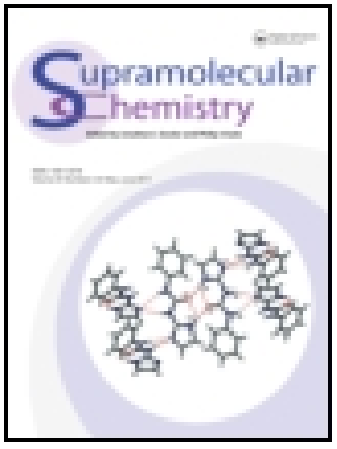

Supramolecular Chemistry

Publication details, including instructions for authors and subscription information:
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Novel copper(II) complexes of p-tert-butylcalix[4]arene diamide derivatives: synthesis, antimicrobial and DNA cleavage activities

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Published online: 19 Nov 2014.

To cite this article: Şeyda Çiğdem Özkan, Aydan Yilmaz, Emine Arslan, Leyla Açık, Ülkü Sayın & Elif Gülbahçe Mutlu (2014): Novel copper(II) complexes of p-tert-butylcalix[4]arene diamide derivatives: synthesis, antimicrobial and DNA cleavage activities, Supramolecular Chemistry, DOI: 10.1080/10610278.2014.978868

To link to this article: http://dx.doi.org/10.1080/10610278.2014.978868

Supramolecular Chemistry, 2014
http://dx.doi.org/10.1080/10610278.2014.978868

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Novel copper(II) complexes of *p-tert*-butylcalix[4]arene diamide derivatives: synthesis, antimicrobial and DNA cleavage activities

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(*Received 22 August 2014; accepted 12 October 2014*)

In this study, two new Cu(II) complexes of *p-tert*-butylcalix[4]arene amide derivatives (**8**, **9**) have been synthesised and investigated their DNA cleavage and antimicrobial activities. On the basis of spectral studies, a smaller distortion of square planar geometry has been proposed for both of the copper(II) complexes. The DNA cleavage studies show that the ligands are not efficacious, whereas the complexes have high activity. Futhermore, in order to determine the site of DNA cleavage, the DNA interactions of these compounds were investigated with some restriction enzymes. In addition, all synthesised compounds were screened for antimicrobial activities against some bacteria and for antifungal activities against yeast strains. The results showed that ethyl ester and furfuryl amide derivatives of the calixarenes are more efficient than other compounds against tested bacteria. However, complexes have not been effective. In case of DNA interaction studies, compounds were very effective against plasmid DNA.

Keywords: calix[4]arene; copper(II) complex; EPR; DNA cleavage activity; restriction enzyme; antimicrobial

1. Introduction

Calixarenes are vase-shaped macrocyclic compounds composed of phenolic units and connected by methylene bridges. They are commonly used to create selective metal cation extractants, sensor materials, catalysts, etc. (*1*). Furthermore, the biological properties of calixarenes such as antimicrobical agents (*2*), cytotoxicity (*3*), DNA binding (*3*), DNA interaction (*4*), protection from ultraviolet radiation (*5*), anticancer agents (*6*), anti-HIV agents (*7*), enzyme and protein inhibitors (*8*, *9*), protein recognition (*10*), antioxidant–antiradical activities (*11*) and other studies (*12*) have been recently investigated. Although many of the metal complexes of calixarenes were synthesised (*13*) until now, they have little field of application in DNA studies (*6a*). In general, metal ions and complexes have great potential in biological studies and applications. Especially, transition metal complexes including that amide and Schiff base ligands are used in biologic fields such as antimicrobial studies (*14*), DNA cleavage and interaction studies (*14b–14d*, *15*), anticancer agents (*16*) and cytotoxicity and apoptosis studies (*17*) because transition metals such as copper, cobalt and manganese are bioessential elements. Copper is an especially important element for humans with its bioessential activity (*17*). Cu(II) complexes have great capacity to DNA cleavage or interaction (*18*). Moreover, these complexes also have activity in the antimicrobial and antifungal studies (*19*). Considering the importance of copper(II) complexes in bacteriology and DNA cleavage studies, we report herein the synthesis and characterisation of two new copper (II) complexes of *p-tert*-butylcalix[4] arenes with amide derivatives. The antimicrobial properties and DNA cleavage activities of ligands and copper(II) complexes were also discussed. In addition, the determination of the site of DNA cleavage with *BamH* I and *Hind* III restriction enzymes was carried out for all compounds.

2. Results and discussion

2.1. Synthesis of calixarene derivatives and the complexes

The *p-tert*-butylcalix[4]arene **1** and derivatives **2**–**4**, **6** and **7** were synthesised according to the literature procedures (*20–27*) (Scheme 1). After synthesising the diethyl-ester derivative of *p-tert*-butylcalix[4]arene, hydrolysis process is carried out by a different method. According to the literature (*24*), this reaction is carried out under reflux and in a 24-h period, but we have accomplished with high efficiency *via* microwave in a very short period of time (10 min). Amide derivatives of calix[4]arene (*4*, *6*, *7*) were synthesised according to literature (*26*, *27*). The compound **5** was obtained by being put into the reaction in the medium of toluene/methanol with the compound **2** and 2-tetrahydrofurfur-

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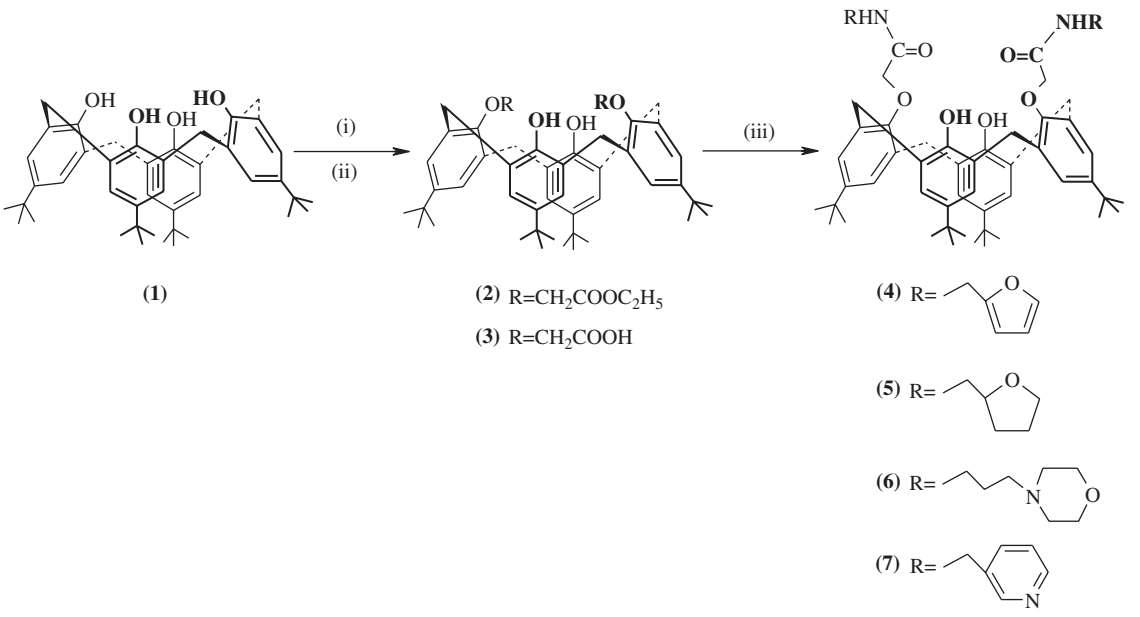

Scheme 1. (i) Ethylbromoacetate, K_2CO_3, acetone, 24 h; (ii) NaOH, ethanol, MW, 600 W (power %100), 10 min; (iii) primary amine, toluene–methanol, 72 h.

ylamine. The complexes (**8** and **9**) were synthesised by the different pathways each other. Compound **8** was synthesised according to the modified literature procedures (*13a*) and compound **9** was synthesised with a different method.

2.2. *Liquid–liquid extraction studies*

In this study, liquid–liquid extraction experiments examined the selectivity of the compounds that synthesised against some transition metal ions [Cu(II), Co(II), Cd(II), Zn(II), Ni(II), Mn(II), Pb(II)]. The extraction activity of the ligands towards transition metal ions was determined by Pedersen's picrate extraction method (*28*). The extraction efficiency of the ligand was carried out by the two-phase solvent extraction of transition metal picrates into chloroform under neutral conditions. The data were obtained using a chloroform solution of these diamide compounds to extract metal picrates from an aqueous phase. The equilibrium concentration of picrate in aqueous phase was determined spectrophotometrically. The data are expressed as percentages of the cation extracted (*E%*) by the ligand (Figure 1).

According to the extraction results, the effect of compound **5** was little, whereas compound **4** showed more activity towards the metals used, Cu^{2+} and Pb^{2+} ions. Compounds **6** and **7** showed the most selectivity against the transition metals. These compounds showed the most efficacy against Cu(II) and Pb(II) ions. Compound **6** extracted Cu(II) ions at a total amount of 83.2%, and Pb(II) ions at the total amount of 90.1% that were the highest, whereas compound **7** extracted Cu(II) ions at a total amount of 52.2%, and Pb(II) ions at a total amount of 38.7%. Compounds **6** and **7** both showed high activity against Cu(II) ions, and due to the biological importance of Cu ions, the Cu (II) complexes of these compounds were synthesised.

2.3. *Characterisation of Cu^{2+}–calixarene complexes*

Characterisation studies of Cu(II) complexes were carried out using elemental analysis, electron pair resonance (EPR), ultraviolet-visible spectroscopy (UV-Vis.), infrared spectroscopy (IR), and atomic absorption spectroscopy (AAS). In addition, the colorimetric method was applied for the determination of nitrate and chloride using a spectrophotometer (*29*, *30*) and suitable reagents were

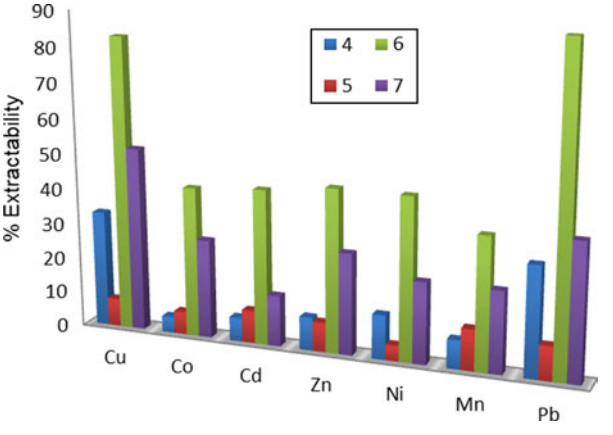

Figure 1. (Colour online) Extraction percentages of the metal picrates with ligand 1:1 (v/v): [picric acid] = 2×10^{-5} M, [ligand] = 1×10^{-3} M, chloroform; [metal nitrate] = 1×10^{-2} M, 298 K, 1-h contact time.

supplied by Hach Lange Company, Dublin, Ireland. The elemental analysis and atomic absorption measurements showed that the metal–ligand ratio of all complexes (**8** and **9**) is 1:1.

IR is one of the important techniques used in the recognition of complexes. Figure 2 shows the IR spectra of ligand and ligand–metal complexes. Although IR spectra of compounds **6** and **7** were observed amide peak (C═O) 1685 and 1682 cm^{-1}, respectively, in the IR spectra of **8** and **9** compounds, these bands were shifted to 1656 and 1651 cm^{-1}, respectively (Figure 3). The amide II band (N—H bending) that belong to ligand **7** in 1577 and 1597 cm^{-1} shifted to 1600 cm^{-1}. The C—N stretching band in 1339 cm^{-1} that belong to ligand **7** disappeared at

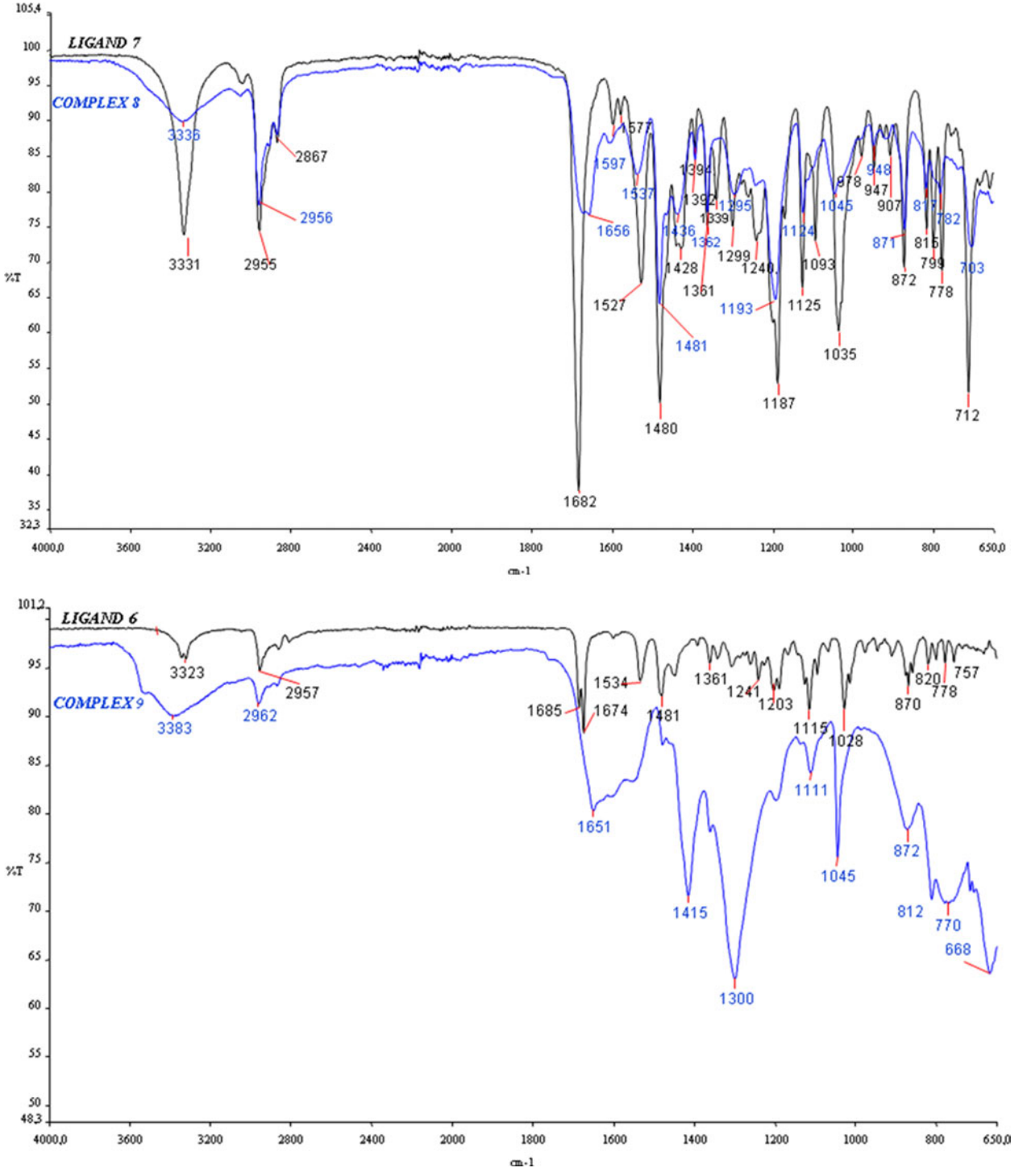

Figure 2. (Colour online) IR spectra of ligands and complexes.

complex **8**. The aliphatic C—H in plane bending band shifted to 1481 cm^{-1} by losing the sharpness (Figure 2). The aliphatic C—H in plane bending band were observed by forming a broad and sharp band, whereas this band does not exist at the ligand **6**. The C—O—C stretching band in 1045 cm^{-1} that belong to complex **9**, were observed more sharply and intensely. In the IR spectrum of complex, **9** was observed to a band that belong to nitrate sharply (Figure 2).

In addition, it was confirmed to the formation of the absorption bands at the different wavelengths by making measurements for ligands, metal salts and both of complexes with UV–vis spectrometer (Figure 3).

The absorption spectra of **8** and **9** (1×10^{-5} M) were recorded in CH_3CN. For compound **8**, increase in absorption intensity of the around 230 nm band is observed (Figure 3). For compound **9** also, differently from ligand **6** and $Cu(NO_3)_2{\cdot}3H_2O$, which used metal salt, showed a new band around 250 nm (Figure 3). In addition, the band around 300 nm that belong to ligand **6** decreased with formed complex (Figure 3).

EPR studies of paramagnetic transition metal ions yield a great deal of information about the magnetic properties of the paramagnetic centre. They also lead to understanding of the nature of bonding of the metal ions with ligands. The $3d^9$ electron configuration of Cu^{2+} is of interest in transition metal complexes because it represents a relatively simple magnetic hole system. The copper(II) ion has an effective spin of $S = 1/2$ and is associated with spin angular momentum, $m_s = \pm 1/2$, leading to doubly degenerate spin state in the absence of a magnetic field. In a magnetic field, the degeneracy is lifted between these states (*31*). X-band EPR spectra of copper(II) complexes **8** and **9** were measured on a JEOL JESFA300 spectrometer (JEOL, Tokyo, Japan) with 100 kHz modulation frequency. EPR spectra of powder samples can sometimes give misleading *g* values because in sufficiently crystalline samples, the orientation of spins in the material are not truly random and many times there are

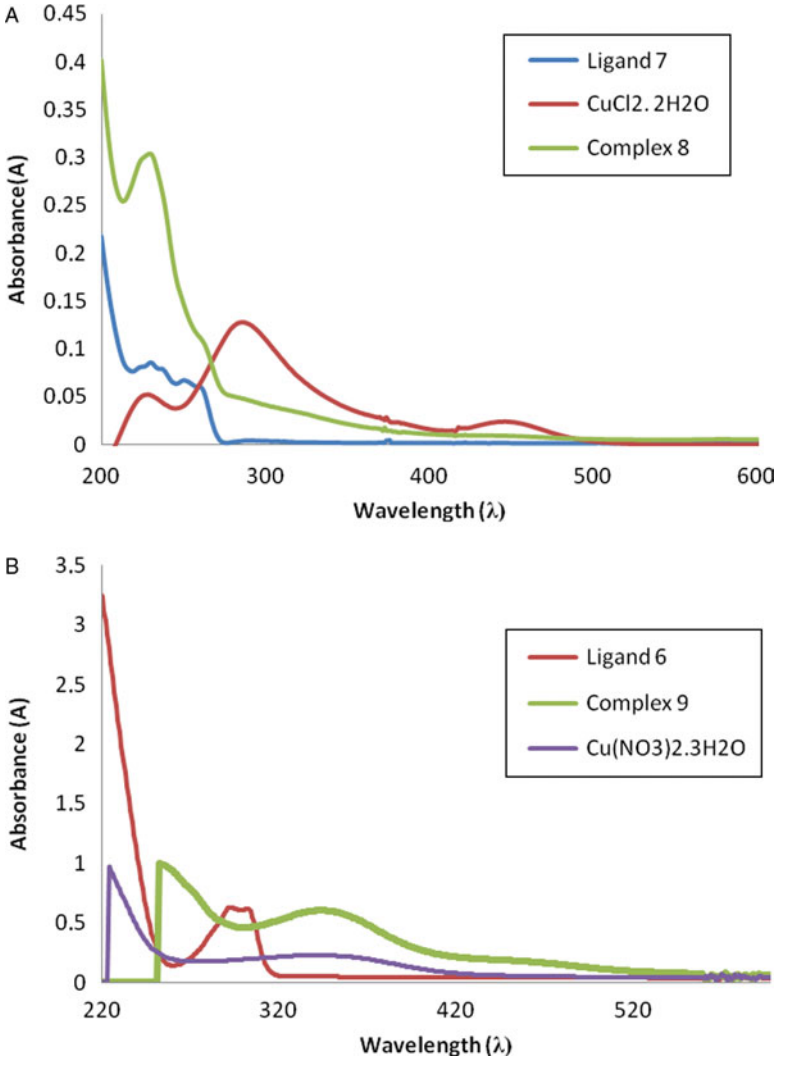

Figure 3. (Colour online) UV–vis spectra of ligands, metal salts and complexes (A and B) (1×10^{-5} M in CH_3CN).

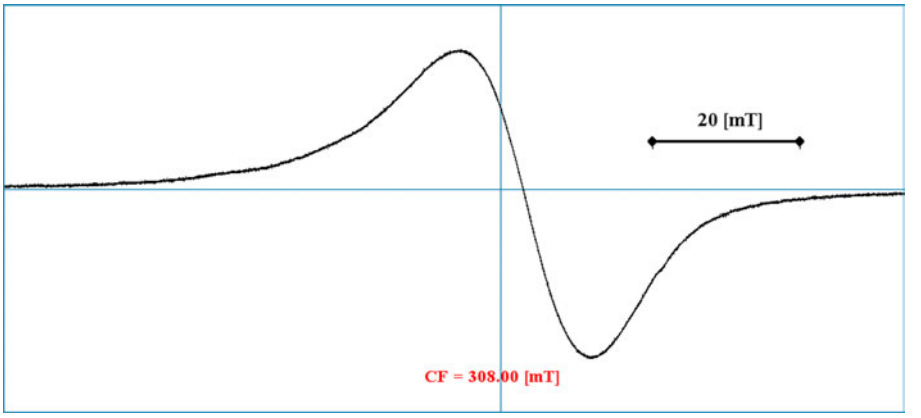

Figure 4. (Colour online) EPR spectra of complex **8** powder at 298 K.

significant dipolar interactions between molecules in the sample (*32*). The EPR spectrum of complex **8** in the polycrystalline state recorded at 298 K given in Figure 4 shows only one broad signal, which is attributable to enhance spin lattice relaxation due to dipolar interaction.

The EPR spectra and relevant simulations of complex **8** and **9** in frozen dimethylformamide (DMF) solution at 123 K temperature are shown in Figure 5. The simulations were done by using calculated ESR parameters of the complexes. The same ESR spectrometer conditions for

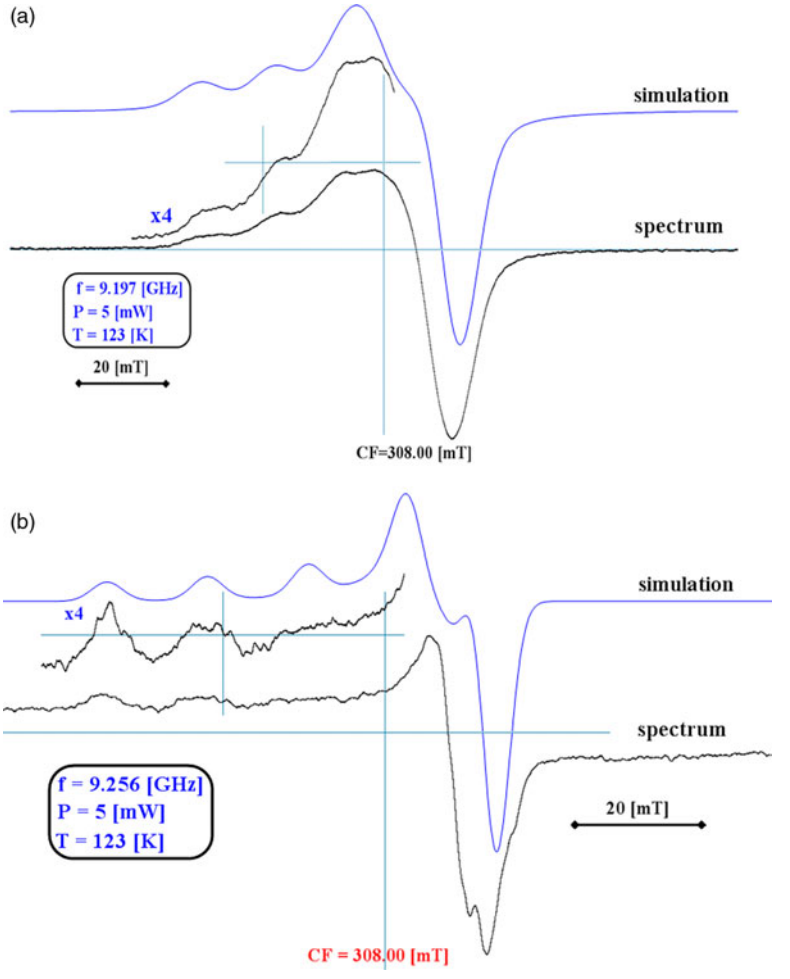

Figure 5. (Colour online) EPR spectra and relevant simulations of complexes in frozen DMF solution: (A) Complex **8** and (B) Complex **9**.

Table 1. EPR parameters of copper(II) complexes.

Complex	$g_{\parallel}$	$g_{\perp}$	g_{av}	$A_{\parallel}$ (10^{-4} cm^{-1})	$A_{\perp}$ (10^{-4} cm^{-1})	a_{av} (10^{-4} cm^{-1})
8	2.2393	2.0745	2.1294	172.5	42.6	85.9
9	2.2912	2.0641	2.1398	174.4	29.8	78.0

two complexes are: microwave power, 5 mW; centre field, 308 mT; sweep width, 200 mT; temperature, 123 K; sweep time, 30 s; time constant, 0.03 s. In addition, the special conditions for complex **8** are: modulation amplitude, 800; modulation width, 0.12 mT; microwave frequency, 9.197 GHz; accumulation, **5**, and for complex **9** are: modulation amplitude, 600; modulation width, 0.3 mT; microwave frequency, 9.206 GHz; accumulation, 15.

The spectra are axial in nature and $g_{\parallel} > g_{\perp} > 2.0023$, indicating $d_{x^2-y^2}(^2B_{1g})$ ground state, which is characteristic of a square planar geometry (*33–36*). The EPR parameters of the complexes are determined and given in Table 1. The g_{av} value calculated from the relation: $g_{av} = 1/3(g_{\parallel} + 2g_{\perp})$ were found to be equal to 2.1294 and 2.1398 for complex **8** and **9**, respectively. The deviations of the g_{av} from that of the free electron (2.0023) are due to the covalent nature (*37*, *38*). The geometric parameter G, a measure of exchange interaction between the copper centres, were calculated for both samples using the equation:

$$G = \frac{g_{\parallel} - 2.0023}{g_{\perp} - 2.0023}$$

If $G > 4$, exchange interaction is negligible, and if it is less than 4, considerable exchange interaction is indicated in the complex (*39*). There exists considerable exchange interaction in the complex **8** with $G = 3.28$ and negligible exchange interaction in the complex **9** with $G = 4.67$.

The ratio $g_{\parallel}/A_{\parallel}(f)$ can be used to predict the geometry adopted by copper complexes. In square planar complexes, this parameter is in the range of 105–135 cm, whereas the value falls to the range of 135–258 cm for distorted tetrahedral complexes (*40–43*). In further detail, f values of 110–120 cm are typical for planar complexes, whereas the range of 130–150 cm is characteristic of slight to moderate distortion and 180–250 cm indicate considerable distortion (*44*, *45*). The calculated f values of complex **8** and **9** are 130 and 131 cm, respectively, indicating smaller distortion of square planar geometry around copper. The covalency parameters α^2 were calculated using the following equation (*46*):

$$\alpha^2 = A_{\parallel}/0.036 + (g_{\parallel} - 2.0023) + 3/7(g_{\perp} - 2.0023) + 0.04$$

If the value $\alpha^2 = 0.5$, it indicates complete covalent bonding, whereas the value of $\alpha^2 = 1.0$ suggests complete ionic bonding. The calculated values of α^2, i.e. 0.79 and 0.84 for complex **8** and **9**, respectively, are less than unity, which indicate that the complexes have some covalent character in the ligand environment (*40*, *46–49*).

So, the proposed structure of the copper(II) complexes of calix[4]arene (**8**, **9**) shown in Figure 6.

2.4. *Antibacterial and antifungal activity*

Table 2 shows the MIC values of the compounds against gram-positive, gram-negative bacteria and two species of fungi. MIC values were ranged from 39 to 10,000 μM. According to the obtained MIC values, while *Bacillus*

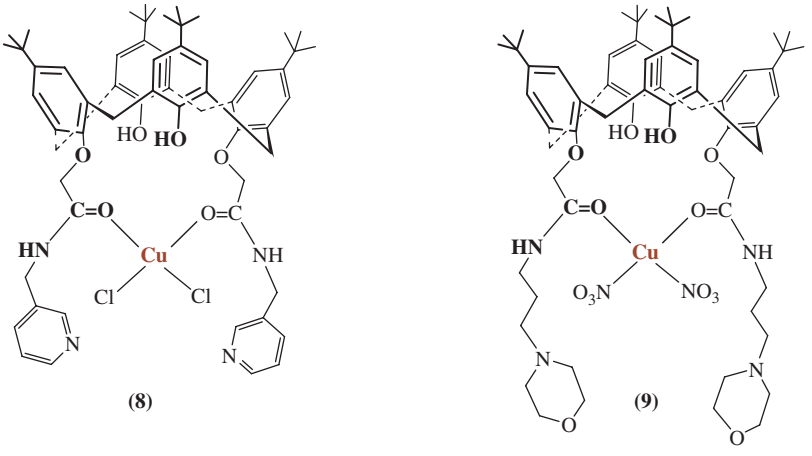

Figure 6. (Colour online) Proposed structures of calix[4]arene copper(II) complexes (**8** and **9**).

Table 2. MIC values ($\mu g\,mL^{-1}$) obtained by broth microdilution method, according to CLSI guideline.

	Gram(+)				Gram(−)				Fungi	
Compounds	*B. subtilis*	*B. cereus*	*E. faecalis*	*S. aureus*	*E. coli* ATCC 25922	*E. coli* ATCC 35218	*P. aeruginosa*	*P. vulgaris*	*C. albicans*	*C. tropicalis*
2	78	39	156	156	39	39	1250	156	[a]	[a]
3	5000	10,000	10,000	10,000	10,000	5000	5000	10,000	10,000	10,000
4	1250	39	39	39	625	78	5000	5000	2500	2500
5	5000	2500	1250	1250	10,000	5000	5000	5000	2500	2500
6	10,000	10,000	10,000	10,000	10,000	2500	2500	2500	2500	5000
7	10,000	10,000	1250	625	1250	2500	5000	2500	10,000	2500
8	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]
9	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]

[a] Not studied.
[b] Not detected to MIC value in the studied concentrations (concentration ≤ 10,000 $\mu g\,mL^{-1}$).

subtilis is sensitive to compound **2** (MIC value 39 $\mu g\,mL^{-1}$), it shows less sensitivity to compound **4**. On the other hand, this bacterium did not show sensitivity to compounds **3** and **5** (MIC values 5000 $\mu g\,mL^{-1}$), whereas it was resistant to compound **6** (MIC value 10,000 $\mu g\,mL^{-1}$). In comparison, *Bacillus cereus* bacteria is sensitive to compounds **2** and **4**, less sensitive to compound **5** and resistant to compounds **3** and **6**. Although *Enterococcus faecalis* and *Staphylococcus aureus* bacteria are quite sensitive to compound **4**, they are less sensitive to both **2** and **5**, and resistant to compounds **3** and **6**.

According to the MIC values, compounds **2** and **4** are effective against *Escherichia coli* ATCC 25922 and *E. coli* ATCC 35218 bacteria. *Pseudomonas aeruginosa* and *Proteus vulgaris* bacteria are more sensitive to compounds **2** and **6**, respectively, and studied concentrations for *P. vulgaris* are resistant to compound **3**. *Candida albicans* fungus shows less sensitivity to compounds **4**–**6**, whereas it is resistant to the other compounds. *Candida tropicalis* fungus shows more sensitivity to compounds **3**–**5** and **7**. The complexes showed no activity against the used bacteria and yeast. It was thought that the reason for this event was that the complexes did not pass through the bacterial cell membrane because of their huge molecular structures.

2.5. *Interaction with pBR322 plasmid DNA and restriction enzyme digestion*

According to some studies, drugs that are developed by targeting DNA to treat infective diseases can destroy bacteria cells (*50*, *51*). It has been reported that copper complexes have cytoxicity against HeLa cancer cells and destroy by passing from the apoptosis period (*52*). Previous studies have shown that copper complexes form DNA cleavages or are bound to DNA due to their high nucleolytic effects.

Interactions of synthesised calixarene compounds and formed Cu(II) complexes with supercoiled pBR322 DNA were investigated using agarose gel electrophoresis. When circular plasmid DNA is subjected to electrophoresis, the fastest migrating supercoiled Form I, the slower moving open circular Form II, and the linear Form III will all be generated with migration in between (*53*). As shown in Figure 7, the mobility of Form I and Form II was found to increase by compound **2**, whereas the mobility of Form I and Form II decreased interaction with compound **3**. Also, the mobility of Form I and Form II DNA decreased for compound **4**. When the plasmid interacted with compound **5**, the mobility of Form I and Form II were decreased due to binding with the compound. The supercoiled and open circular DNA disappeared completely in the two highest concentrations, and linear DNA was observed to interact with compound **5**. In the case of compound **6**, the intensity

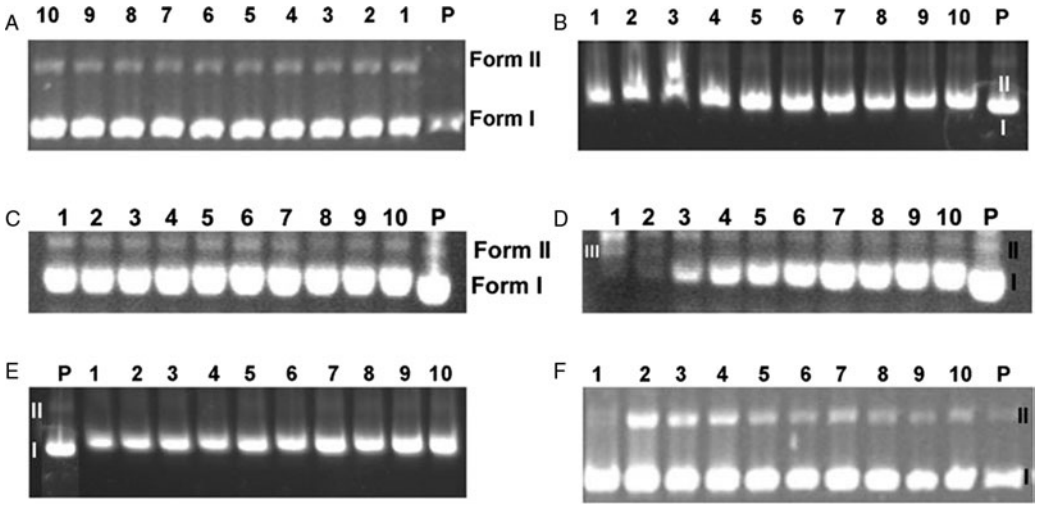

Figure 7. Modification of gel electrophoretic mobility of pBR322 plasmid DNA after treated with various concentrations of compounds **2**–**7**. Lane 1 (P), DNA control, P: untreated plasmid DNA; lane 1 → 10, 10,000, 5000, 2500, 1250, 625, 312.5, 156, 78, 39, 19.5 μM, respectively. (A) Compound **2**; (B) compound **3**; (C) compound **4**; (D) compound **5**; (E) compound **6**; (F) compound **7**.

and mobility of supercoiled Form I DNA decreased, whereas the mobility of Form I and Form II DNA decrease for compound **7** in the highest four concentrations. In decreasing concentrations for compound **7**, its mobility closed to the control plasmid DNA. At the highest concentration, compound **8** and in the first eight concentrations of compound **9** destroyed the DNA completely (Figure 8).

The mobility of Form I and Form II decreased in the other decreasing concentrations of the compound **8**. This shows that compound **8** connected to the plasmid DNA and slowed down due to the increasing molecular weight. As shown in Figure 8, the mobility of Form I and Form II decreased interaction with compound **9** in the other decreasing concentrations. Even containing trace amounts of compounds such as the 15th dilution 0.6 μM did not give a profile of the control plasmid DNA.

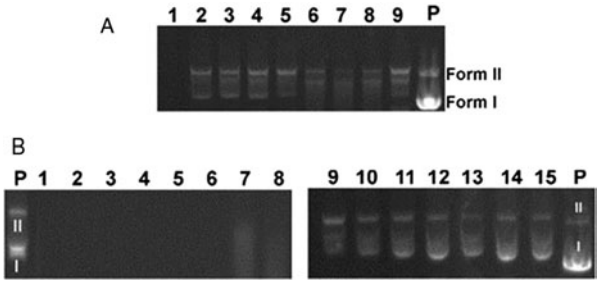

Figure 8. Modification of gel electrophoretic mobility of pBR322 plasmid DNA after treated with various concentrations of compound **8** (A) and **9** (B). Lane 1 (P), untreated pBR322 plasmid DNA; (A) lane 1–9; 10,000, 5000, 2500, 1250, 625, 312.5, 156, 78, 39 μM, respectively. (B) Lane 1–15; 10,000, 5000, 2500, 1250, 625, 312.5, 156, 78, 39, 19.5, 9.7, 4.8, 2.2, 1, 0.6 μM, respectively.

DNA cleavage was studied using electrophoresis by displaying the conversion of supercoiled Form I open circular DNA into Form II and Form III linear DNA. In order to assess whether calixarene compounds and formed Cu(II) complexes show affinity for guanine–guanine (GG) and/or adenine–adenine (AA) regions of DNA, the compound–DNA adducts were digested with *BamH* I and *Hind* III enzymes. *BamH* I and *Hind* III enzymes recognise DNA at the G/GATCC and A/AGCTT and hydrolyse phosphodiester bonds between nucleotides, respectively (*54*). The digestion of plasmid DNA with these enzymes converts supercoiled plasmid Form I DNA into linear Form III DNA. However, when the compound binds to nucleotides at the restriction site, enzyme digestion will be prevented. Figures 9 and 10 give the electrophoretograms as applied to incubated mixtures of plasmid DNA and compounds that were digested with *BamH* I and *Hind* III enzymes for a period of 1 h at 37°C. When the incubated mixtures of plasmid DNA and compounds **2**–**9** were digested with *BamH* I, two bands corresponding to Form I and II were observed for **2** and **4** because of not cutting whereas only one band was observed for compounds **3** due to cut. Enzyme digested targeting only the supercoil-structured Form I in compound **5** and **6** and two bands were observed. There was not a full cut in compound **7**; therefore, Form I, Form II and Form III were observed. When the incubated mixtures of plasmid DNA and compounds **2**–**9** were digested with *Hind* III, two bands (Form I and Form II) were observed for compounds **2**, **3** and **4** and only one band was observed for compounds **5** (Figures 9 and 10). Enzyme digested targeting only the supercoil-structured Form I in compound **6** and **7** and transformed into the linear structure (Form III).

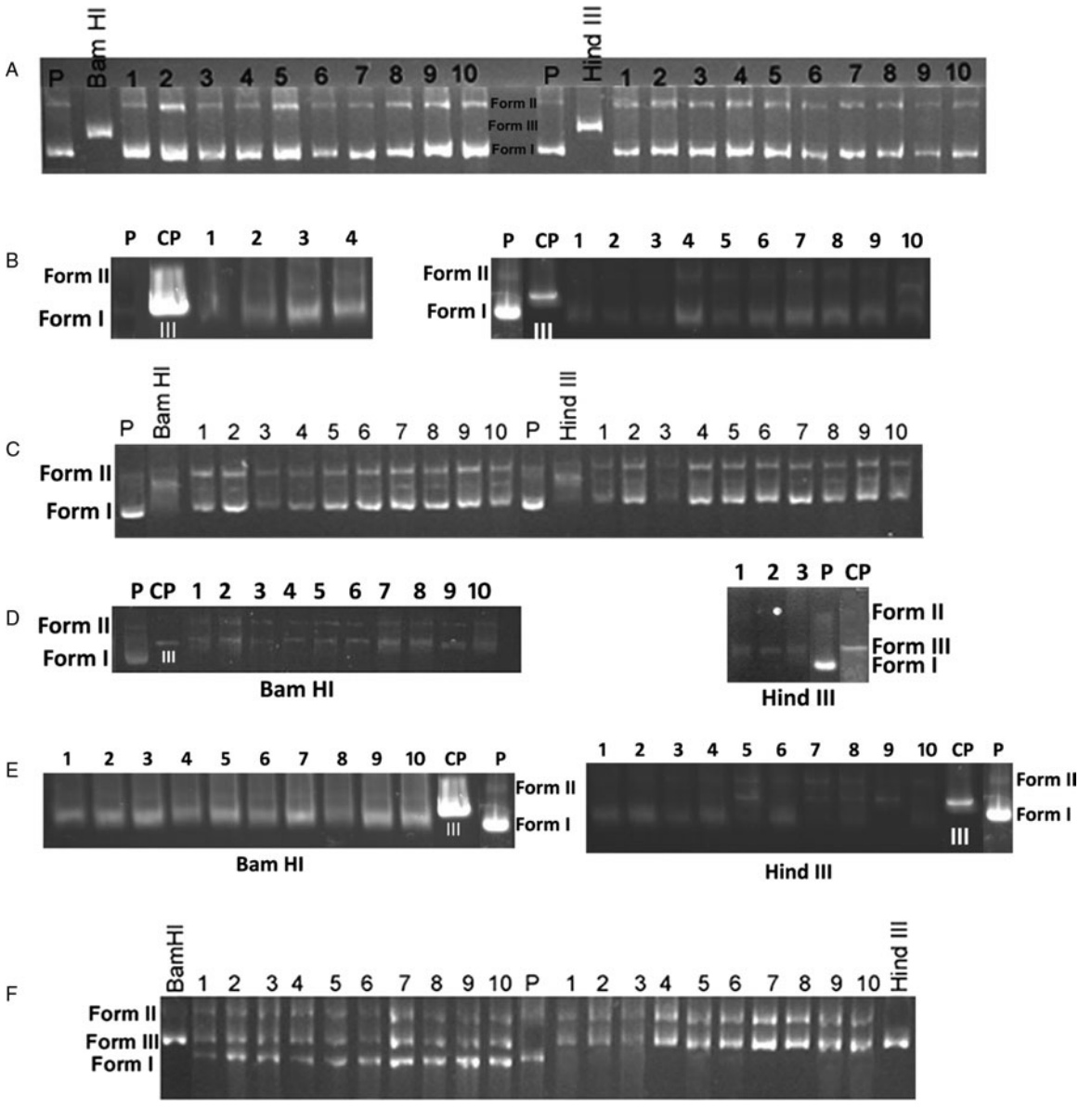

Figure 9. Digestion of pBR322 plasmid DNA–compounds **2**–**7** with Restriction Endonucleases (*BamH* I and *Hind* III, left to right). P: untreated pBR322 plasmid DNA; CP: cut pBR322 plasmid DNA, (A) compound **2** lane 1–10; 10,000–19.5 μM, (B) compound **3** lane 1–4; 10,000–1250 μM, lane 1–10; 10,000–19.5 μM, (C) compound **4** lane 1–10; 10,000–19.5 μM, (D) compound **5** lane 1–10; 10,000–19.5 μM, lane 1–3; 10,000–2500 μM, (E) compound **6** lane 1–10; 10,000–19.5 μM, (F) compound **7** lane 1–10; 10,000–19.5 μM, respectively.

Restriction enzymes *BamH* I and *Hind* III in compound **8**, its cut was carried out by targeting only the supercoil-structured Form I. As shown in Figure 10, the open circular plasmid DNA was not cut. As DNA fragmented in the first eight concentrations of compound **9**, the results of cut with restriction enzymes were not able to be observed. As a result of the cutting of both restriction enzymes in the dilutions of 9th and 15th, the enzymes targeted only Form I; therefore, the DNA that was transformed into the linear structure was observed as Form III in Figure 10. The open circular structure of the plasmid, Form II was not cut.

3. Conclusion

In this study, calix[4]arene derivatives such as ester, acid and four amides have been synthesised. The copper complexes of amide derivatives with pyridine and morpholino groups were synthesised and characterised by different techniques. The spectroscopic results indicated that the ligands provided a smaller distortion of square planar geometry around the copper centre. Antimicrobial studies were carried out with prepared ligands (**2**–**7**) and metal complexes (**8** and **9**). It was established that compounds **2** and **4** are more effective than

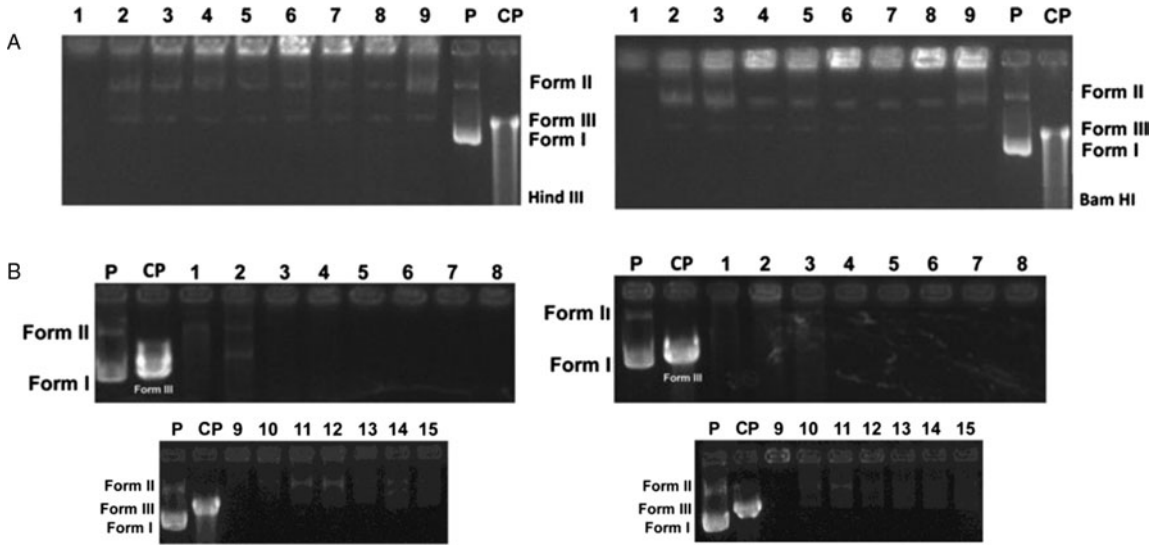

Figure 10. Digestion of pBR322 plasmid DNA–compound **8** (A) and **9** (B) with Restriction Endonucleases (*Hind* III and *BamH* I, left to right). P: untreated pBR322 plasmid DNA; CP: cut pBR322 plasmid DNA; (A) lane 1–9; 10,000–39.5 μM, (B) lane 1–8; 10,000–78.125 μM, lane 9–15; 39.5–0.6 μM, respectively.

other compounds. The DNA cleavage studies were performed with synthesised calix[4]arene derivatives (**2**–**7**) and metal complexes (**8** and **9**). Some chemical substances, binding to DNA and creating fractures in a DNA strand, cause changes that affect the structural and electrophoretic mobility of DNA such as the damages of DNA that inhibited DNA replication and transcription (*55*–*58*). As a carrier of genetic information, DNA is a major target for drug interactions because of the ability to interfere with transcription (gene expression and protein synthesis) and replication, a major step in cell growth and division. The results of DNA interaction studies suggest that each of compounds **2**–**9** was able to cause damage to the plasmid DNA. The DNA cleavage results showed that copper complexes are very effective, whereas calix[4] arene derivatives are not effective. The complexes cleaved the supercoiled pBR322 DNA into much smaller fragments. Although it showed that plasmid DNA cleaved to the highest concentrations of compounds **3**, **5** and complexes (**8**, **9**), it was concluded that compound **9** is the most effective on DNA. *BamH* I and *Hind* III digestion were used to gain further knowledge about the binding of compounds with plasmid DNA. The results of the studies showed that some compounds bind to G/G (compounds **2**, **4**, **6** and **7**) and A/A (compounds **2**, **3** and **4**) nucleotides.

4. Experimental section

4.1. Reagents and techniques

Chemicals and solvents were obtained from commercial sources [Fluka (St. Gallen, Switzerland), Merck (Darmstadt, Germany) and Sigma-Aldrich (Steinheim, Germany)] and used without further purification. DNA (supercoiled pBR322) and restriction enzymes (*BamH* I and *Hind* III), bacteria and funguses were purchased from Fermantas (Vilnius, Lithuania). Measurements of ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian MR 400 MHz spectrometer (Varian, Abington, UK) using TMS as an internal standard. A Perkin Elmer 1605 FT-IR spectrophotometer (Perkin-Elmer, Massachusetts, USA) was used to record the infrared spectra of all compounds (4000–400 cm^{-1}). Elemental analyses (C, H and N) were performed using a Leco 932 CHNS (LECO corporation, Michigan, USA). The UV–vis measurements were performed on a Shimadzu 1700 UV–vis spectrometer (Shimadzu Scientific Instruments, Columbia, USA). AAS measurements were performed on a continuum source atomic absorption spectrometer (ContrAA 300, Analytik Jena, Jena/Germany). X-band EPR was measured on a JEOL JESFA300 spectrometer with 100 kHz modulation frequency. Microwave irradiated reactions were performed by using a CEM MDS-2000 (Matthews, NC, USA). The determination of the melting points was performed using a Büchi B-540 (Flawil, Switzerland). Analytical TLC was performed on precoated silica gel plates (SiO_2, Merck PF254).

4.2. Synthesis of calixarene derivatives

4.2.1. Synthesis of compound 5

Yield: 75%. M.p: 251–254°C. IR: 1677 cm^{-1} (C=O). ^{1}H NMR ($CDCl_3$): δ (ppm) 1.02 (s, 18H, Bu^t), 1.26 (s, 18H, Bu^t), 1.52–1.58 (m, 4H, $OCH_2CH_2\underline{CH_2}$), 1.83 (p, 4H, $J = 7.1$ Hz, $OCH_2\underline{CH_2}CH_2$), 3.22–3.47 (overlapped, 6H, $ArCH_2Ar$, $NHCH_2$) 3.62–3.85 (overlapped, 6H, $ArCH_2$-

Ar, $NHCH_2$), 3.97–4.07 (m, 2H, OCH), 4.10–4.21 (m, 4H, $O\underline{CH_2}CH_2$), 4.46–4.65 (m, 4H, OCH_2CO), 6.90 (bs, 4H, $Ar\underline{H}$), 7.06 (bs, 4H, ArH), 7.70 (bs, 2H, OH), 9.08 (t, 2H, $J = 5.2$ Hz, NH). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm): 168.8, 149.8, 149.4, 148.1, 142.6, 132.4, 126.8, 126.1, 125.4, 75.1, 68.0, 65.6, 43.6, 34.2, 33.9, 32.1, 31.6, 31.0, 29.3, 25.6. FABMS *m/z*: 954.07 $(M + Na)^+$. Anal. calc.: $C_{58}H_{78}O_8N_2$ (931.25): C, 74.8%; H, 8.4%; N, 3.0%. Found: C, 75%; H, 8.2%; N, 2.9%.

4.2.2. Synthesis of Cu(II) complexes of calixarenes (**8** and **9**)

4.2.2.1. Synthesis of complex ***8***. A solution of $CuCl_2$-$2H_2O$ (36 mg, 0.21 mmol) in MeOH (5 mL) was added to a stirred solution of **7** (100 mg, 0.106 mmol) in CH_2Cl_2 (5 mL). The solution was filtered after 30 min of stirring and concentrated by a slow evaporation of solvents. The crude product was washed with MeOH and water and then dried in air. The product was obtained as a green solid with yield 55%. M.p: > 300°C IR (υ, cm^{-1}): 3336 (OH, stretching), 2956 (aliphatic C—H stretching), 1656 (C═O stretching), 1600 (second amide band), 1481 (aliphatic C—H bending). Anal. calc. for $C_{60}H_{72}CuO_6N_4Cl_2$: C, 66.7%; H, 6.7%; N, 5.2%. Found: C, 66.1%; H, 6.6%; N, 5.0%.

4.2.2.2. Synthesis of complex ***9***. A solution of $Cu(NO_3)_2{\cdot}3H_2O$ (35 mg, 0.196 mmol) in MeOH (5 mL) was added to a stirred solution of **6** (100 mg, 0.098 mmol) in acetone (5 mL). Then the solution was refluxed for 1 day, filtered and concentrated by slow evaporation of solvents. The crude product was washed with water and MeOH and then dried in air. The product was obtained as a green-brownish solid (55 mg, 50%). M.p: > 300°C IR (υ, cm^{-1}): 3383 (OH stretching), 1651 (C═O stretching), 1415 (aliphatic C—H bending) 1300 (NO_3), 1045 (C—O—C stretching). Anal. calc. for $C_{62}H_{88}CuO_{14}N_6$: C, 61.8%; H, 7.3%; N, 6.9%. Found: C, 60.8%; H, 7.2%; N, 6.5%.

4.3. Antibacterial and antifungal assays

The microdilution method is used in antimicrobial activity studies according to CLSI standards. Bacterial strains used in this study were *P. vulgaris* ATCC 8427, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *B. subtilis* ATCC 6633, *S. aureus* NTCT 8325, *E. faecalis* (a clinic sample), *B. cereus* NRRl-B-3711, and standard yeast strains were *C. albicans* ATCC 90028 and *C. tropicalis* ATCC 13803. Stock solutions of the compounds were prepared by dissolving with DMF and loaded onto micro-plates. After the dilution with DMF, different concentrations (10,000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.0625, 19.53125 $\mu g\,mL^{-1}$) were obtained. After micro-plates were prepared and inoculated, they were incubated for bacteria at 37°C for 24 h and for *C. albicans* at 30°C for 48 h; by determining the last well in which there was no reproduction, MIC values were identified.

4.4 DNA binding and cleavage experiments

The DNA cleavage studies were determined by using plasmids pBR322 with gel electrophoresis technique. The supercoiled pBR322 plasmid DNA was treated with different concentrations (10,000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.0625, 19.53125 $\mu g\,mL^{-1}$) of compounds for 24 h at 37°C. In the assays performed with cutting enzymes using gel electrophoresis technique, an enzyme and its buffer were added to the incubated DNA-compound mixture and it was incubated in an oven for 1 h more at 37°C. Then, the DNA–compound mixture and the mixture of DNA–compound incubated with restriction enzymes were electrophoresed for 3 h at 70 V on 1% agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed under UV light.

Funding

We thank the Research Foundation of Selcuk University, Konya-Turkey [grant number BAP 2009/10201020] for its financial support of this work produced from a part of Ş.Ç. Özkan's MS thesis.

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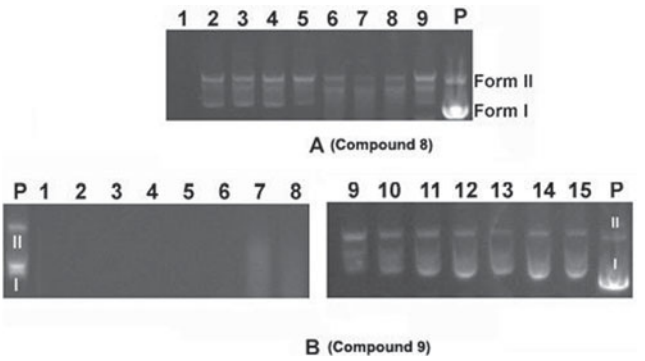

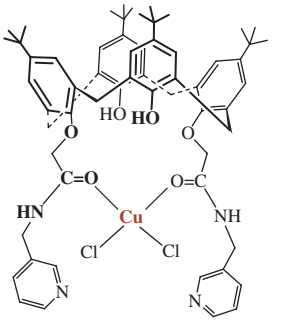

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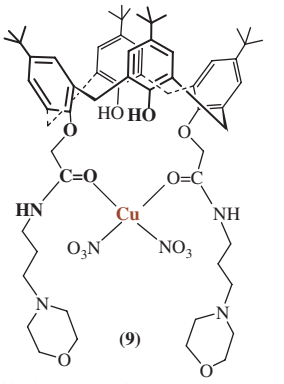

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Şeyda Çiğdem Özkan, Aydan Yilmaz, Emine Arslan, Leyla Açık, Ülkü Sayın and Elif Gülbahçe Mutlu

Novel copper(II) complexes of *p-tert*-butylcalix[4]arene diamide derivatives: synthesis, antimicrobial and DNA cleavage activities

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