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Cytotoxicity and DNA binding of copper (II) and zinc (II) complexes of flavonoids: quercitrin, myricitrin, rutin

B. Atabey-Ozdemir[1], O. Demirkiran[2], U. Yildiz[1], I. O.Tekin[3], B. Coban*[1]

[1]*Bülent Ecevit University, Faculty of Arts and Sciences, Department of Chemistry, Zonguldak, 67100, Turkey.*
[2]*Trakya University, Faculty of Pharmacy, Department of Pharmacognosy, Edirne, 22100, Turkey*
[3]*Bülent Ecevit University, Faculty of Medicine, Department of Immunology, Zonguldak, 67100, Turkey.*

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Flavonoids: quercitrin, myricitrin and rutin and their metal complexes were comparatively investigated for binding to DNA by means of spectrophotometric methods and DNAse activities were evaluated via agarose gel electrophoresis of pBR322. Free flavonoids bind to DNA in an intercalative mode, but Cu(II) complexes of these flavonoids bind even stronger due to the electrostatic interaction of the metal in addition to the intercalation. Flavonoids show protective effect against DNA cleavage in the presence of peroxide. However, Cu(II) and Zn(II) complexes of these flavonoids cause multiple scissions on the DNA backbone. In addition, Cu(II) complexes of the flavonoids have stronger DNAse activity. Moreover, myricitrin was found two times more cytotoxic when combined with metal ions (Cu^{2+} or Zn^{2+}) than when used alone against peripheral blood mononuclear cells.

Keywords: Flavonoids, DNA binding, DNAse activity, Cytotoxicity, Cu(II) complex, Zn(II) complex.

INTRODUCTION

Interaction between DNA and drug molecules is a very popular subject [1-3] especially for the designing of new DNA-targeted drugs and their *in vitro* screening.

Flavonoids are non-nutritive compounds present in plants, and have a broad spectrum of pharmacological activities. Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-chromen-4-one), and myricetin 2-(3,4,5-trihydroxyphenyl)-3,5,7-trihydroxy-chromen-4-one) (Table 1), some of the most abundant natural flavonoids, are present in various vegetables and fruits, and their average human daily intake is estimated to be 16–25 mg/person [4]. They are also some of the main active components of many natural Chinese traditional medicines (CTM). The flavonoids frequently occur as glycosides, quercitrin (quercetin-3-O-*L*-rhamnoside), myricitrin (myricetin-3-O-*L*-rhamnoside) and rutin (quercetin-3-O-*D*-rutinoside) (Fig. 1) being the most common flavonol glycosides in the human diet [5]. Flavonoids, especially quercitrin (Q), myricitrin (M) and rutin (R), are capable of scavenging reactive oxygen species (ROS) and chelating iron ions which play a vital role in initiating free radical reactions, and suppressing the generation of hydroxyl radicals in the Fenton reaction [6-9]. Myricitrin and rutin have been found to have anticancer properties against prostate cancer and malignant gliomas [10, 11].

In this context, it is generally considered that these flavonoids form coordination complexes with some essential trace metals, and it is believed that this is the active form of the compounds, which is medicinally beneficial [12]. Flavonoids are known to react with various metal cations to form stable compounds which have demonstrable antibacterial properties and antitumor activity [13-15]. Given that some metal ions, especially transition metals, not only play vital roles in a vast number of widely differing biological processes, but also may be potentially toxic in their free state, the studies of interactions between flavonoid–metal complexes and DNA are important to further the understanding of the pharmacology of flavonoids. Complexes of different transition metals including copper and zinc were investigated and all metal complexes showed stronger DNA binding ability and were more active against cancer cell lines in respect to free flavonoid [16-23].

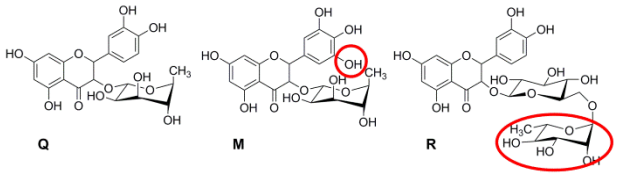


Fig. 1. Flavonoids, especially quercitrin (Q), myricitrin (M) and rutin (R),

Many techniques, such as UV–Vis spectrophotometry [24], fluorescence spectroscopy [25, 26], circular dichroism spectroscopy [27], mass spectrometry [28], and voltammetry [29] have been used to study the binding between metal complexes and DNA.

* To whom all correspondence should be sent:
E-mail: burakcoban@yahoo.com

In this work, the interactions of flavonoids: Q, M and R and their metal complexes with calf thymus (CT)-DNA were comparatively investigated with the aid of absorption and fluorescence spectrophotometry and their DNAse activity was investigated against pBR322 plasmid DNA *via* agarose gel electrophoresis assays. In addition, cytotoxicity of myricitrin alone and in combination with metal ions was evaluated.

EXPERIMENTAL

All reagents and solvents were of commercial origin and were used without further purification unless otherwise noted. Solutions of calf thymus DNA (CT-DNA purchased from Sigma) in 50 mM ammonium acetate (pH 7.5) had a UV–Vis absorbance ratio of 1.8–1.9: 1 at 260 and 280 nm (A_{260}/A_{280} = 1.9), indicating that the DNA was sufficiently free of protein [30]. The concentration of DNA was determined spectrophotometrically using a molar absorptivity of 6,600 M^{-1} cm^{-1} (260 nm) [30]. Double-distilled water was used to prepare buffers. Stock solution of CT-DNA was stored at 4 °C and used within 4 days.

Physical measurements

UV–Vis spectra were recorded with a Varian Cary 100 spectrophotometer and emission spectra were recorded with a Perkin Elmer LS 55 fluorescence spectrometer at room temperature.

Absorption and emission titrations

For the absorption and emission titrations, flavonoids and metals (about 1 mmol) were dissolved in a minimum amount of DMSO (0.5 mL), and were then diluted in 5 mM ammonium acetate buffer, pH 7.5 to a final concentration of 20 μM. Titrations were performed in a 10-mm stoppered quartz cell by using a fixed concentration of the compound (20 μM; flavonoid alone and in combination with (1:1) Cu^{2+} or Zn^{2+} ion), to which the CT-DNA stock solution was added in increments of 1 μL to a DNA-to-compound concentration ratio of 6:1. Analysis was performed by means of a UV–Vis or fluorescence spectrophotometer by recording the spectrum after each addition of DNA. Compound–DNA solutions were incubated for 10 min before the spectra were recorded. A control solution of 20 μM of the drug in the same buffer was treated in the same manner. Cell compartments were thermostated at 25 ± 0.1°C.

For emission intensity measurements, the excitation wavelength was fixed and the emission range was adjusted before the measurements. Ammonium acetate (5 mM), pH 7.5 buffer was used as a blank to make preliminary adjustments. All measurements were performed with a 5-nm entrance slit and a 5-nm exit slit. The complexes were excited at 383 and 400 nm, respectively; the emission spectra were monitored between 710 and 740 nm.

Competitive studies

The competitive behavior of each compound with ethidium bromide (EB) was investigated by fluorescence spectroscopy in order to examine whether the compound is able to displace EB from the DNA–EB complex.

DNA was pretreated with EB at a DNA-to-EB concentration ratio of 50:1 at 27 °C for 30 min to prepare the initial DNA-EB complex. The intercalating effect of the compounds with the DNA-EB complex was studied by adding a certain amount of a solution of the compounds in increments to the solution of the DNA-EB complex. The influence of each addition of compounds to the solution of the DNA-EB complex was estimated by recording the change in the fluorescence peak at 640 nm. To study the competitive binding of the compounds with EB, the latter was excited at 453 nm in the presence of DNA alone, as well as in the presence of the compounds.

DNAse activity by gel electrophoresis

Gel electrophoresis experiments were performed using pBR322 negatively supercoiled plasmid DNA and 1 % agarose gels together with a tris(hydroxymethyl)aminomethane-borate-EDTA running buffer solution. Reaction mixtures (10 mL) containing 0.1 μg pBR322 together with different amounts of **1** and **2** (0, 20, 40, 60, 100 and 200 μM) in 50 mM ammonium acetate buffer, pH 7.5 were prepared at 0 °C and incubated at 36 °C for 1 h in the dark. Prior to the samples being loaded onto the gel, 2.5 mL of 0.25 % bromophenol blue loaded buffer and sucrose in water (40 % w/v) was added to the reaction mixtures. Gels were obtained at room temperature by using a Thermo midi horizontal agarose gel electrophoresis system and applying a potential of 35 V for 4 h. The resulting gels were stained with EB solution (0.5 μg mL^{-1}) for 45 min, after which they were soaked in water for further 20 min. Gels were visualized under UV light and photographed.

Cytotoxicity assays

Peripheral blood mononuclear cells from healthy individuals were activated with phytoheamagglutinin for proliferation and treated with 150 and 1,500 μg of myricitrin in combination with (1:1) metal ion (Cu^{2+} or Zn^{2+}). Cells were

incubated at 37 °C for 48 h in a humidified incubator in 5 % CO_2 atmosphere in RPMI-1640 supplemented with 10 % fetal bovine serum, 100 U /mL penicillin, and 100 µg/m L streptomycin for viability analysis using propidium iodide (PI) staining and flow cytometry. A 100-µl cell suspension was used for each sample. Cells were stained with PI according to the manufacturer's instructions, and their viability was measured in a Coulter FC500™ flow cytometer (Beckman-Coulter).

RESULTS

All three flavonoids have very similar UV spectra with an absorption peak at 360 nm wavelength. Addition of metal ions caused the absorption peak to shift to around 400 nm and a slight hypochromic effect is seen in Fig. 2.

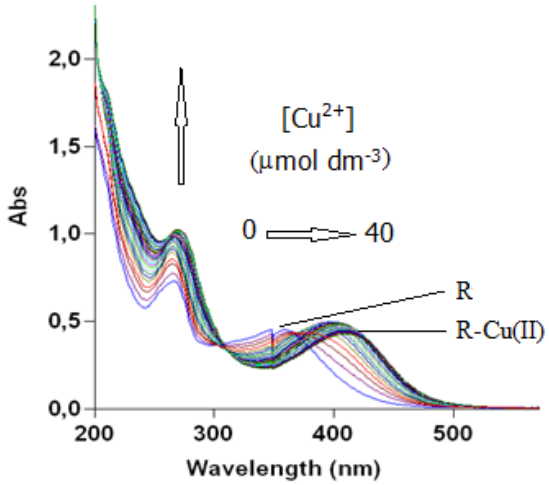


Fig. 2. Absorption spectra of rutin in 50 mM ammonium acetate buffer on gradual additions of Cu^{2+} at pH 7.0 at 298 K. [Rutin]= 20 µM, [Cu^{2+}]= 0-40 µM. Arrows indicate the absorbance and wavelength changes with increase in Cu^{2+} concentration.

Very low hypochromic and bathochromic effects were seen after the addition of DNA to the free flavonoid solutions (Fig. 3). The hypochromicity and bathochromic shift increased when Cu(II) complexes of flavonoids were titrated with DNA solution (Fig. 4). Their intrinsic binding constants (K_b) were calculated as Q–Cu(II): 2.62 ±0.25 × 10^5 M^{-1}; M–Cu(II): 1.80 ±0.20 × 10^5 M^{-1}; R–Cu(II): 1.02 ±0.10 × 10^5 M^{-1}.

The competitive titrations of ethidium bromide (EB) dye and flavonoid–Cu(II) species in the presence of DNA are shown in Fig. 5. According to the spectra, EB fluorescence intensity was decreased by the addition of flavonoid-Cu(II) complexes.

The potential of the Cu(II) and Zn(II) complexes to cleave DNA was studied by agarose gel electrophoresis using pBR322 plasmid DNA. When circular DNA is subjected to gel electrophoresis,

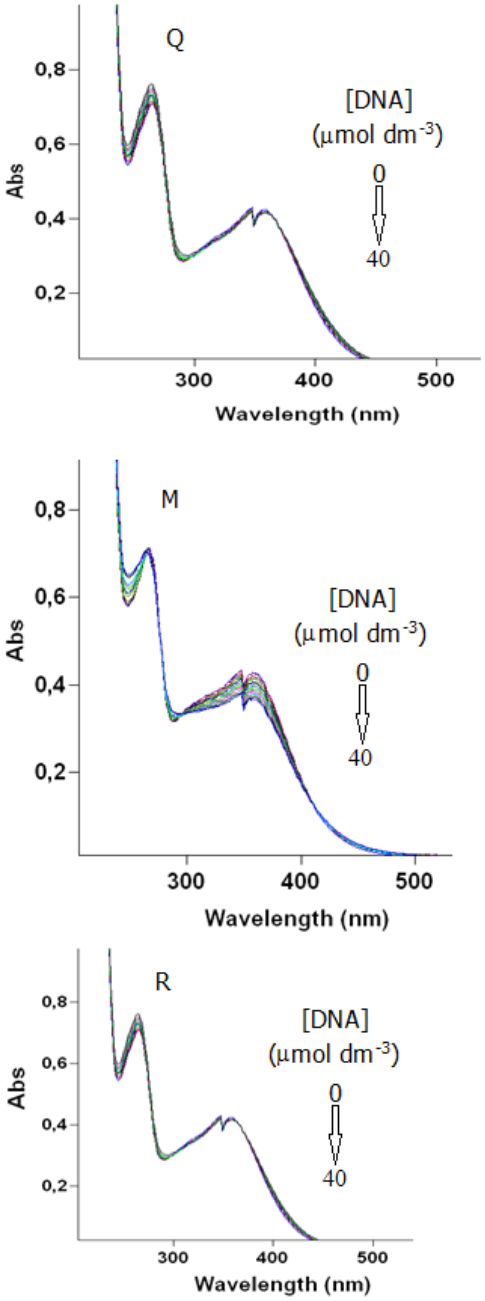


Fig. 3. Absorption spectra of free flavonoids **Q**, **M** and **R** in 50 mM ammonium acetate buffer on gradual additions of calf thymus DNA at pH 7.0 at 298 K. [flavonoid]= 20 µM, [ct-DNA]= 0-40 µM. Arrows indicate the absorbance changes with increase in ct-DNA concentration.

relatively fast migration will be observed for the supercoiled form (form I). If scission occurs on one strand (nicked circular), the supercoiled form will relax to generate a slower moving open circular form (form II). If both strands are cleaved, a linear form (form III) that migrates between the two forms will be generated [31].

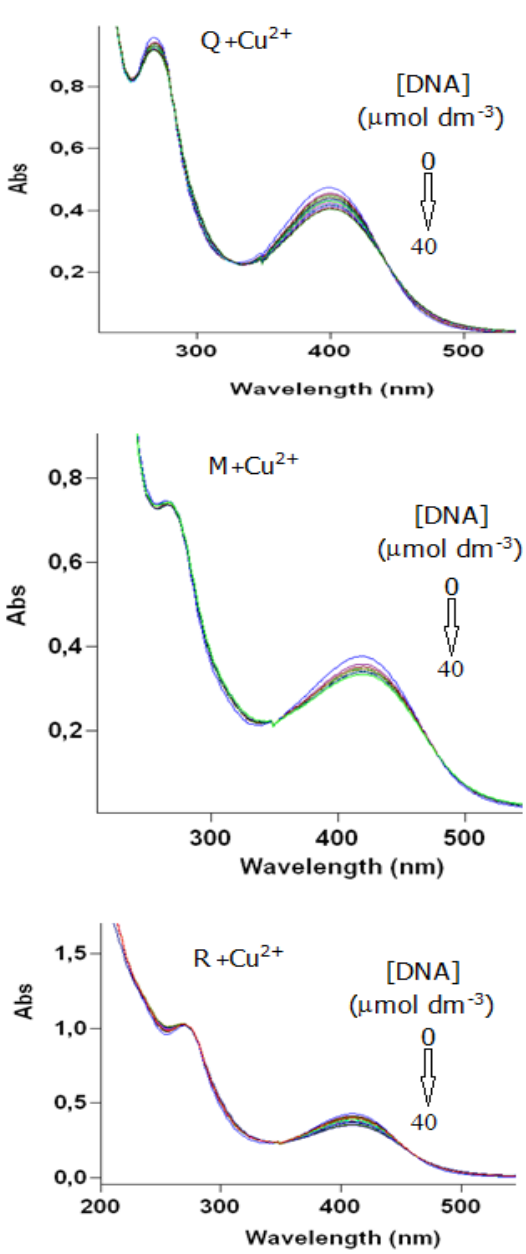


Fig. 4. Absorption spectra of Cu(II) complexes of Q, M and R in 50 mM ammonium acetate buffer on gradual additions of calf thymus DNA at pH 7.0 at 298 K. [Cu(II) complex]= 20 μM, [ct-DNA]= 0-40 μM. Arrows indicate the absorbance changes with increase of ct-DNA concentration.

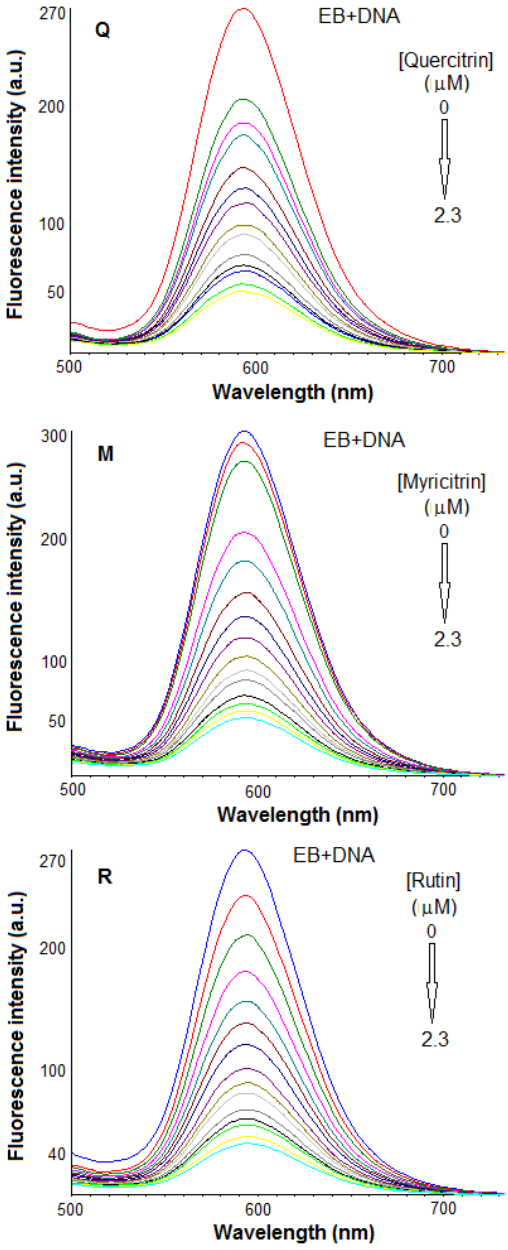


Fig. 5. Fluorescence spectra of the competition between Cu(II) complexes of Q, M and R and ethidium bromide (EB) at 298 K. [EB] = 20.0 μM and [ct-DNA] = 100.0 μM at λ_{exc} = 363 nm. [Complex] = 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.55, 1.7, 1.9, 2.1, 2.3 μM.

Fig. 6 shows the separation of pBR322 by gel electrophoresis after incubation with Q, M and R, respectively. Lane 1 is the control having only DNA. The flavonoids, as expected, were unable to cleave the DNA as shown in lanes 2-4. Lane 5 is negative control DNA incubated with peroxide solution, a small proportion of DNA cleavage can be seen as formation of form II. Addition of flavonoids will reduce the DNA cleavage in the lanes 6-8 indicating that free flavonoids protect DNA from peroxide damage.

When plasmid DNA was treated with Cu(II) and Zn(II) complexes of flavonoids, a very small fraction of form II was formed indicating a mild DNA damage (not shown on the pictogram). Peroxide addition makes the comparison easier to understand.

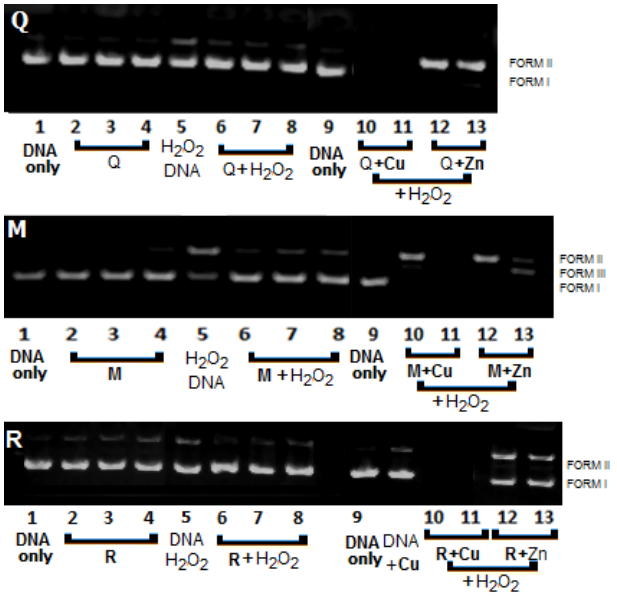


Fig. 6. Agarose gel electrophoresis pictogram showing the cleavage pattern of pBR322 plasmid DNA (50μg) with varying concentrations of flavonoids **Q**, **M** and **R** or their Cu^{2+} or Zn^{2+} complexes at 37 °C for 12 h of incubation. Lanes 1 and 9: DNA control, lanes 2-4: 50, 100, 500 μM flavonoid, lane 5: peroxide, lanes 6-8: 50, 100, 500 μM flavonoid+peroxide, lanes 10,11: 50, 500 μM flavonoid-Cu^{2+} complex+peroxide, lanes 12,13: 50, 500 μM flavonoid-Zn^{2+} complex+peroxide.

Following lanes cover the metal complexes of the flavonoids incubated with DNA (10-13) in the presence of peroxide. Cu(II) complexes caused multiple scissions on the plasmid DNA backbone and therefore small DNA fragments cannot be seen in the gel (lanes 10 and 11) whereas, Zn(II) complexes also cleave plasmid DNA producing form II of all DNA incubated with the Q-Zn(II) and R-Zn(II) complexes (for Q and R: lanes 12 and 13) [32]. With the addition of higher concentrations of M-Zn(II) complex a fraction of form III was also formed (M: lane 13). These observations suggest that Cu(II) complexes caused greater damage to plasmid DNA in comparison to the Zn(II) complexes of the three flavonoids.

Table 1 Cytotoxicity assay results of Myricitrin at two different concentrations in presence and absence of Cu and Zn.

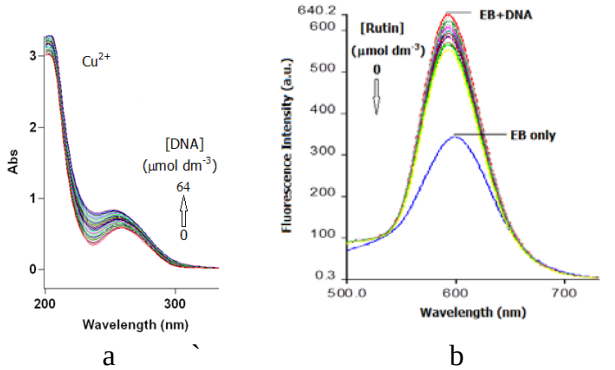
	M (g/l)		M+Zn (g/l)		M+ Cu (g/l)		Zn (g/l)	Cu (g/l)	Control*
	0.15	1.5	0.15	1.5	0.15	1.5	1.5	1.5	-
Dead cells (%)	9.1 ±0.2	10.8 ±0.5	16.5 ±1.0	22.5 ±1.9	17.7 ±1.8	26.8 ±2.8	12.1 ±0.6	14.9 ±0.3	0.1 ±0.1

*No compounds were used. Results are expressed as means ± SD (n = 3)

In the cytotoxicity assay results shown in Table 1, dead cells were presented as percentages and concentrations of the compounds were chosen as 0.15 and 1.5 g L^{-1} and in the control try no compounds were used (no dead cells were expected). Dose-dependent cell death was registered for this assay.

DISCUSSION

The spectral changes (the absorption peak shifted to 400 nm) upon addition of Cu(II) ions (or Zn(II) ions) to the flavonoid solutions indicate that 1:1 stable complexes were formed (Fig. 1) [14, 23]. The interaction of the free flavonoids with DNA was classical intercalation revealed by the observation of very low hypochromic and bathochromic effects in the spectrum (Fig. 2) and this is in accordance with literature findings [18]. In addition, Cu(II) complexes of these flavonoids interacted with DNA in the same manner but stronger hypochromic and bathochromic effects were measured (a decrease in the absorption peak at ~400 nm wavelength with a small red shift ~1-4 nm) (Fig. 3). This may be explained by allowing the planar flavonoid part of the complex to bind DNA by intercalation between the base pairs, as well as by addition of the Cu(II) cation electrostatically interacting with the negatively charged phosphate backbone. The binding values are comparable with the literature values [20]. The most hydrophobic flavonoid Q has the highest binding constant; the additional -OH group in M and the additional monosaccharide in R slightly reduced the binding constants of the latter flavonoids. As a control experiment, the interaction between DNA and the Cu(II) ions was studied (Fig. 7a) and the resulting hyperchromic effect on the spectra indicates that Cu(II) ions bind DNA electrostatically by an external binding mode.



a b

Fig.7. a: (left) The interaction between DNA and the Cu(II) ions; b: (right) The displacement of EB by the R studied by using florescent spectroscopy.

The intercalated EB was displaced from the EB-DNA complex by the flavonoid:Cu(II) species (Fig. 4). The strength of the displacement can be summarized as Q-Cu(II) > M-Cu(II) > R-Cu(II). This displacement was in accordance with the absorption spectral results, as well as with the

literature data [20]. In addition, when the free flavonoid (R) was used in place of metal complex the displacement of EB by the flavonoid was very weak (Fig. 7b). This exchange reaction was supported by the results from cyclic voltammetry studies from which the equilibrium formation constant for the Q-Cu(II) complex binding to DNA was found to be about 2.05 times larger than that of its free form [20]. This result is also in accordance with the UV titration findings.

DNAse activities of the flavonoids and their metal complexes were compared and the same displacement was found as Q > M > R. The results suggested that the Cu(II) complexes were more reactive than the Zn(II) complexes (Fig. 5). This may be due to the advanced redox cycling activity of Cu(II) *vs* Zn(II), where cuprous ions (Cu^{+}) formed *in situ* can convert H_2O_2 to hydroxyl radicals and binding of the complex to DNA provides centers for generation of hydroxyl radicals close to sites susceptible to breakage [33]. Thus, the quercitrin-Cu(II) complex has caused the highest level of damage and the rutin-Zn(II) complex produced the lowest level of damage to plasmid DNA. Similar results were obtained in the literature [34].

According to the results in Table 1, there is a concentration-dependent raise of cell death for each trial and the flavonoid (M) was two times more potent when it was in combination with a metal ion (Cu^{2+} or Zn^{2+}) compared to the flavonoid used alone. This result supports the results of spectrophotometric titrations and electrophoretic assay. Cell deaths may probably be due to DNA binding of the compounds.

DNA protective effects of the flavonoids have been extensively studied [35, 36]. However, cytotoxicity of these flavonoids against cancer cells has also been reported as mainly due to their binding ability to DNA [11, 37, 38]. The anticancer activities of flavonoid metal complexes were found to be even greater than those of the sole flavonoid. The binding abilities of flavonoid-metal complexes to DNA were studied and their intercalative binding mode and cytotoxicity to certain cancer cell lines were reported [22, 39, 40].

CONCLUSIONS

In conclusion, absorption spectroscopy studies demonstrated that a 1:1 Q:Cu(II) complex was formed in appropriate mixtures of the ligand and the metal ion, and that this complex was stabilized by intercalation between the base pairs of DNA (binding constant, $K_b = (1.82 \pm 0.20) \times 10^5\ M^{-1}$). A competitive reaction, monitored by fluorescence spectroscopy, between fluorescent probe, ethidium bromide (EB) dye, DNA and Q-Cu(II) showed that the intercalated EB was displaced from the EB–DNA complex by the Q-Cu(II) species. In both spectrophotometric studies flavonoid-Cu(II) complexes bind DNA stronger than the actual flavonoid. Very similar results were found by electrophoresis of the plasmid DNA in the presence of the flavonoids, flavonoid-Cu(II) complexes and flavonoid-Zn(II) complexes with or without peroxide addition. While free flavonoids do not show DNA damage they even protect against peroxide damage, as indicated in the literature. Both metal complexes of the flavonoids create much stronger DNA damage while Cu(II) complexes show higher DNAse activities. In the cytotoxicity assay myricitrin showed two times higher potency when combined with metal ions (Cu^{2+} or Zn^{2+}) which is consistent with the other results reported here.

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ЦИТОТОКСИЧНОСТ И ДНК-СВЪРЗВАНЕ НА МЕДНИ (II) И ЦИНКОВИ (II) КОМПЛЕКСИ НА ФЛАВОНОИДИ: КУЕРЦИТРИН, МИРИЦИТРИН, РУТИН

Б. Атабей-Оздемир[1], О. Демиркиран[2], У. Йилдиз[1], И.О. Текин[3], Б. Джобан*[1]

[1]Университет „Бюлент Еджевит“, Факултет за изкуство и наука, Департамент по химия, Зонгулдак, Турция
[2]Тракийски университет, Факултет по фармация, Департамент по фармакогнозия, Одрин 22100, Турция.
[3]Университет „Бюлент Еджевит“, Факултет по медицина, Департамент по имунология, Зонгулдак 67100, Турция

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(Резюме)

Направено е сравнително изследване на флавоноидите куерцитрин, мирицитрин и рутин и техните метални комплекси за свързването им с спектрофотометрични методи и ДНК-азната активност с агарозна електрофореза на pBR322. Свободните флавоноиди се свързват с ДНК по интеркалационен механизъм, но медните комплекси Cu(II) се свързват по-здраво поради електростатични взаимодействия. Флавоноидите имат защитно действие срещу разкъсването на ДНК в присъствие на водороден пероксид. Обаче комплексите с Cu(II) и Zn(II) с тези флавоноиди предизвиква срязващо на скелета на ДНК. Освен това, медните комплекси с флавоноидите имат силна ДНК-азна активност. Мирицитринът е два пъти по-цитотоксичен, когато е комбиниран с метални йони (Cu2+ и Zn2+) отколкото самостоятелно използван спрямо кръвни моно-нуклеарни клетки.