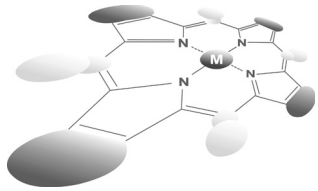
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Antimicrobial and antioxidant properties of novel octa-substituted phthalocyanines bearing (trifluoromethoxy) phenoxy groups on peripheral positions

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ABSTRACT: This study presents a novel phthalonitrile derivative (**2**) bearing (trifluoromethoxy) phenoxy groups in 4,5 positions. Cyclotetramerization of (**2**) in *N*,*N*-dimethylaminoethanol (DMAE) gave a series of peripherally octa-substituted metallophthalocyanines (**3-Zn, 3-Co** and **3-Cu**). The newly synthesized phthalocyanines have been characterized by a combination of various spectroscopic techniques. Effects of solvent nature on aggregation behavior of **3-Zn** were studied using different solvents such as acetone, $CHCl_3$ and dichloromethane (DCM). In addition, the aggregation behavior of the phthalocyanine complex **3-Zn** was examined in DCM at different concentrations ranging from 4×10^{-6}–14×10^{-6} M. Antimicrobial activities of synthesized compounds were tested by using the thin layer chromotography (TLC)-direct bioautography and disk diffusion methods. In both assays, the molecules showed activity on the tested Gram (+) bacteria. Antioxidant activities of the molecules, on the other hand, were determined by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method and a reducing power assay. The highest activity was obtained with **3-ZnPc** for both methods.

KEYWORDS: phthalocyanine, (trifluoromethoxy)phenoxy, aggregation behavior, antimicrobial and antioxidant activity, TLC-direct bioautography.

INTRODUCTION

Phthalocyanines (Pcs), as full-aromatic 18π-electron structures, have high electron transfer abilities making them useful in numerous areas [1–4] such as non-linear optical applications [5], molecular electronics [6], photodynamic therapy (PDT) [7], chemical sensors [8], Langmuir–Blodgett films [9], liquid crystals [10], photovoltaic cells [11], nanotechnology [12], electrochromic display devices [13] and in the dyes and pigments industry [1–4]. In addition, some Pcs can play a significant role in photosensitizers owing to their photostability, long lifetimes of the photoexcited triplet states and high molar absorption coefficients [14].

All these applications require highly soluble and pure compounds. Unsubstituted Pcs are nearly insoluble in common organic solvents and aqueous media due to intermolecular interactions, causing a drastic degradation of their optical properties and thereby minimizing their uses [1–4, 15]. In this respect, an important target of Pc chemistry is to synthesize soluble Pcs. In addition, Pcs have many significant physical, chemical and biological properties depending on their peripheral and axial substitution patterns. The presence of diverse substituents (such as long chain alkyl, alkylthio or alkoxy groups) on peripheral and/or non-peripheral positions of the Pcs can increase the distance between the bulky planar structures, subsequently resulting in improved solubility and optical properties of these compounds [16–22].

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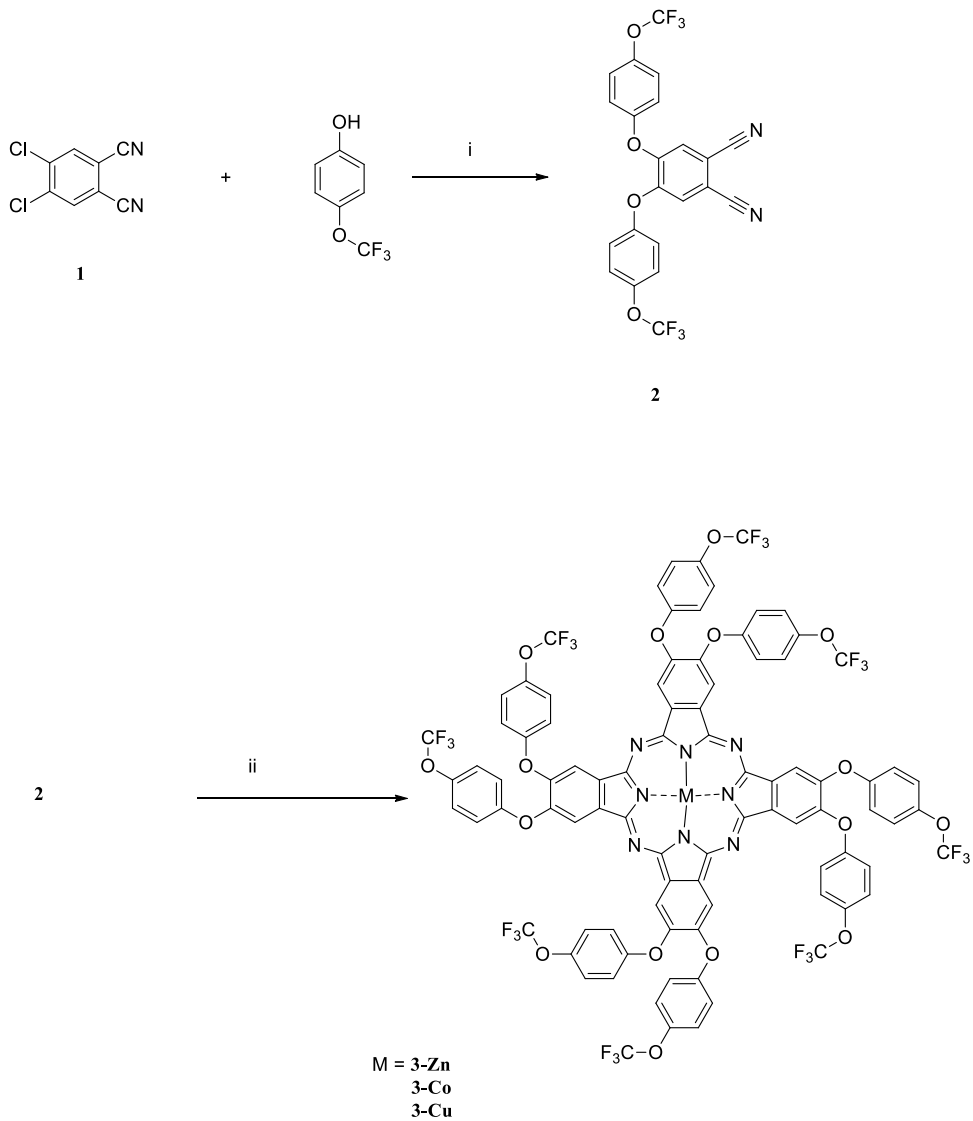
There is a great increase in the number of studies concentrated on the development of fluorine-containing Pcs to detect novel materials resulting from the unique properties of fluorine such as high stability, chemical resistance, lipophilicity and electron transport characteristics. In general, multi-fluoro-substituted Pcs are considered because of their good solubility even in polar and aprotic solvents [23–30]. In recent years, researchers have also studied the antioxidant [31–33] and antimicrobial [34, 35] activities of Pc molecules. Reactive oxygen species (ROS) are produced in the metabolisms of living organisms and these ROS decrease the normal defensive mechanisms of an organism by readily reacting with most of the cellular biomolecules [36]. Due to the broad use of commercial antibiotic drugs resulting in multi-drug resistance, the synthesis of new Pcs with antimicrobial activity becomes important.

In this study, we present the synthesis and characterization of novel octa-substituted Pcs bearing (trifluoromethoxy)phenoxy groups on the peripheral positions. We report here the aggregation behavior of the metallophthalocyanine (**3-Zn**) in different concentrations of DCM. In addition, antimicrobial and antioxidant activities of molecules were studied and TLC-direct bioautography method was used for the first time for the determination of antimicrobial activities of Pcs.

RESULTS AND DISCUSSION

Synthesis and characterization

The synthetic pathway of MPcs (**3-Zn**, **3-Co** and **3-Cu**) is shown in Scheme 1. The precursor phthalonitrile derivative, namely 4,5-bis(4-(trifluoromethoxy)phenoxy)



Scheme 1. Synthetic route for phthalonitrile (**2**) and phthalocyanines (**3-Zn**, **3-Co** and **3-Cu**). (i) DMSO, K_2CO_3, 80 °C, 4h. (ii) DMAE, 135 °C, 24 h

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phthalonitrile (**2**), was achieved by aromatic nucleophilic substitution of **1** with 4-(trifluoromethoxy)phenol [21, 37, 38]. The resulting phthalonitrile derivative **2** was crystallized from ethanol to obtain a yield of 55%. Cyclotetramerization of **2** with anhydrous metal salts $Zn(CH_3COO)_2$, $CoCl_2$ and $CuCl_2$ led to the formation of peripherally octa-substituted zinc (II), cobalt (II) and copper (II) Pcs (**3-Zn**, **3-Co** and **3-Cu**) (Scheme 1). The purified product was chromatographed using chloroform/THF; 50:1 as the eluent on the silica-gel column. The target Pcs were characterized using UV-vis, FT-IR, ^{1}H NMR, MS spectroscopic data, and the purity of the compounds were checked by elemental analysis. Satisfactory yields were obtained for the synthesis of all the Pcs **3-Zn** (29.01%), **3-Co** (32.75%) and **3-Cu** (30.49%). The newly synthesized Pcs show good solubility in a number of solvents such as $CHCl_3$, DCM, acetone, tetrahydrofuran (THF), ethylacetate, *N*,*N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO).

The FT-IR spectrum of phthalonitrile derivative **2** includes the characteristic peaks of Ar–H, CN, CF_3, and Ar–O–Ar appearing at 3119, 2233, 1369, and 1104 cm^{-1}, respectively. In the ^{1}H NMR spectrum of compound **2**, the aromatic protons of the phthalonitrile were observed as singlets at 8.00 ppm and aromatic protons of the substituent (F_3CO–ArH–O) appeared at 7.39–7.38 ppm as doublets and 7.21–7.19 ppm as doublets. The ^{13}C NMR spectrum of compound **2** was in accord with the structure. In the ^{13}C NMR spectrum of compound **2**, benzene carbon atoms appeared at a range between 152.43 and 111.36 ppm, while C–F carbons and nitrile carbons were observed at 123.26 and 120.69 ppm, respectively. ^{19}F NMR spectroscopy is an appropriate method to investigate the fluorinated structures. The ^{19}F NMR spectrum of **2** showed a peak at -58.19 ppm as a singlet corresponding to 6F. In the mass spectrum of derivative **2**, presence of the molecular ion peak at m/z = 480.54 $[M]^+$ verified the synthesis of the desired structure.

In the FT-IR spectra of **3-Zn**, **3-Co** and **3-Cu**, the disappearance of the intense absorption of the C≡N group confirmed cyclotetramerization of compound **2**. The IR spectra of the newly synthesized Pcs are very similar. For the Pcs **3-Zn**, **3-Co**, and **3-Cu**, the vibrations corresponding to aromatic ether groups (Ar–O–Ar) were observed at 1089, 1093 and 1091 cm^{-1} and characteristic substituted CF peaks appeared at 1365, 1349 and 1344 cm^{-1}, respectively. Only the **3-Zn** including a diamagnetic metal was employed for ^{1}H NMR. The ^{1}H NMR spectrum of **3-Zn** showed aromatic Pc ring protons at δ 7.40 ppm as singlets, while the (trifloromethoxy)phenoxy protons were observed in the region between 7.24 and 7.07 ppm as multiplets. In the MALDI-TOF mass spectra of purified products, the characteristic peaks appearing at 1985 $[M]^+$, 1979 $[M]^+$, and 1983.50 $[M]^+$ respectively evidenced the formation of the proposed products **3-Zn**, **3-Co** and **3-Cu**.

Electronic absorption spectra

UV-vis spectra of Pcs demonstrate an intense absorption in the Q-band region at approximately 650–700 nm which is referred to as the π–π* transition from the HOMO to the LUMO of the Pc^{2-} ring. The B band appears at around 300–400 nm (UV region) resulting from the deeper π–π* transition [1–4]. The position and band width of the absorption bands can be changed by altering the central metal atom, size and symmetry of π-conjugated system or the type, number and positions of substituents on periphery [39]. For instance, addition of aromatic rings (polybenzennulation) increases the wavelength of absorbed electromagnetic radiation by enhancing the conjugation of π-electrons [40].

The electronic spectra of **3-Zn**, **3-Co** and **3-Cu** exhibited a sharp single Q band at 674, 661 and 673 nm, respectively. The Q-band maxima of the synthesized MPcs were in the following order Zn > Cu > Co due to the central metal ion of the molecules (Fig. 1). The B band of these Pcs was observed in the UV region between 336 and 358 nm. Log ε values of the related peaks are given in Table 1. In addition, for compounds **3-Zn**, **3-Co** and **3-Cu**, the maxima of the vibronic bands correlated with *n*–π* transitions, appearing at 608, 600, 607 nm correspondingly [19, 41, 42].

Aggregation studies

Aggregation has a vital effect on the bioavailability, the *in vivo* distribution and singlet oxygen production efficiency, optical and PDT properties of highly delocalized π-electron systems [7]. On the other hand, non-aggregated Pcs with bulky substituents have good solubility which can be useful for the purification and characterization processes. Due to the many applications of Pcs derived from the optical properties, aggregation

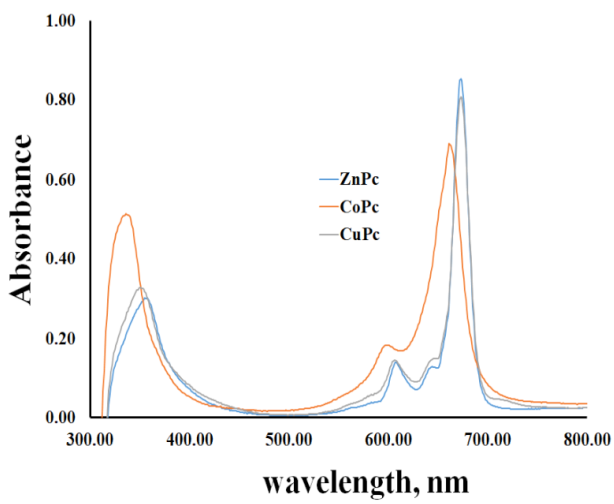


Fig. 1. UV-vis spectra of **3-Zn**, **3-Co** and **3-Cu** in THF (concentration; 6×10^{-6} M)

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Table 1. UV-vis data for **3-Zn, 3-Co** and **3-Cu** in THF

Compd.	B Band λ_{max}/nm (log ε)	Q Band λ_{max}/nm (log ε)
3-Zn	358 (4.63)	674 (5.13)
3-Co	336 (4.90)	661 (5.03)
3-Cu	351 (4.68)	673 (5.11)

and control of that are of critical importance as well. Generally, characteristics such as concentration, solvent type, nature of substituent and temperature can influence the aggregation behavior of Pcs [1–4, 15, 17–22].

In this study, the aggregation behavior of **3-Zn** was investigated in DCM at different concentrations (Fig. 2a). As shown, increase in concentration of **3-Zn** was followed by increase in intensity of the Q band and no new bands resulting from the aggregated species were observed. Due to non-aggregation behavior, the compound **3-Zn** obeyed the Lambert–Beer law in DCM at different concentrations ranging from 4×10^{-6} to 14×10^{-6} M (Fig. 2b). Moreover, the electronic absorption of compound **3-Zn** in diverse organic solvents consisting of acetone, $CHCl_3$, THF, DMF, DCM and DMSO were studied (Fig. 2c). According to Fig. 2c, as the ascending refractive index of the solvent is enhanced, the Q band shift to the red region increases. In this study, the bathochromic shift of the Q band based on solvent nature for compound **3-Zn** increased in the following order: acetone < THF < DCM < DMF < $CHCl_3$ < DMSO. The related spectra were analyzed with the Bayliss method [23, 38, 43] (Fig. 2d). As shown, the linearity of the Q-band frequency-refractive index plot reveals that the red shifts in the Q band mainly result from solvation effects.

TLC-direct bioautography

In TLC-direct bioautography assays, the developed TLC plate is dipped into the bacterial suspension and the chromatogram is incubated overnight. For visualization of antimicrobial activities, tetrazolium salts are used and colored formazen result from the conversion of tetrazolium salts with dehydrogenases of living organisms [44]. This

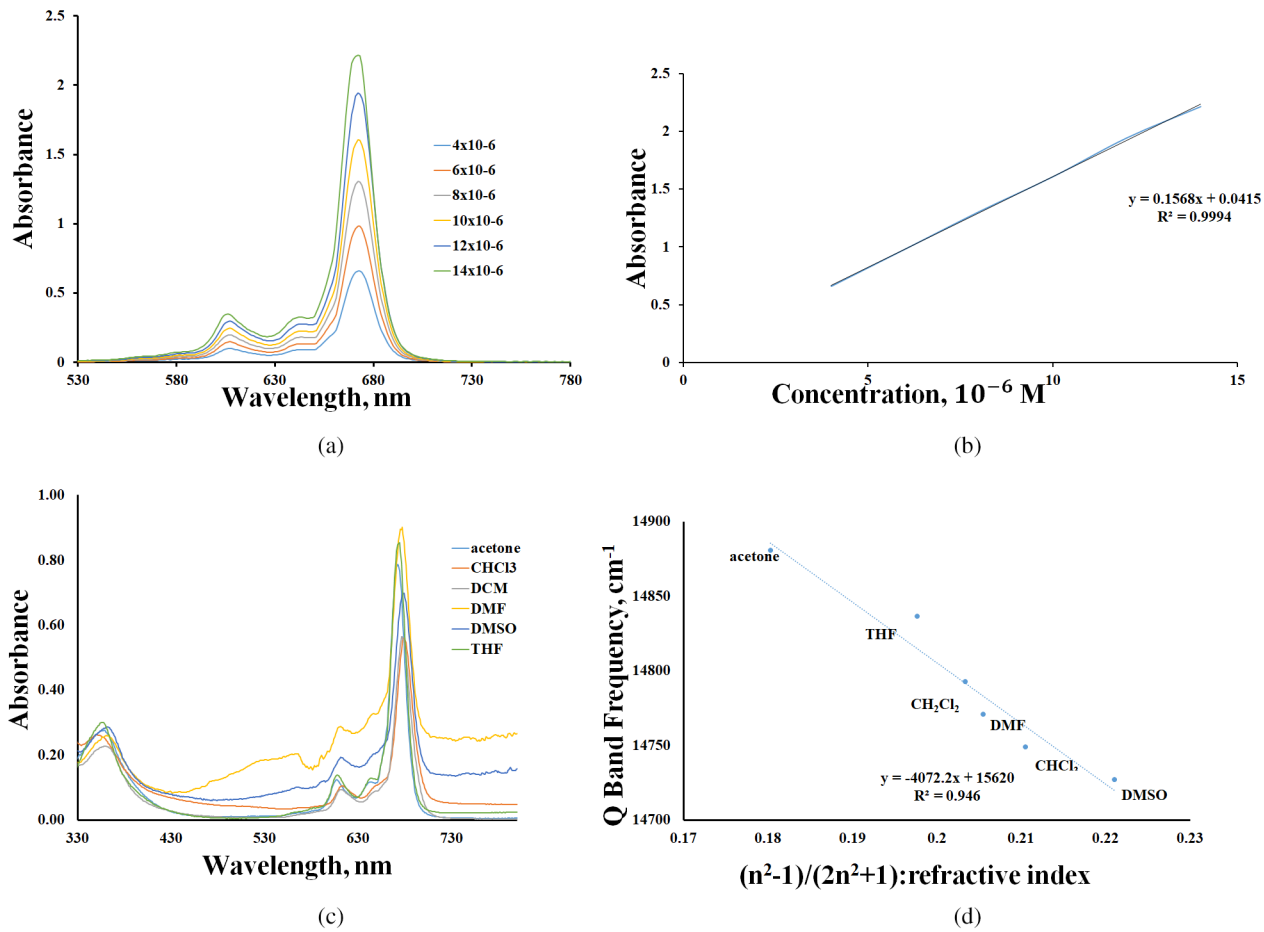


Fig. 2. (a) Electronic spectra of **3-Zn** in DCM at different concentrations from 4×10^{-6}–14×10^{-6} M, (b) Plot of the absorbance of **3-Zn** against to concentration, (c) UV-vis spectra of **3-Zn** in different solvents (concentration; 6×10^{-6} M), (d) Plot of the Q band frequency of **3-Zn** against $(n^2 - 1)/(2n^2 + 1)$

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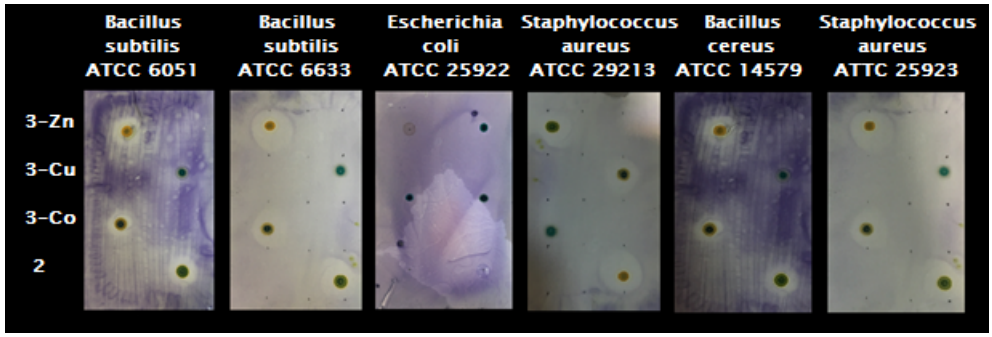
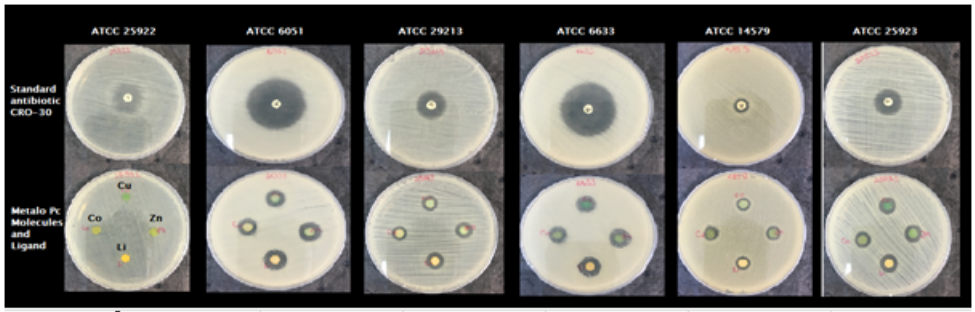


Fig. 3. Antimicrobial activities of the tested molecules against tested microorganisms



	Escherichia coli (ATCC 25922)	*Bacillus subtilis* (ATCC 6051),	*Staphylococcus aureus* (ATCC 29213),	*Bacillus subtilis* (ATCC 6633)	*Bacillus cereus* (ATCC 14579)	*Staphylococcus aureus* (ATCC 25923)
2	0	14	13	16	10	13
3-Zn	0	13	12	15	9	12
3-Co	0	12	12	12	9	11
3-Cu	0	11	11	12	9	12
Ceftriaxone (CRO-30)	20	38	21	38	11	20

Fig. 4. Antimicrobial activity of the molecules (0.005M) against the bacterial strains tested based on disk-diffusion method (inhibition zones given in mm)

technique has been commonly used to determine the antimicrobial properties of plant extracts [45] and it is here applied for the first time for the evaluation of potentials of Pc molecules. This method is very useful in terms of giving preliminary information about the antimicrobial activity of molecules.

In our study we examined compound **2**, **3-Zn**, **3-Co** and **3-Cu** using a TLC-direct bioautography assay. Figure 3 shows the TLC biograms of tested bacteria and white areas against purple backgrounds indicate inhibition on the TLC plates. Compound **2** and **3-Zn** both showed inhibitory activity against all tested gram (+) bacteria; **3-Co** was found to inhibit the growth of *Staphylococcus aureus* (ATCC 29213) only and **3-Cu** possessed activity against all tested gram (+) bacteria except *Staphylococcus aureus* (ATCC 29213). None of the molecules was effective on the gram (-) bacteria of *Escherichia coli* (ATCC 25922).

In this study, metal-containing Pcs without cationic substituents at R groups showed antimicrobial activity against all tested gram positive microorganisms. Mikula *et al*. investigated the antimicrobial activities of some novel Pcs against *E. coli* and found that positively charged molecules showed inhibitory effects on bacteria while negatively charged and neutral ones showed no inhibitory effect [46]. There are also other studies in which the antimicrobial activities of Pcs against both gram (+) and gram (-) bacteria have been investigated [47–49].

Disk diffusion assay

Antimicrobial activities of molecules were determined by using a disk diffusion assay against one gram negative and five gram positive bacteria. All tested compounds showed inhibition activity against all tested gram (+) bacteria species. The inhibition areas obtained from the molecules were in the range of 9–16 mm, and standard antibiotic ceftriaxone showed the highest inhibition activity in all tested microorganisms. Compound **2,** among all the molecules tested, showed the highest inhibition area in all tested gram (+) bacteria, and MPcs did not vary significantly according to their metal atom (Fig. 4).

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Some of the studies on the antimicrobial activity of Pc molecules are summarized in Table 2. In these studies, researchers have identified the antimicrobial activity of Pcs using a variety of methods such as viable cell count, disk diffusion, agar diffusion, biomass accumulation and minimum inhibitory concentration (MIC). In addition to these methods, we applied TLC-direct bioautography method for the first time to the Pc molecules.

DPPH assay

DPPH radical scavenging activities of all the tested molecules and control compounds are given in Fig. 5. In the DPPH assay, results were expressed as the ratio percentage of the absorbance decrease of DPPH radical solution in the presence of tested molecules [55]. All molecules showed DPPH scavenging activity in all tested concentrations. When the concentration increased from 5 $mg \cdot mL^{-1}$ to 100 $mg \cdot mL^{-1}$, the percentage of DPPH scavenging activity increased from 0.87 to 38.27 for compound **2**, 4.71 to 63.76 for **3-Zn**, 1.03 to 40.34 for **3-Co** and 3.01 to 43.08 for **3-Cu**. The standard compound *Trolox* showed the highest activity among all the tested molecules in all tested concentrations.

Reducing power

One of the most significant indicators of potential antioxidant activity is the reducing capacity of a compound. In this assay, conversion of Fe^{+3} complex to ferrous form in the presence of reductants was used. Thus, the concentration of Fe^{2+} is obtained by measuring the absorbance at 700 nm [56] and an increase in absorbance indicates an increase in reducing power. Reducing capacity test results of the tested molecules are given in Fig. 6. The standard compound α-*tocopherol* showed the highest reducing capacity at 25, 50 and 100 $mg \cdot mL^{-1}$ concentrations. Compound **2** showed the highest reducing capacity at all tested concentrations, followed by compounds **3-Zn**, **3-Co** and **3-Cu**.

The literature on antioxidant activity studies of Pc molecules synthesized in recent years is given in Table 3. In this context, DPPH removal activities obtained in the studies vary between 30–70%, and reducing power activities of Pc molecules vary between 2 to 3 absorbance values (700 nm) at 100 $mg \cdot mL^{-1}$ concentration. These values are consistent with the results in our study.

EXPERIMENTAL

Apparatus

IR spectra were recorded on a Perkin–Elmer Spectrum One spectrometer and electronic spectra were performed on a Scinco Lab Pro Plus UV/vis spectrophotometer. ^{1}H NMR, ^{13}C and ^{19}F spectra were recorded on an Agilent VNMRS 500 MHz spectrometer using a Thermo Finnigan Flash EA 1112 apparatus to measure elemental data at 950–1000 °C. Mass spectra were determined on a Perkin–Elmer Clarus 500 GC-MS and a Bruker Microflex MALDI-TOF mass spectrometer.

Synthesis

4,5-*bis*(4-(*trifluoromethoxy*)*phenoxy*)*phthalonitrile* (2). 1,2-Dichloro-4,5-dicyanobenzene (**1**) [60] (1.00 g, 5.08 mmol) and 4-(trifluoromethoxy) phenol (2.26 g, 12.69 mmol) were dissolved in 20 mL DMSO. Then 7.00 g of K_2CO_3 (50.76 mmol) was added during 2 h and stirred for further 6 h at 80 °C. After cooling, it was poured into ice water (200 mL). The precipitate was filtered and washed several times with cold water until the filter became neutral. The crude product was crystallized using ethanol. Yield 1.27 gr (% 52.30), mp 104 °C. Anal. calcd. for $C_{22}H_{10}N_2O_4F_6$: C, 55.01; H, 2.10; N, 5.83. Found: C, 55.12; H, 2.18; N, 5.75. ^{1}H NMR (500 MHz; d_6-DMSO): δ, ppm 8 (2H, s, Ar–H (Pc ring)), 7.39–7.38 (4H, d, F_3CO–ArH), 7.21–7.19 (4H, d, F_3CO–ArH). ^{13}C NMR (d_6-DMSO): δ, ppm 152.43 (aromatic C), 151.88 (aromatic C), 146.46 (aromatic C), 123.26 (aromatic C–F), 122.82 (aromatic C), 121.82 (aromatic C), 120.69 (C≡N), 114.59 (aromatic C), 111.36 (aromatic C). ^{19}F NMR (d_1-$CDCl_3$): δ, ppm -58.19 (6F, s). FT-IR: ν_{max}, cm^{-1} 3119 (CH aromatic), 2233 (-C≡N), 1369 (CF_3), 1104 (Ar–O–Ar). MS (MALDI-TOF): m/z 480.54 (calcd. For $[M]^+$ 480.31).

General procedures for metallophthalocyanines (3-Zn, 3-Co and 3-Co)

Compound **2** (0.100 g, 0.208 mmol), anhydrous metal salts [$Zn(CH_3COO)_2$ (0.013 g, 0.069 mmol), $CoCl_2$ (0.009 g, 0.069 mmol), $CuCl_2$ (0.009 g, 0.069 mmol)] and DMAE (1.5 mL) were stirred at 135 °C in a glass sealed tube for 24 h under an inert nitrogen atmosphere. The green-blue product was cooled to room temperature and poured into an ice and water mixture (80 mL) and stirred for 1 h. The precipitate was filtered off, washed with hot *n*-hexane and finally dried *in vacuo*. The purified solid was achieved by utilizing silica gel chromatography (chloroform/THF; 50 : 1) as the eluent. Color: dark blue. Solubility: $CHCl_3$, DCM, acetone, THF, ethylacetate, DMF and DMSO.

***Zinc*(*II*) *phthalocyanine* (3-*Zn*).** Yield 0.040 g (29.01%), mp > 250 °C. Anal. calcd. for $C_{88}H_{40}N_8O_{16}F_{24}Zn$: C, 53.20; H, 2.03; N, 5.64%. Found: C, 53.58; H, 2.28; N, 5.42%. ^{1}H NMR (500 MHz; d_1-$CDCl_3$, d_5-pyridine): δ, ppm 7.4 (8H, s), 7.24 (16H, m), 7.07 (16H, m). FT-IR: ν_{max}, cm^{-1} 3067 (aromatic CH), 1365 (CF_3), 1089 (Ar–O–Ar). UV-vis (THF): λ_{max}, nm (log ε) 358 (4.63), 674 (5.13). MS (MALDI-TOF): m/z 1985 (calcd. for $[M]^+$ 1985).

***Cobalt*(*II*) *phthalocyanine* (3-*Co*).** Yield 0.045 g (32.75%), mp > 250 °C. Anal. calcd. for $C_{88}H_{40}N_8O_{16}F_{24}Co$: C, 53.38; H, 2.04; N, 5.66%. Found: C, 53.63; H, 2.32; N,

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Table 2. Antimicrobial activity researches from the literature

Pc	Metal	Concentration	Photo inactivation	Assay	Strain	Result	Reference
SiPc-PEG	Si	2500 $\mu g \cdot mL^{-1}$	Yes	Viable cell count	*E. Coli*	$<5 \times 10^{9}$ cfu/mL	50
2,10,16,24-Tetrakis[methyl 2-(oxy)-2,2-diphenylacetate Phthalocyaninato] cobalt(II)	Co	500 $mg \cdot L^{-1}$	No	disk diffusion	*P. aeriginosa* *E. coli* *B. subtilis*	15 mm 13 mm 13 mm	51
2,10,16,24-Tetrakis[methyl 2-(oxy)-2,2-diphenylacetate Phthalocyaninato] copper(II)	Cu	500 $mg \cdot L^{-1}$	No	disk diffusion	*S. aureus*	12 mm	
Metal (II) 2, 9, 16, 23-tetra-phenyliminophthalocyanines-Cu-PhImPc	Cu	500 ppm	No	agar diffusion	*Xanthomonas compstris*	16 mm	52
Metal (II) 2, 9, 16, 23-tetra-phenyliminophthalocyanines-Zn-PhImPc	Zn	500 ppm	No	agar diffusion	*Xanthomonas compstris*	19 mm	
1(4), 8(11), 15(18), 22(25)-Tetrakis-a-(8-(2,3-dicyanophenoxy) quinoline-5-sulfonic acid)-zinc(II) phthalocyanine	Zn	3 μg–6 $\mu g \cdot mL^{-1}$	No	biomass accumulation	*Arthrospira platensis-M2*	Non toxic	53
4-(3-Methoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate	Co	—	No	MIC	*E. Coli* *S. aureus* *Salmonella Typhimurium* *Listeria monocytogenes* *Micrococcus luteus*	0.00091 J/cm^2 2.5 mg/mL 5 mg/mL 0.625 mg/mL 1.25 mg/mL	54

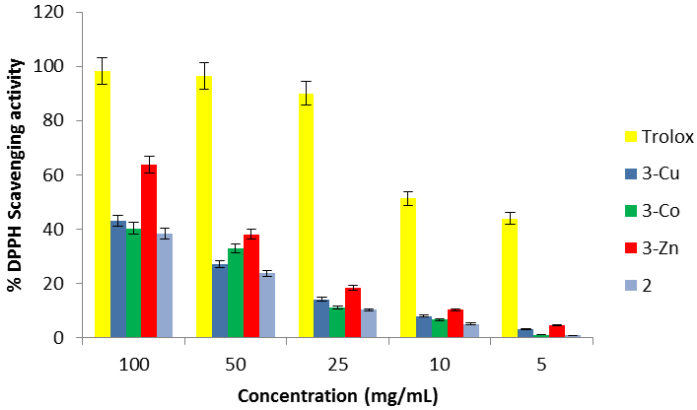


Fig. 5. Radical scavenging activity on DPPH radical (%) of the tested compounds

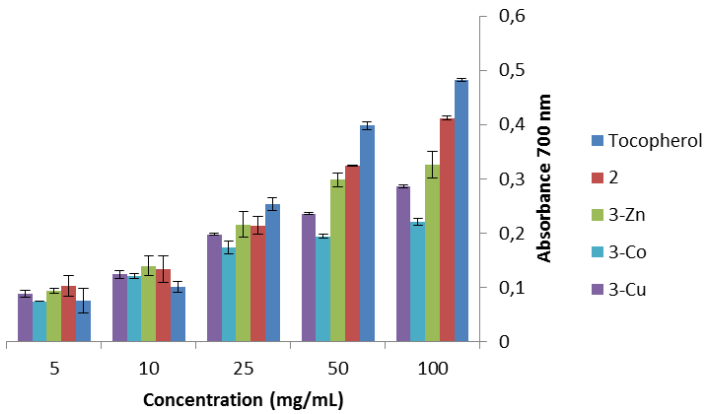


Fig. 6. Reducing power assay of tested molecules at different concentrations (vertical bars represent the SD)

5.48%. FT-IR: υ_{max}, cm^{-1} 3075 (Ar–H), 1349 (CF_3), 1093 (Ar–O–Ar). UV-vis (THF): λ_{max}, nm (log ε) 336 (4.90), 661 (5.03). MS (MALDI-TOF): m/z 1979 (calcd. for $[M]^+$ 1979).

***Copper(II) phthalocyanine* (3-*Cu*).** Yield 0.042 g (30.49%), mp > 250 °C. Anal. calcd. for $C_{88}H_{40}N_8O_{16}F_{24}Cu$: C, 53.25; H, 2.03; N, 5.65%. Found: C, 53.61; H, 2.21; N, 5.45%. FT-IR: υ_{max}, cm^{-1} 3067 (Ar–H), 1344 (CF_3), 1091 (Ar–O–Ar). UV-vis (THF): λ_{max}, nm (log ε) 351 (4.68), 673 (5.11). MS (MALDI-TOF): m/z 1983.50 (calcd. for $[M]^+$ 1983.50).

Microorganisms and media

The microorganisms used in this study were purchased from Microbiologics Inc. Antimicrobial activities of the molecules were examined against one Gram (-) (*Escherichia coli* ATCC 25922) and five Gram (+) bacteria (*Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 14579 and *Staphylococcus aureus* ATCC 25923).

For disk-diffusion assays, all the stock cultures were inoculated in Mueller–Hinton Broth (MHB). After 24 h incubation at 37 °C, microorganisms were added to the 5 mL deionized-water-containing sterile tubes and adjusted to 0.5 McFarland standard (1.5×10^8 CFU/mL) by using UV-spectrophotometer (Optimizer) [51].

In the TLC-direct bioautography method, bacterial colonies were put into 10 mL MHB-containing sterile tubes and incubated at 37 °C overnight. After incubation, obtained bacterial suspensions were diluted 1 : 200 portions in MHB. Then 5 mL of diluted bacterial suspension were added to the 20 mL MHB and stirred on a magnetic stirrer at 37 °C for 5 h. A 20 mL amount of MHB (pH 7.2 ± 0.2) was inoculated with 1 mL bacterial suspension obtained directly from the additional pre-incubation and put on the magnetic stirrer at 37 °C for 7 h [61].

Disk diffusion assay

Antimicrobial activities of the molecules were determined by using the disk diffusion assay described by Saki *et al.* [56]. Samples from prepared bacterial suspensions were inoculated by using sterile swabs to the petri dishes containing Mueller–Hinton Agar (MHA). DMSO was used to dissolve the test compounds with a final concentration of 0.005 M. Blank disks were impregnated with 20 µL of prepared solutions of molecules and put on the inoculated-MHA-containing petri dishes. Blank disks impregnated with DMSO (20 µL) and ceftriaxone loaded disks (CRO-30) were used as the negative and positive controls, respectively. After incubation of inoculated petri dishes at 37 °C for 24 h, inhibition zones against the tested bacteria were evaluated.

TLC-direct bioautography

5 µL solutions of molecules at 0.005 M concentration were loaded on the plate (6 × 4 cm) by using micropipette and dried at room temperature. The bacterial suspensions were prepared according to the previously described procedure. The dried plates were immersed in prepared bacterial solutions for 8 s, then, placed in 5 × 8 cm polyethylene containers containing cotton wet with sterile deionized water and incubated for one night at 37 °C. After the incubation, chromatograms were sprayed with 0.2% MTT (3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyltetrazolium bromide) aqueous solution, re-incubated at 37 °C for 0.5 to 3 h, and the photos of bioautograms were taken [62].

DPPH assay

To evaluate the antioxidant activity, 100 $mg \cdot mL^{-1}$ stock solutions of molecules were prepared in CH_3Cl.

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Table 3. Antioxidant activity researches from the literature

PC	Metal	Concentration	Antioxidant activity			Reference
			DPPH activity	Reducing power	Chelating activity	
[Tetrakis (3,4,5-trimethoxybenzyloxy)phthalocyaninato] nickel (II)	Ni	100 mg · mL^{-1}	67.90%	—	—	57
4-(3,4,5-Trimethoxybenzyloxy)phthalonitrile	—	100 mg · mL^{-1}	—	—	96.70%	
[Co] 2(3),9(10),16(17),23(24)-tetrakis(4-(methylthio)phenylthio) phthalocyanine	Co	500 µg.mL^{-1}	54%	—	—	55
2(3),9(10),16(17),23(24)-tetrakis(4-(methylthio)phenylthio) phthalocyaninato metal free	—	100 µg · mL^{-1}	—	2.81 nm	79.86%	
1(4),8(11),15(18),22(25)-Tetrakis-3′-(thiophen-3-ylmethoxy)phthalocyaninatocobalt (II)	Co	100 mg · mL^{-1}	37.94%	—	—	
2(3),9(10),16(17),23(24)-Tetrakis-4′-(thiophen-3-ylmethoxy)phthalocyaninatocobalt (II)	Co	500 µg · mL^{-1}	—	—	81	58
2(3),9(10),16(17),23(24)-Tetrakis-4′-(thiophen-3-ylmethoxy)phthalocyaninatozinc(II)	Zn	100 mg · mL^{-1}	—	2.78 nm	—	
4-[(4′-(*Tert*-butyl)phenoxy)phenoxy]phthalonitrile	— Zn Co	100 mg · mL^{-1}	— 30% 40%	— — —	— — —	59

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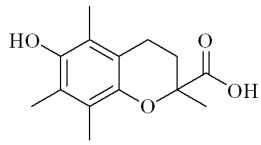
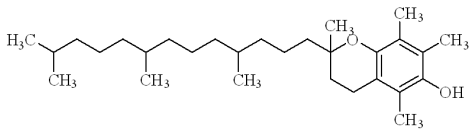
	Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble analog of vitamin E with antioxidant properties. Prevents peroxynitrite-mediated oxidative stress and apoptosis in rat thymocytes.
	Tocopherols (TCP) (vitamin E) are a series (α, β, γ and δ) of chiral organic molecules that vary in their degree of methylation of the phenol moiety of the chromanol ring. Tocopherols are lipid soluble anti-oxidants that protect cell membranes from oxidative damage. α-Tocopherol is the form of tocopherol preferentially absorbed by homo sapiens.

Fig. 7. Structures of *Trolox* and *Tocopherol*

As test solutions, molecules at different concentrations in CH_3Cl, *Trolox* (positive control, Fig. 7) in methanol and CH_3Cl (negative control) were prepared. DPPH activities of the compounds were evaluated according to the procedure earlier given by Pascoal *et al.* [63]. 1 mM DPPH solution in methanol was prepared and 0.5 mL of this solution was added to the 1 mL of test solutions in the test tubes and mixed gently. This solution was incubated at room temperature in the dark for 1 h and the absorbance was read at 517 nm spectrophotometrically (Optimizer). The percentage scavenging activity was calculated by following formula:

$$DPPH\ scavenging\ activity\ (\%) = \frac{A\,control - A\,sample}{A\,control} \times 100$$

where A control is the absorbance of the control reaction mixture and A sample is the absorbance of the sample.

Reducing power

1 mL solutions of molecules at different concentrations were mixed with 2.5 mL of 200 mM sodium phosphate buffer and 2.5 mL of 1% potassium ferricyanide. Then the mixture was incubated at 50 °C for 20 min. After addition of 2.5 mL 10% trichloroacetic acid, the mixture was centrifuged at 3000 rpm for 10 min. and 0.5 mL 0.1% ferric chloride along with 2.5 mL deionized water added to the upper layer. Finally, the absorbance was measured at 700 nm using a UV-vis spectrophotometer (Optimizer). α-*Tocopherol* (Fig. 7) was chosen as the control [63].

CONCLUSION

In this study, a novel peripherally disubstituted phthalonitrile derivative bearing 4-(trifluoromethoxy) phenoxy groups was prepared and utilized for synthesis of new MPcs. All the analyzed data of newly synthesized Pcs showed good agreement with the proposed structure. Remarkably, they show good solubility in common organic solvents. The spectroscopic properties of **3-Zn** were examined in diverse solvents such as DCM, acetone, THF, ethylacetate, DMF, and etc. According to the Lambert–Beer law, no aggregation was observed for compound **3-Zn** in DCM at concentrations ranging from 4×10^{-6} to 14×10^{-6} M. Compound **3-Zn** also showed the highest antioxidant activity on both DPPH scavenging activity and reducing power assays. For the determination of antimicrobial activity of synthesized molecules, TLC-direct bioautography assay and disk diffusion assays were used. In all performed assays, test molecules showed antimicrobial activity against gram (+) bacteria.

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