. Recent studies have indicated that different metals cause discrete and distinct types of injuries to microbial cells as a result of oxidative stress, protein dysfunction or membrane damage (Lemire et al. 2013). The same pattern was obtained and observed in this study.

Different antimicrobial activity of P_1-P_4 and their complexes suggested different mechanism and/or biocidal property. Further studies are required to explore the exact mechanism of antibacterial and antifungal potency. Overall, the solvent used to prepare the compound solutions (DMSO) did not show inhibition against the tested organisms (negative control), the antibacterial and antifungal activity of free porphyrins P_1-P_4 increased upon coordination to metal ions. They inhibited the growth of bacteria and fungi more than the parent uncomplexed ligands under identical experimental conditions (Figs. 1-6).
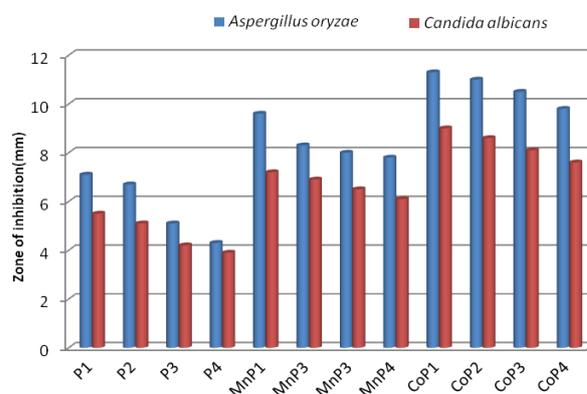

Figure 4 - Antifungal activity of porphyrins and their complexes (25 µg/mL) by disc-diffusion method.
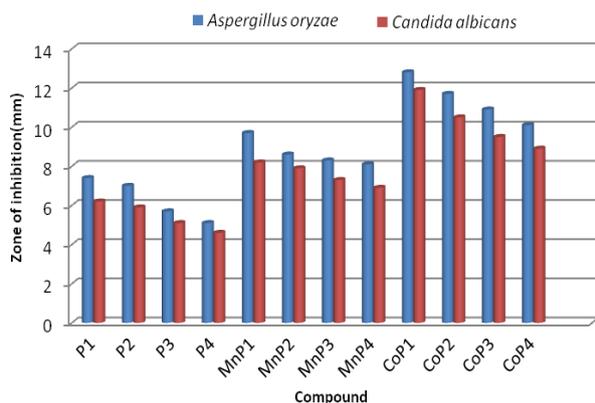
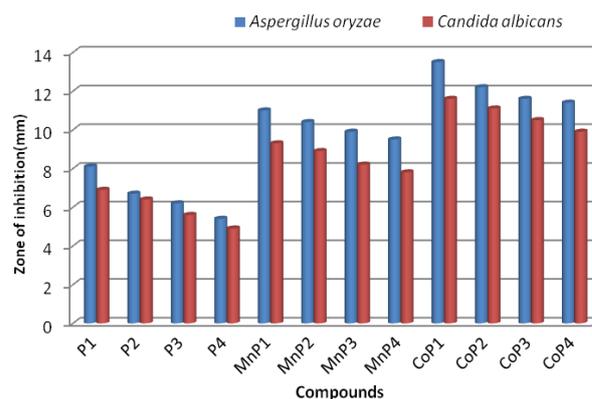**Figure 5 -** Antifungal activity of porphyrins and their complexes (50 µg/mL) by disc-diffusion method.

Figure 6 - Antifungal activity of porphyrins and their complexes (100 µg/mL) by disc-diffusion method.
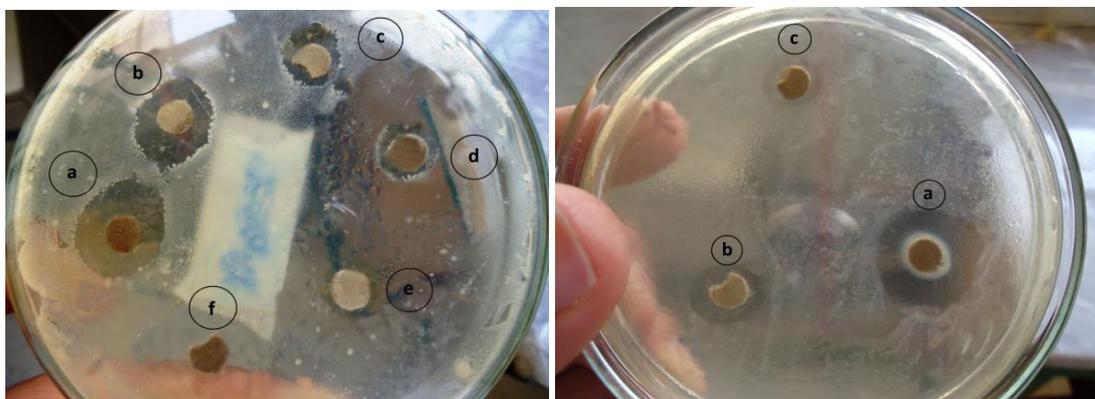

Figure 7 - Zone of inhibition for antimicrobial activity of CoP_1 (right pic: (a) *S. aureus*, (b) *B. subtilis* and (c) *P. aeruginosa*) and antifungal activity of CoP_1 ((left pic: (a) *A. oryzae*, (b) *C. albicans*); CoP_2 (left pic: (c) *A. oryzae*, (d) *C. albicans*) and CoP_3 (left pic: (e) *A. oryzae* (f) *C. albicans*).

Such increase in the activity of the complexes compared to that of ligands could be explained on the basis of Overtone's concept (Overton 1901) and Tweedy's Chelation theory (Tweedy 1964). According to Overtone's concept of cell permeability, the lipid membrane that surrounds the cell favors the passage of only the lipid-soluble materials, which makes liposolubility as an important factor that controls the antibacterial and antifungal activity. Upon chelation, the polarity of cation is reduced to a greater extent due to the overlap of the ligand orbital and partial sharing of the positive charge of the metal ion. The chelation increases the delocalization of p-electrons over the whole chelate ring and enhances the lipophilicity of the complexes. It is plausible that MnP complexes contain Mn^{3+} and P^{2-} and an OAc^- as counter ion that makes it less lipophilic and less able to act as antibacterial and antifungal agents. However, in CoP complexes, Co^{2+} is almost completely neutralized by the porphyrin ligands (P^{2-}). Therefore, the polarity is minimized and the liposolubility of CoP complexes is significantly increased.

This increase in lipophilicity enhances the penetration of the complexes into lipid membranes, and blocks the metal binding sites of the enzymes of the microorganism. Metal complexes also disturb the respiration process of the cell and, block the synthesis of the proteins and can restrict further growth of the organism. In this study, MnP and CoP complexes were more reactive than the corresponding uncomplexed P_1-P_4 ligands. Among different porphyrin complexes, CoP_1 showed the highest antibacterial and antifungal activity. The literature survey revealed

that antibacterial and antifungal activity of these 5,10,15-tris(4-aminophenyl)-20-(*N*,*N*-dialkyl/ diaryl-*N*-phenylurea) porphyrins (P$_1$-P$_4$) and their Mn and Co complexes (MnP and CoP) were comparable and in some cases, even superior to previously synthesized porphyrins and metalloporphyrins in terms of the growth inhibitory effects (Li et al. 1997; Beirão et al. 2014; Prasanth et al. 2014; Meng et al. 2015; Zoltan et al. 2015).

CONCLUSIONS

The present work studied the synthesis and characterization of some new 5,10,15-tris(4-aminophenyl)-20-(*N*,*N*-dialkyl/diaryl-*N* phenylurea) porphyrin ligands (P$_1$-P$_4$) and their Mn(III) and Co(II) complexes (MnP, CoP) and evaluation of their antibacterial activity against *E coli, P. aeruginosa, S. aureus, B. subtilis* and antifungal activity against *A. oryzae* and *C. albicans* in *vitro* by agar-disc diffusion method. The peripheral urea derivative substituents in the *para* position of phenyl ring affected the antibacterial and antifungal activity of these porphyrin ligands. The order of activity for the ligands was P$_1$ > P$_2$ > P$_3$ >P$_4$, which was consistent with the order of their liposolubility. However, MnP and CoP complexes showed higher antibacterial and antifungal activities than P$_1$-P$_4$ ligands and markedly inhibited the growth of most bacteria and fungi tested. The result also showed that the order of activity for the Mn(III) complexes was MnP$_1$ > MnP$_2$ > MnP$_3$ > MnP$_4$ and for the Co(II) complexes, it was CoP$_1$ > CoP$_2$ > CoP$_3$ > CoP$_4$. Generally, the Co(II) complexes virtually exhibited enhanced antibacterial and antifungal activities compared to MnP complexes. This could be related to lower polarity of the Co(II) complexes (higher lipophilicity), which enhanced the penetration of the complexes into lipid membranes of the microorganism. Among the Co(II) complexes, CoP$_1$ showed significant inhibitory effects against both Gram-positive (*S. aureus, B. subtilis*) and Gram-negative (*E. coli, P. aeruginosa*) bacterial and fungus species (*A. oryzae, C. albicans*) and, therefore, could have a potential to be used as drug. Having established antibacterial and antifungal activity of these aminoporphyrins having urea derivative substituents and their metal complexes, the next goal could be to synthesis aminoporphyrins with two or more biological active functions and evaluate their antibacterial, antifungal activity and DNA binding affinities.

ACKNOWLEDGEMENTS

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