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SYNTHESIS, CHARACTERIZATION, AND BIOLOGICAL ACTIVITY OF 1,3-DISUBSTITUTED BENZIMIDAZOLIUM SALT AND ITS Ag-NHC COMPLEX

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In this study, a 1,3-dioxane functionalized benzimidazole salt and its Ag-complex were synthesized. The prepared compounds were structurally verified by several spectroscopic techniques including ^{1}H NMR, ^{13}C NMR, FT-IR, LC-MS, and C, H, N analysis. Compounds were evaluated for their *in vitro* antioxidant activity. The salts and their Ag-NHC complexes were further evaluated for their *in vitro* anticancer activities against HCT-116 human colon cancer cells. The results showed that complex **2** exhibited high activity (4.1 times) against the cancer cell lines studied when compared to the reference drug cisplatin. Stability studies demonstrated that these compounds are stable in aqueous and organic solutions.

Keywords: silver complexes; *N*-heterocyclic carbene; benzimidazole; 1,3-dioxane; antioxidant and anticancer activity

СИНТЕЗА, КАРАКТЕРИЗАЦИЈА И БИОЛОШКА АКТИВНОСТ НА 1,3-ДИСУПСТИТУИРАНА БЕНЗИМИДАЗОЛНА СОЛ И НЕЈЗИНИОТ Ag-NHC-КОМПЛЕКС

Во оваа студија е синтетизирана бензимидазолна сол функционализирана со 1,3-диоксан и нејзиниот Ag-комплекс. Приготвеното соединение е структурно верифициарно со неколку спектроскопски техники вклучувајќи ги ^{1}H NMR, ^{13}C NMR, FT-IR, LC-MS, како и со C, H, N анализа. Соединенијата беа евалуирани за нивната *in vitro* антиоксидациска активност. Покрај тоа, тие беа евалуирани за нивната *in vitro* антиканцерогена активност во однос на канцерогени HCT-116 клетки на дебелото црево. Резултатите покажува дека комплексот **2** покажува 4,1 пати поголема активност во однос на испитаните канцерогени клетки споредено со референтниот цисплатин. Студиите за стабилност покажаа дека овие соединенија се стабилни во водни и во органски раствори.

Клучни зборови: сребрени комплекси; *N*-хетероцикличен карбен; бензимидазол; 1,3-диоксан; антиоксидациска и антиканцерогена активност

1. INTRODUCTION

Heterocyclic compounds are cyclic organic compounds containing one or more heteroatoms in the ring. The most common heteroatoms are nitrogen, oxygen, and sulfur, but heterocyclic rings containing other heteroatoms are also commonly known. Heterocyclic structures containing nitrogen are included in the structure of natural and synthetic drugs.[1] Benzimidazole, which is found in the structure of some natural compounds, forms an important part of nitrogen-containing heterocyclic structures.[2] In addition, it has antimicrobial, antitumor, antiulcer, and antiviral properties, as well as an important place in anticancer drugs.[3–7]

Carbenes are divalent neutral species containing six electrons in the valence shell. The declaration of the first free and stable carbene in 1991

has guided the study of many new chemical compounds. Compared to their counterparts such as phosphine, their steric and electronic properties (strong σ-donating and weak π-accepting) make these compounds unique ligands for coordination chemistry because they form more stable complexes with metals.[8] In addition, NHCs have been successfully used in metal-free chemical reactions.[9] 1,3-disubstituted benzimidazole salts with an acidic hydrogen in the 2-position are widely used as carbene precursors in the preparation of metal-NHC complexes. Benzimidazolium salts and their metal complexes have important biological properties, such as antimicrobial and anticancer activity, as well as successful applications in enzyme inhibition.[10–12] In addition, these compounds are used as strong and reactive catalysts in arylation, alkylation, transfer hydrogenation, amination, etc. reactions.[13–16]

Although pharmaceutical research studies are generally based on compounds containing gold and platinum, complexes of many metals such as rhodium, ruthenium, and rhenium have been extensively investigated in recent years. In addition, silver carbene complexes are receiving increasing attention due to their low toxicity, better stability, and fewer side effects.[17–19] Ag-NHC compounds have antibacterial, anticancer, anti-inflammatory, and antiseptic properties.[20,21] Besides, they exhibit good biocompatibility.[22] Ag(I) NHC complexes, which are used as carbene transfer agents, have been also used in the synthesis of other metal complexes such as palladium, platinum, and ruthenium.[23,24]

In this study, the 1,3-disubstituted benzimidazole salt **1** and its Ag-complex **2** were synthesized (Fig. 1). The structures of the synthesized compounds were characterized by (^{1}H NMR, ^{13}C NMR, FT-IR, LC-MS, and C, H, N analysis) spectroscopic methods. Afterward, the necessary studies were conducted to determine the antioxidant capacity of the synthesized compounds.

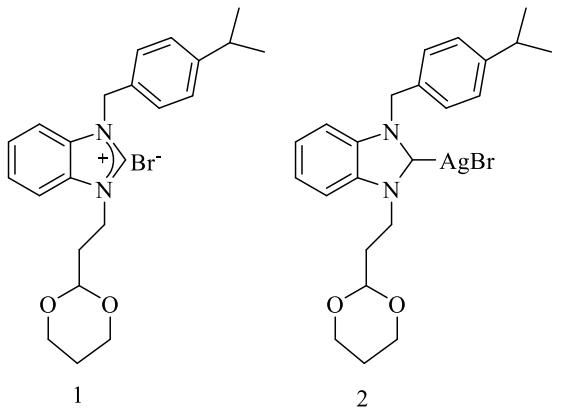

Fig. 1. Chemical structure of the synthesized compounds **1** and **2**

Additionally, the stability of the compounds synthesized in aqueous and organic solvents was investigated by using UV-vis and NMR spectroscopy. Most importantly, the synthesized compounds were tested for anticancer activity against human colon cancer (HCT-116). All of these studies pointed out that compound **2** has promising anticancer activity.

2. MATERIALS AND METHODS

2.1. *General information*

All reagents and solvents were purchased commercially and used directly without purification. All experiments were performed using the standard Schlenk technique under an argon atmosphere. NMR spectra of compounds were recorded with a Bruker Avance III 400 MHz NMR spectrometer using $CDCl_3$ as the solvent and tetramethylsilane as the internal reference. The data are shown as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). Melting points (m.p.) were determined with an Electrothermal 9100 melting point detection apparatus and are uncorrected. LC–MS spectra of the Ag(I) complex were recorded in an Agilent 1100 LC/MSD SL mass spectrometer equipped with an electrospray ion source. FT-IR spectra of all compounds were recorded with Perkin Elmer Spectrum 100 spectrophotometer.

2.2. *Synthesis and characterization of 1-(4-isopropylbenzyl)-3-(2-(1,3-dioxane-2-yl)ethyl)benzimidazolium bromide, **1***

Substitution of the isopropylbenzyl group with a different benzimidazolium salt was carried out according to a study previously mentioned in the literature.[25] Briefly, 1-(4-isopropylbenzyl)benzimidazole (2.5 g, 1 mol) was dissolved in 5 ml of DMF, then the alkyl bromide (1.95 g, 1 mol) was added to the solution. The mixture was stirred for 5 h at room temperature and then heated at 80 ºC for 8 h. The reaction mixture was allowed to cool. After adding diethyl ether (10 ml), the solid formed was filtered and washed with diethyl ether (3 × 10 ml). The product was recrystallized with alcohol/diethyl ether (1:2 ratio) after drying under a vacuum. Yield: 83 %, M_r: 445 g/mol, m.p.: 104–105 ºC, FT-IR $\nu_{(CN)}$: 1561 cm^{-1}, LC-MS, calculated for [L$^+$]: 365.1; found: 365.2. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.19 (d, 6H J = 8 Hz, C_6H_4-4-CH(CH_3)$_2$); 1.22 (m, 2H, NCH_2CH_2-1,3-dioxane-5-CH_2); 2.31 (h, J = 8 Hz, 2H, NCH_2CH_2-1,3-dioxane); 2.86 (m, 1H, C_6H_4-4-CH(CH$_3$)$_2$); 3.73 and 3.88 (m, 4H, NCH_2CH_2-1,3-dioxane-4,6-CH_2); 4.83 (m, 3H,

NC*H*$_2$CH$_2$-1,3-dioxane and NCH$_2$C*H*$_2$-1,3-dioxane-2-C*H*); 5.86 (s, 2H, C*H*$_2$C$_6$H$_4$); 7.20–8.02 (m, 8H, Ar-H,); 11.38 (s, 1H, NC*H*N). ^{13}C NMR (100 MHz, CDCl$_3$) δ (ppm): 23.8 (C$_6$H$_4$-4-CH(*C*H$_3$)$_2$); 25.4 (NCH$_2$CH$_2$-1,3-dioxane-5-*C*H$_2$); 31.4 (C$_6$H$_4$-4-*C*H(CH$_3$)$_2$); 33.8 (NCH$_2$*C*H$_2$-1,3-dioxane); 42.6 (N*C*H$_2$CH$_2$-1,3-dioxane); 50.9 (*C*H$_2$C$_6$H$_4$); 66.6 (NCH$_2$CH$_2$-1,3-dioxane-4,6-*C*H$_2$); 99.2 (NCH$_2$CH$_2$-1,3-dioxane-2-*C*H); 113.1, 113.7, 126.9, 127.3, 128.4, 130.4, 131.4, 150.0 (Ar-*C*); 143.4 (N*C*HN). Elemental analysis, calcul. for C$_{23}$H$_{30}$N$_2$BrO$_2$·H$_2$O: C: 59.48, H: 6.95, N: 6.03 (%); found: C: 58.27, H: 6.77, N: 6.28 (%).

2.3. *Synthesis and characterization of bromo-1-(4-isopropylbenzyl)-3-[2-(1,3-dioxalane-2-yl)ethyl]benzimidazole-2-ylidinesilver(I),* ***2***

The Ag(I)-NHC complex **2** was obtained from the reaction of benzimidazolium salt **1** (0.44 g, 1 mmol) and Ag$_2$O (0.12 g, 0.5 mmol) in DCM (15 ml) at room temperature and in the dark. The resulting mixture was filtered through celite and the colorless filtrate was crystallized with diethyl ether. Yield: 77 %, M_r: 551 g/mol, m.p.: 127–128 ºC, FT-IR $\nu_{(CN)}$: 1393 cm^{-1}, LC-MS, calculated for [AgL$_2$]$^+$ = 837; found: 837.4. ^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 1.20 (d, 6H *J* = 4 Hz, C$_6$H$_4$-4-CH(C*H*$_3$)$_2$); 1.32 (m, 2H, NCH$_2$CH$_2$-1,3-dioxane-5-C*H*$_2$); 2.20 (q, *J* = 8 Hz, 2H, NCH$_2$C*H*$_2$-1,3-dioxane); 2.86 (q, *J* = 8 Hz, 1H, C$_6$H$_4$-4-C*H*(CH$_3$)$_2$); 3.73 and 4.09 (m, 4H, NCH$_2$CH$_2$-1,3-dioxane-4,6-C*H*$_2$); 4.61 (m, 3H, NC*H*$_2$CH$_2$-1,3-dioxane and NCH$_2$CH$_2$-1,3-dioxane-2-C*H*); 5.58 (s, 2H, C*H*$_2$C$_6$H$_4$); 7.16-7.62 (m, 8H, Ar-H,). ^{13}C NMR (100 MHz, CDCl$_3$) δ (ppm): 23.9 (C$_6$H$_4$-4-CH(*C*H$_3$)$_2$); 25.6 (NCH$_2$CH$_2$-1,3-dioxane-5-*C*H$_2$); 33.8 (C$_6$H$_4$-4-*C*H(CH$_3$)$_2$); 35.2 (NCH$_2$*C*H$_2$-1,3-dioxane); 44.6 (N*C*H$_2$CH$_2$-1,3-dioxane); 53.1 (*C*H$_2$C$_6$H$_4$); 66.9 (NCH$_2$CH$_2$-1,3-dioxane-4,6-*C*H$_2$); 99.1 (NCH$_2$CH$_2$-1,3-dioxane-2-*C*H); 111.6, 112.2, 124.1, 124.2, 127.1, 127.3, 132.4, 133.8, 133.9, 149.3 (Ar-*C*). Elemental analysis, calculated for C$_{23}$H$_{29}$N$_2$BrAgO$_2$: C: 49.93, H: 5.28, N: 5.06 (%); found: C: 50.12, H: 5.19, N: 5.21 (%).

2.4. *Antioxidant and oxidant properties*

TAS and TOS levels of compounds were assayed with the commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey) according to the instructions provided by the manufacturer.[26] TAS and TOS results were expressed in mmol Trolox equivalent/l, and μmol H_2O_2 equivalent/l, respectively. All experiments were carried out in triplicates and at 37 ºC.

2.5. *In vitro cytotoxicity assay*

The *in vitro* cytotoxicity of the synthesized compounds was examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay using human colon cancer (HCT-116) cells.[27] The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, supplemented with 10 % fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 37 °C under a 5 % CO_2 atmosphere. For each cell line, when 70–80 % confluent cell culture was reached, cells were trypsinized and seeded into 96-well plates. The concentrations of 12.5, 25, 50, and 100 μg/ml of compounds **1** and **2** were added to the wells with cells and allowed to grow in a CO_2 incubator (37 °C, 5 % CO_2) for 24 h. After 24 h treatment, 10 μl of MTT solution (5 mg/ml in phosphate-buffered saline (PBS, pH 7.4)) was introduced to each well, and the mixture was incubated at 37 °C for 4 h to form the formazan crystals. Then, 100 μl of DMSO was introduced to each well and the plates were incubated in a CO_2 incubator for 4 h. Finally, the optical density of plates was read at 540 nm via an Eon Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Untreated cells, DMSO, and cisplatin were used as positive control, negative control, and reference drug, respectively. Data were recorded and analyzed to evaluate the effects of test samples on growth inhibition and cell viability. Besides, the half-maximal inhibitory concentrations (IC50) were calculated using GraphPad Prism 8.4.2 (GraphPad Software, La Jolla, CA, USA).

2.6. *Stability studies*

Stabilities of compounds in both aqueous and organic media were investigated by spectrophotometric measurements through a microplate reader. For this purpose, the 10^{-4} M solutions of compounds were prepared in PBS (50 mM, pH 7.4), DMSO, and DMF. All the experiments were carried out at the wavelength range of 200–400 nm at 25 ºC. In addition, to confirm the stability, the NMR spectra of compound **2** were recorded in different time intervals (0, 6, and 24 h) in a DMSO-d_6.

3. RESULTS AND DISCUSSION

3.1. *Characterization of benzimidazolium salt and Ag-NHC complex*

In this work, 4-isopropylbenzyl bromide was used to synthesize the 1-(4-isopropylbenzyl)benzimidazole ligand. As a result of the reaction of 1-

(4-isopropylbenzyl)benzimidazole and 2-(2-bromoethyl)-1,3-dioxane, benzimidazolium salt **1** was obtained with excellent yield (83%). The general synthesis of benzimidazole salt **1** and its Ag-complex **2** is shown in Fig. 2. In the ^{1}H NMR spectra of compound **1**, the acidic C2 proton peak was observed at 10.11 ppm. In the ^{13}C NMR spectra, a carbon resonance peak was observed at 143.4 ppm, corresponding to the benzimidazolium salt C2 proton. Benzimidazolium salt **1** exhibits a characteristic υ(NCN) band typically at 1561 cm^{-1}. NMR data and IR absorption values of the benzimidazolium salt **1** are compatible with the literature.[28]

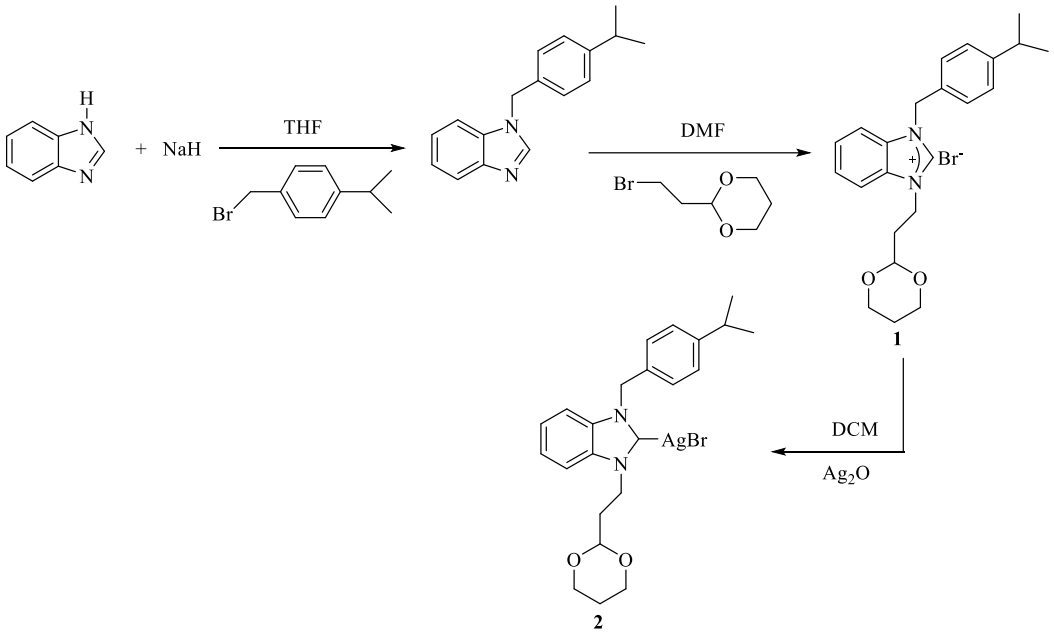

Fig. 2. General synthesis of compounds **1** and **2**

Due to the neutral nature of electron donors, NHCs can bind to metal ions via σ-donation. In addition, due to their stability, they have an important research area, especially in organometallic chemistry and catalysis. Ag-NHC complexes attract attention due to their pharmaceutical properties, as well as their applications in organometallic chemistry. Also, they are used as carbene transfer agents in the synthesis of different metal complexes. The Ag-NHC complex **2** was obtained by the reaction of benzimidazolium salt **1** and 0.5 mol Ag_2O at room temperature and in the dark. The reaction mixture was filtered through the celite and a white product was obtained with a good yield (77 %). All of the synthesized compounds are soluble in organic solvents such as ethanol, methanol, *N*,*N*-dimethylformamide, and dimethyl sulfoxide. In the NMR spectrum of the Ag-NHC complex **2**, the disappearance of the peak corresponding to the C2 carbon of the salts indicates that the C2 carbon is coordinated with the metal ion. However, the resonance of the carbene carbon in the Ag(I)-NHC complex was not detected due to the fluxional behavior of the NHC complex.[20] In the FT-IR spectrum of compound **2**, the C–N absorption band was observed at 1393 cm^{-1}. NMR data and IR absorption values of the Ag-NHC complex **2** are compatible with the literature.[20]

The halide ions and solvent effects on the structures of Ag-NHC complexes have been reported in the literature.[29,30] The peaks seen in the LC-MS data indicate that the Ag(I)-NHC complex is molecular ions ($[Ag(NHC)_2]^+$) in solution. The weak Ag–C bond in the Ag-NHC complex allows the formation of ($[Ag(NHC)_2]^+$) in solution.

3.2. *Antioxidant and oxidant properties*

A TAS value greater than or equal to 1.0 mmol Trolox equiv./l indicates a high antioxidant profile.[31] However, compounds **1** and **2** exhibited less antioxidant activity than 1 mmol of Trolox. Compounds **1** and **2** displayed values of 0.22 ± 0.04 and 0.05 ± 0.02 mmol Trolox equiv./l, respectively (Table 1). On the other hand, a structure with anticancer properties is expected to have oxidative properties. The oxidative effects of structures with ≥20 μmol H_2O_2 equivalent/l activity are considered strong. Based on this reference value, compounds **1** and **2** displayed negligible oxidative activi-

ty. From Table 1, it was observed that these compounds showed oxidative activity with 7.05 ± 2.02, and 8.11 ± 0.76 μmol H_2O_2 equiv./l, respectively. The differences in the antioxidant and oxidative activities of these compounds were mainly affected by the number and position of substituents.[32]

T a b l e 1

*TAS and TOS values of compounds **1** and **2***

Compound	TAS (mmol Trolox equiv./l)	TOS (μmol H_2O_2 equiv./l)
1	0.22 ± 0.04	7.05 ± 2.02
2	0.05 ± 0.02	8.11 ± 0.76

3.3. *In vitro cytotoxicity assay*

The *in vitro* cytotoxicity of the compounds was tested against the HCT-166 cell line using the MTT assay, and the cell viability results obtained are shown in Figure 3. In addition, the concentrations of the agents inhibiting 50 % cell growth (IC_{50}) are summarized in the inset of Figure 3. It was observed that compound **2** showed promising cytotoxicity against the HCT-166 cell line, while compound **1** showed almost no activity against the tested cell line. In addition, the cytotoxicity of the compounds was found to be concentration-dependent. IC_{50} values for these compounds were determined as 21.95 ± 3.81 μg/ml and 577.84 ± 5.97 μg/ml, respectively. Compared with cisplatin (IC_{50} = 90.70 ± 3.37 μg/ml), it was found that compound **2** is 4.1-fold more cytotoxic to HCT-116 cancer cells. These values revealed that HCT-116 cells were more sensitive to the Ag(I)-NHC complex (compound **2**) and less sensitive to the NHC salt (compound **1**). Probably, the coordination of Ag to the carbene carbon of ligands enhanced the anticancer effect of Ag(I)-NHC complexes because of the participation of Ag ions in the cell death mechanism. Similar results were previously reported.[33] Taken together, it is apparent from this work that the Ag-NHC complex could be a potential anticancer drug candidate in the future.

Furthermore, optical microscopy images of HCT-116 cell morphology are shown in Fig. 4. According to these images, compounds **1** and **2** lead to morphological changes consistent with apoptotic cell death. Particularly, compound **2** affected cell growth, and the cell confluent level was observed to be significantly reduced.

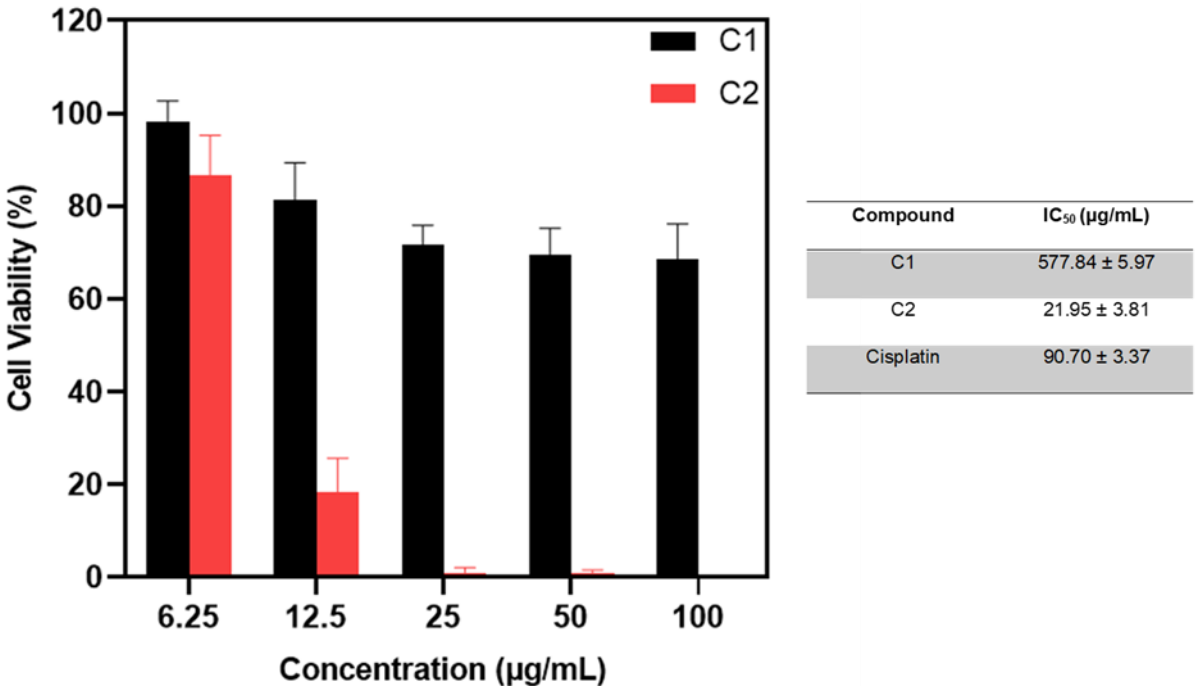

Fig. 3. The percentage cell viability of investigated compounds towards human colon cancer (HCT-116) and their IC_{50} values in μg/ml (inset)

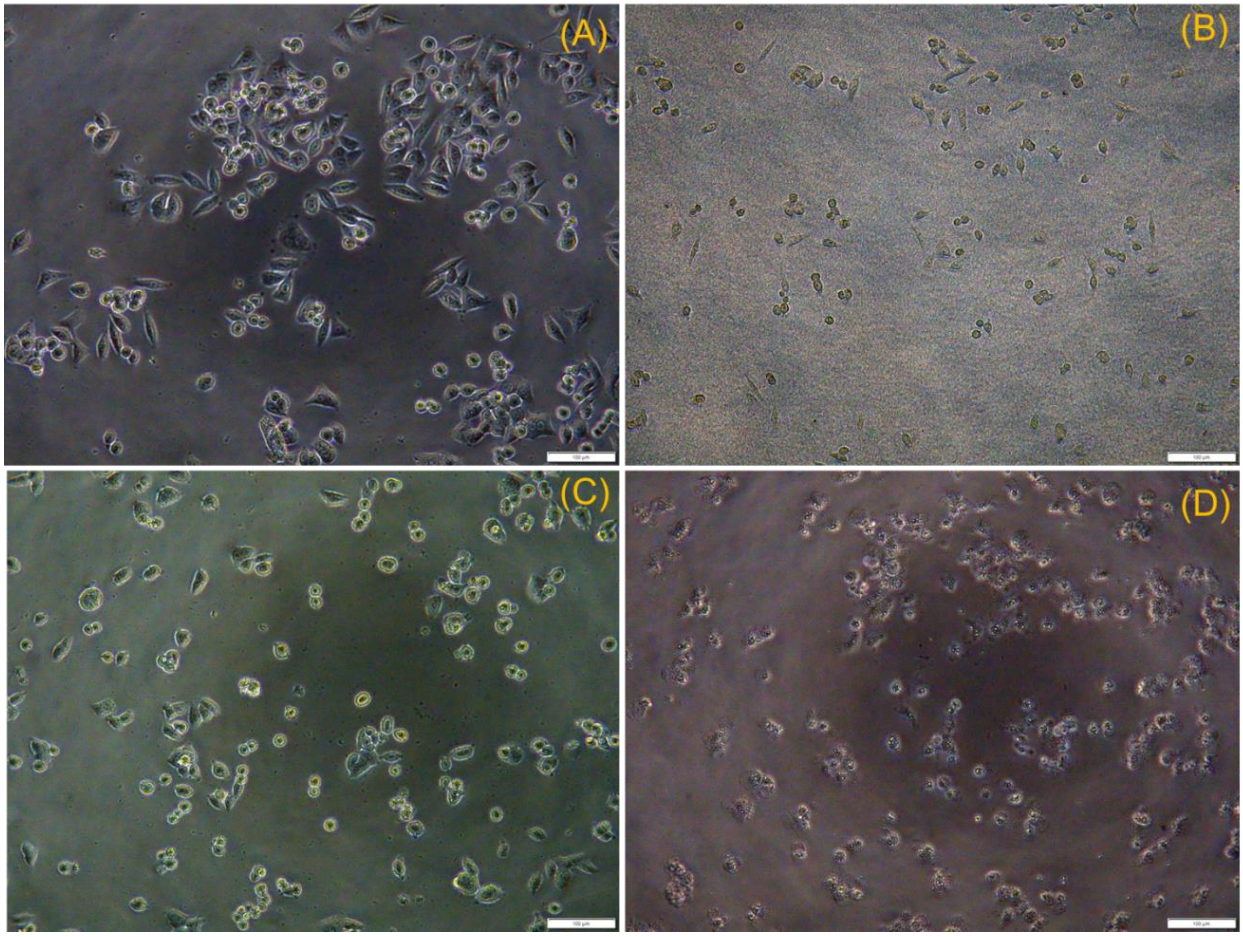

Fig. 4. Optical microscopy images of HCT-116 cell morphology (A) control, (B) DMSO, (C) compound **1,** and (D) compound **2** after 24 h of incubation

3.4. *Stability studies*

To test the stability of compounds **1** and **2** with respect to dissociation in an aqueous solution, we recorded the UV absorption spectra of **1** and **2** in PBS. As shown in Fig. 5, we found that both **1** and **2** exhibited similar curves of absorbance in the region λ = 200–400 nm. The maximum absorptions of **1** and **2** were 215 and 208 nm in PBS with molar extinction coefficients of 25757 cm^{-1} M^{-1} and 6271 cm^{-1} M^{-1}, respectively. Additionally, a negligible shift appeared in the UV spectra of compounds, and no new peak was observed in 24 h. Therefore, these findings suggested that compounds **1** and **2** were stable within 24 h in water at ~25 °C. We also investigated the stability of these compounds in common organic solvents (DMSO, and DMF). The UV spectra did not show any change within 24 h, testifying to the stability in DMSO (Fig. 6) and DMF solutions (Fig. 7). On the other hand, to confirm the stability, the NMR spectra of compound **2** were recorded in different time intervals (0, 6, and 24 h) in DMSO-d6. The spectra retained all the peaks in the respective regions over a period of 24 h, suggesting that compound **2** may be the compound responsible for any observed activity (Fig. S5). These outcomes demonstrated that the proposed compound **2** is suitable for biological and cellular studies.

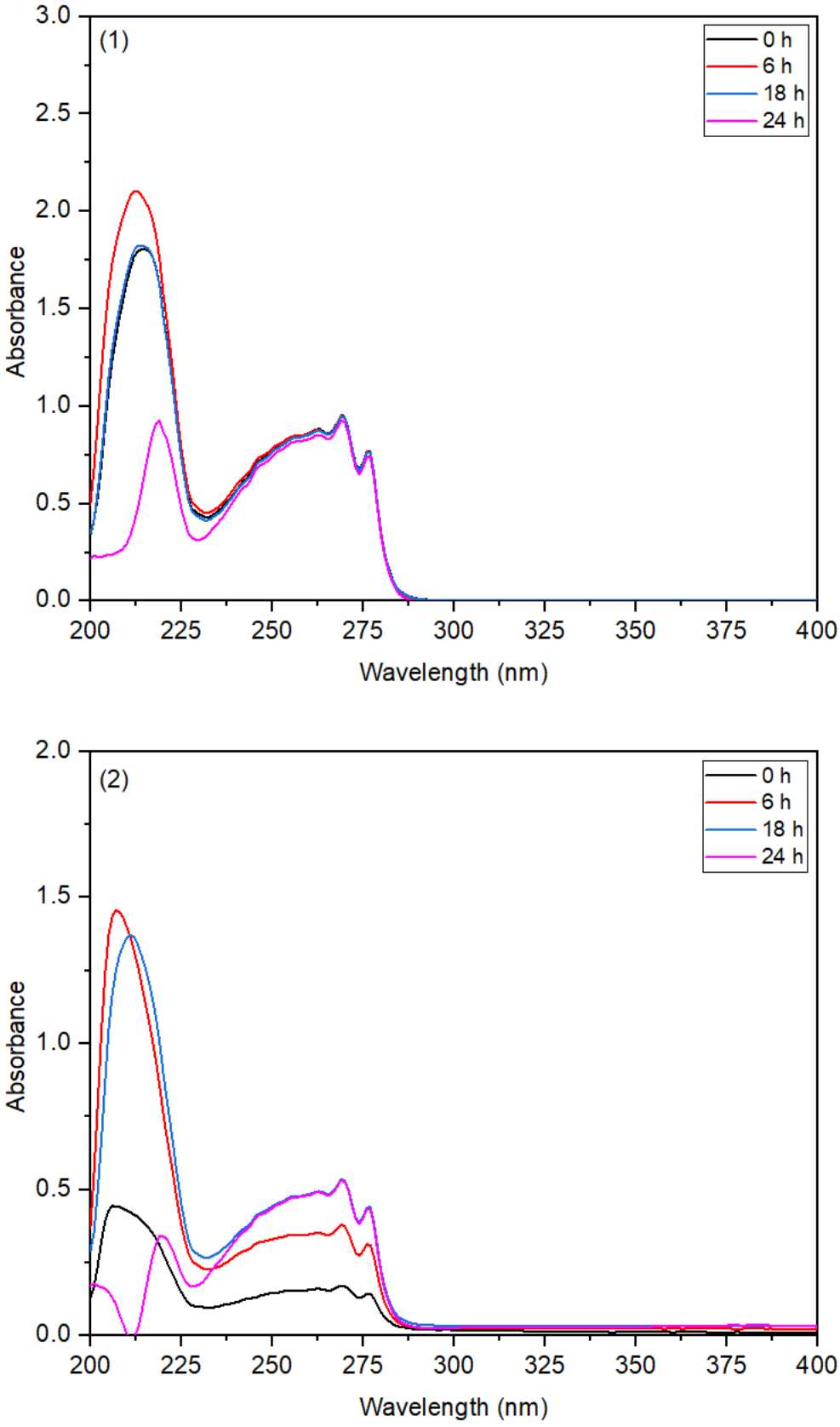

Fig. 5. UV spectra of compounds **1** and **2** in PBS at different time intervals

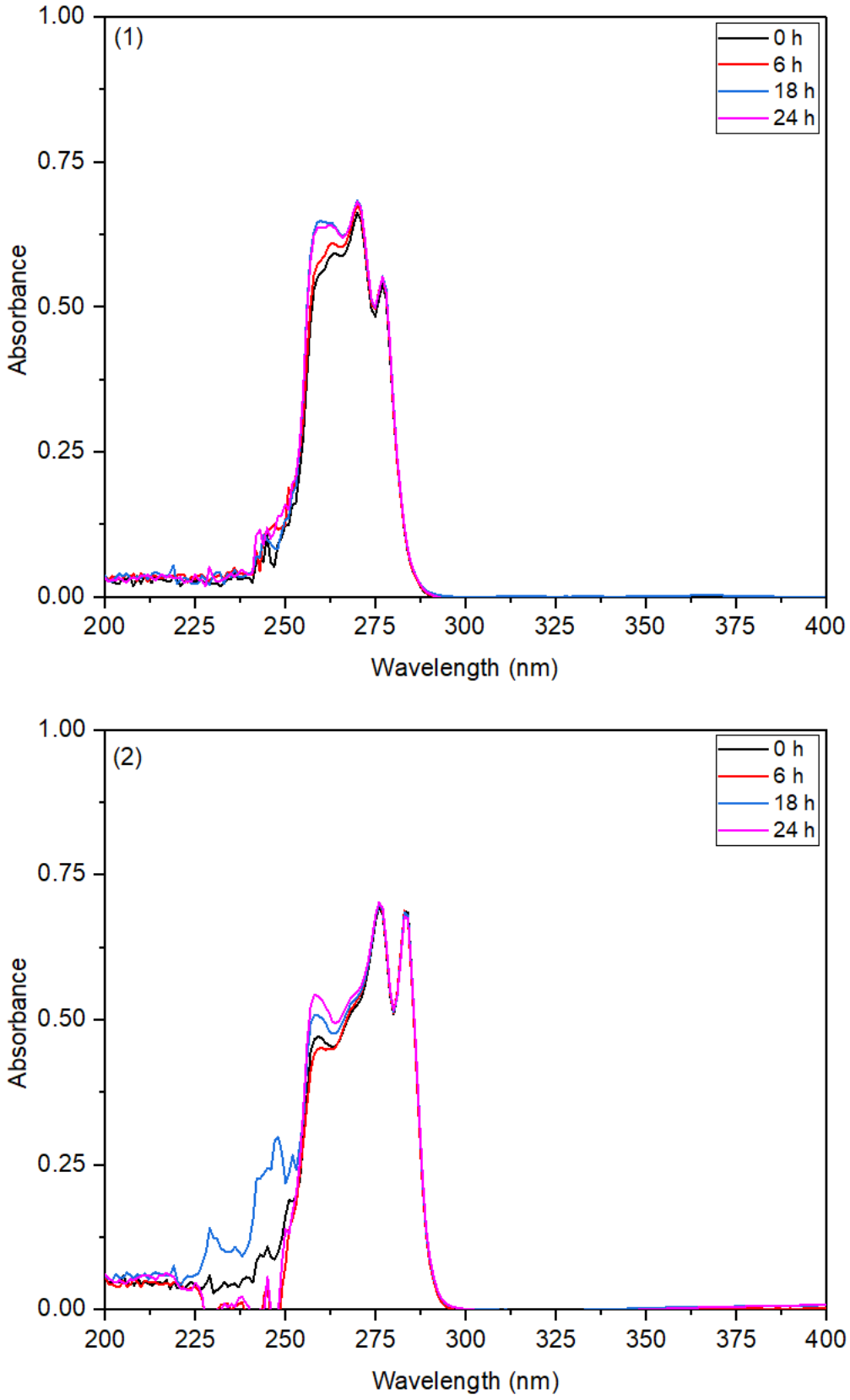

Fig. 6. UV spectra of compounds **1** and **2** in DMSO at different time intervals

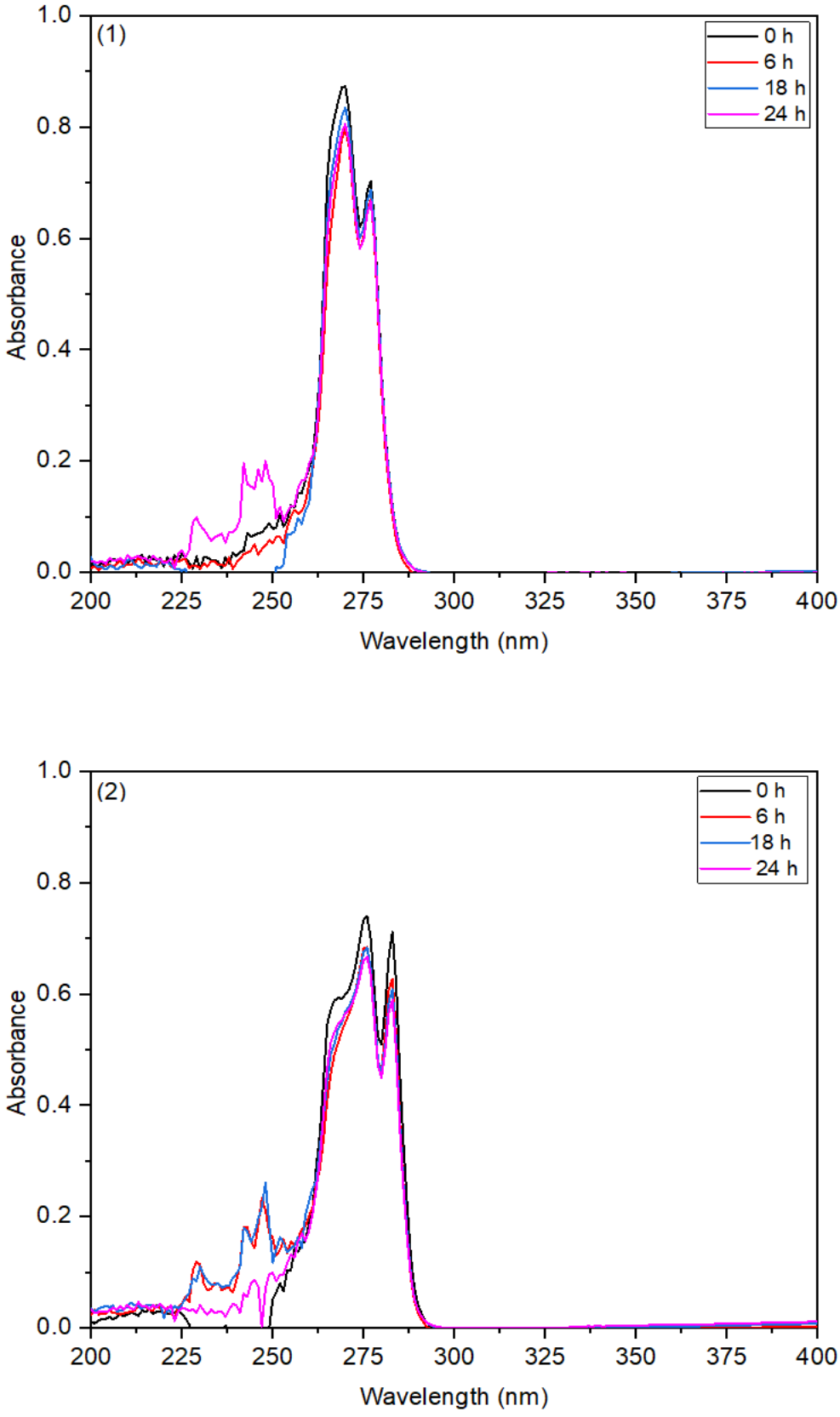

Fig. 7. UV spectra of compounds **1** and **2** in DMF at different time intervals

4. CONCLUSION

In summary, a new 1,3-disubstituted benzimidazolium salt was synthesized. Afterward, the Ag(I)-NHC complex was synthesized by the procedure involving the reaction of the benzimidazolium salt and Ag_2O in DCM at ambient temperature. The structures of the prepared compounds **1** and **2** were elucidated by ^{1}H NMR, ^{13}C NMR, FT-IR, LC-MS spectroscopic methods and elemental analysis. In addition, the biological properties including the antioxidant and anticancer activity of the synthesized compounds were investigated. The biological results showed that the compounds showed negligible antioxidant and oxidative effects. However, compound **2** displayed promising activity against the cancer cell lines studied. In addition, the UV absorption and NMR spectra acquired confirmed good stability of the compounds in aqueous and organic media. Therefore, compound **2** represents a good candidate for further studies currently under development in our laboratory.

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