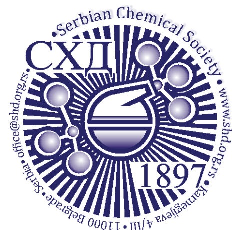

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Original scientific paper

In vitro and *in silico* analysis of the effect of fluconazole, an antifungal drug, on DNA

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Abstract: Fluconazole is an important antifungal drug used worldwide for the treatment of peritonitis and cryptococcal meningitis, urinary tract infections, esophageal tract infections and vaginal candidiasis. In this research, pUC19 plasmid DNA was treated with different concentrations of fluconazole in the presence of ascorbic acid, H_2O_2, iron, iron plus H_2O_2, copper and copper plus ascorbic acid, followed by agarose gel electrophoresis. Fluconazole–DNA interactions were investigated by UV–Vis spectrophotometeric titration and *in silico* methods. Even in the presence of an oxidative agent or a reducing agent, at higher concentrations of fluconazole, double stranded DNA was not broken more than usually found in human plasma. Fluconazole concentrations ≥ 88 μM could protect 46 μM of DNA against hydroxyl radicals produced in the reaction between 1.5 mM of $FeSO_4$ and 6 mM of H_2O_2 while drug concentrations ≤ 44 μM could not provide the protection. In addition, the drug could not protect DNA against ROS originating from the reaction between copper and ascorbic acid. The binding constant of fluconazole–DNA in UV-Vis spectrophotometry analysis and docking analysis was estimated to be 1.087×10^3 and 6.22×10^5 M^{-1}, respectively.

Keywords: DNA; fluconazole; antifungal; docking; interactions.

INTRODUCTION

Fluconazole (FCZ), an antifungal agent, is used against a vast spectrum of pathogenic fungi, such as dermatophytosis, histoplasmosis, cryptococcosis, coccidioidomycosis, blastomycosis, candidiasis, *etc.*[1] Fluconazole exerts its antifungal effect by inhibiting the lanosterol 14α-demethylase enzyme (Erg11) which is responsible for the conversion of lanosterol to ergosterol, a very important part of the fungal plasma membrane. On the other hand, lanosterol is potentially a toxic sterol and accumulation of sterol exert fungistatic activity.[2]

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The study of drug–DNA interactions is very interesting because DNA is an important material in maintaining cell life and it includes all genetic information of cells. Interactions of drugs and chemicals with DNA and the resulting DNA damage are often associated with cancer.[3] Binding of chemical substances to double-strand DNA can be by covalent, groove binding or intercalation ways. Different binding modes of chemicals to DNA can expose different effects to cells, which depend on the cell type and tissue.[4] The minor groove of DNA is sensitive to the attack of small drug molecules. Some of the minor groove bindings molecules (MGBs) damage DNA while some of them inhibit DNA dependent functions. Depending on the chemical structure of drugs, they show selectivity for some nucleophilic parts of DNA.[5] Cooper and iron are important elements in the maintenance of chromosome structure.[6] They are transition elements and show two oxidation states, oxidized states (Cu^{2+} and Fe^{3+}) and reduced states (Cu^{+} and Fe^{2+}). In the reduced state, they catalyze the generation of damaging reactive oxygen species (ROS) that have a toxic effect.[7] There are many studies showing the correlation between iron intake and the risk of multiple cancers, such as colorectal cancer, breast cancer, esophagus cancer and lung cancer.[8] Furthermore, there are several reports showing that some drugs, for instance chloramphenicol, can induce DNA breakage in the presence of iron and/or copper.[9]

In one research, fluconazole caused dose-dependent cytotoxicity in rat hepatocytes with high lactate dehydrogenase (LDH) as assessed by an *in vitro* test.[10] Yuzbasioglu and his coworkers[11] using an *in vitro* test showed that 12.5, 25 and 50 μg mL^{-1} of fluconazole increased chromosomal aberrations (CA), sister chromatid exchange (SCE) and micronuclei frequency in human lymphocytes in a dose-dependent manner, while the drug was not clastogenic in an *in vivo* test. Correa and her coworkers[12] reported that 3, 81.6, 326.5 and 1306 μM of fluconazole increased micronuclei frequency and the DNA damage index (as assessed by the comet test) in African green monkey kidney (Vero) cell line. In the study conducted by Silva,[13] fluconazole concentrations ranging from 6 to 120 μg mL^{-1} caused a concentration-dependent increase in micronuclei frequency in cultured human peripheral blood mononuclear cells (PBMCs). Docking analysis is one of the mainstream *in silico* methods that is used on a daily basis to predict different binding modes of proteins, DNA and different small molecules. Key interactions between docked molecules, binding site, binding conformation of molecules and estimated binding energy are some of the data provided by molecular docking analysis.[14] Therefore, this study aimed at investigating the effect of fluconazole on DNA by treating supercoiled double-strand DNA with the drug in the presence and absence of a reducing agent, an oxidative agent, iron and copper. In addition, in this study, drug–DNA interaction was investigated by docking analysis and the UV absorption titration method.

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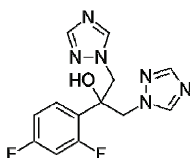
MATERIAL AND METHODS

Reagents

In this research pUC19 plasmid DNA, previously purified from transformed *Escherichia coli* NEB5α strain in the laboratory, was used. To facilitate the dissolution of fluconazole, 0.5 % of absolute ethanol in water was used. Fluconazole was purchased from Sigma–Aldrich (PHR1160-1G) and used as the test substance. The molecular weight of fluconazole (FCZ) is 306.27 g mol^{-1} and its chemical structure:



Effect of fluconazole on DNA

To study the DNA-breaking effect of fluconazole, 46 μM bases of pUC19 plasmid DNA was treated with different concentrations of fluconazole (8800, 880, 88, 44 and 22 μM) at 37 °C for 45 min. After incubation, the reaction mixtures were analyzed by 1 % agarose gel electrophoresis at 90 V for 55 min. The test was repeated in the presence of an oxidative agent (H_2O_2, 6 mM and 6 μM) and in the presence of a reducing agent (ascorbic acid, 88 μM). Untreated pUC19 plasmid DNA was also used as a control sample. All tests were performed in triplet in phosphate saline buffer (PBS) (pH 7.5).

Effect of fluconazole on DNA in the presence of iron

The protective effect of fluconazole against hydroxyl radicals resulting from the reaction between $FeSO_4$ and H_2O_2 was studied according to the method used by Ul-Haq and his coworkers.[15] Briefly, 46 μM bases of pUC19 plasmid DNA was incubated with a solution including different concentrations of fluconazole (8800, 880, 88, 44 and 22 μM), 1.5 mM of $FeSO_4$ and 6 mM of H_2O_2 at 37 °C for 60 min. A sample incubated with $FeSO_4$ (1.5 mM) plus H_2O_2 (6 mM) was used as the positive control and untreated DNA was used as the negative control.

In order to analyze the effect of the drug on DNA in the presence of iron, the DNA was treated with an H_2O_2-free solution containing fluconazole (8800, 880, 88, 44 and 22 μM) and $FeSO_4$ (1.5 mM). All tests were performed in triplet in phosphate buffered saline (PBS) buffer (pH 7.4).

Effect of fluconazole on DNA in the presence of copper

To analyze the effect of fluconazole on copper-mediated DNA breaking, pUC19 plasmid DNA (46 mM bases) was treated with different concentrations of fluconazole (8800, 880, 88, 44 and 22 μM) and $CuCl_2$ (60 μM) for 45 min at 37 °C followed by analysis in agarose gel (1 %) electrophoresis. In addition, another test was conducted by treating the DNA with the solution containing different concentrations of fluconazole (8800, 880, 88, 44 and 22 μM) and $CuCl_2$ (60 μM) plus ascorbic acid (60 μM). All tests were performed in triplet in PBS buffer, pH 7.4.

DNA binding analysis by UV absorption measurements

For this purpose, calf thymus DNA (ctDNA) and fluconazole were dissolved at Tris-HCl buffer (50 mM Tris, pH 7.2). Absolute ethanol (0.5 %) was used to facilitate the dissolution of fluconazole. The DNA solution was titrated using the fluconazole solution followed by recording their spectra at wavelengths between 220 and 320 nm using an Optizen 2120 UV–Vis

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spectrophotometer at every 1 nm. The mixtures were incubated 5 min at room temperature before recording their spectra. Binding constant (K_b) was calculated using Eq. (1):

$$\frac{1}{A-A_0}=\frac{1}{A_\infty-A_0}+\frac{1}{K_b\left(A_\infty-A_0\right)}\frac{1}{C_{\text{ligand}}} \tag{1}$$

where A_0 is the absorbance of DNA at 260 nm in the absence of ligand. A_∞ is the final absorbance value of the saturated state of DNA and fluconazole. A is the absorbance value recorded at different fluconazole concentrations. C_{ligand} is the fluconazole concentration.[16]

Molecular docking analysis

The crystal structure of B-DNA (PDB ID: 1BNA) was downloaded from the Protein Data Bank (RCSB)[17] in PDB format. The 3D structure of the drug was downloaded from the PubChem database[18] in SDF format and converted to PDB format by Discovery Studio 3.0 software.[19] Macromolecule (DNA) and ligand (drug) files in PDB format were prepared using AutoDock tools version 1.5.4. Molecular docking studies were calculated using the Autodock Vina program.[20] B-DNA was the rigid receptor molecule, whereas fluconazole was used as the flexible ligand. The number of active torsions was set to 6. The receptor was enclosed in a script box which had 52x×48y×98z grid points and a grid spacing of 0.375 Å. The amount of independent docking runs was set to 10. The lowest-energy docked conformer, considered to be the best result, was used to further analyze the molecular interactions of fluconazole with DNA. LIGPLOT (version .4.5.3) was used to plot the two dimensional (2D) schematics of the fluconazole–DNA interaction.[21]

RESULTS

Effect of fluconazole concentrations on DNA

The supercoiled plasmid DNA treated with different drug concentrations (8800, 880, 88, 44 and 22 µM) showed no changes in agarose gel electrophoresis compared to untreated DNA, even in the presence of ascorbic acid and H_2O_2, while in the presence of H_2O_2 plus $FeSO_4$, the DNA was converted to the linear form (Fig. 1).

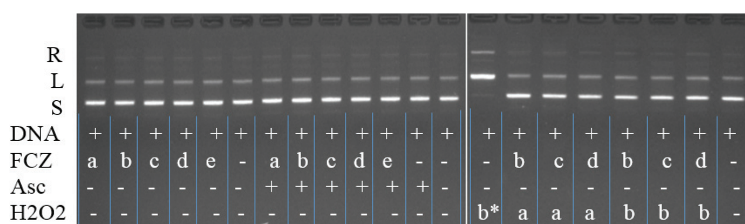

Fig. 1. Treatment of 46 µM bases of pUC19 plasmid DNA with fluconazole (FCZ), FCZ plus H_2O_2 and FCZ plus ascorbic acid (Asc). a, b, c, d and e represent 8800, 880, 88, 44 and 22 µM concentrations of FCZ, respectively. For H_2O_2, a and b show the concentration of 6 mM and 6 µM, respectively and b* represents H_2O_2 (6 µM) plus $FeSO_4$ (1.5 mM). The concentration of ascorbic acid was 88 µM. Plus and minus signs represent the presence or absence of components, respectively. R, L, and S represent relaxed, linear and supercoiled form of the plasmid DNA, respectively.

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Effect of fluconazole on DNA in the presence of iron

When the pUC19 plasmid DNA was incubated with 8800, 880, 88, 44 and 22 μM of fluconazole plus 1.5 mM of $FeSO_4$, no breakage of DNA occurred. Treatment of the supercoiled pUC19 plasmid DNA with $FeSO_4$ (1.5 mM) plus 6 mM of H_2O_2 converted the supercoiled form of the DNA into a relaxed form and linear form because of breakages. Fluconazole concentration ≥ 88 μM could protect 46 μM of DNA against radical hydroxyl while the drug concentration ≤ 44 μM could not provide the protection (Fig. 2).

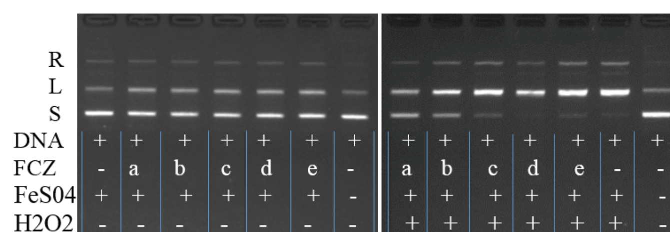

Fig. 2. Effect of fluconazole (FCZ) on DNA in the presence of iron. pUC19 plasmid DNA was used as 46 μM. The different concentrations of fluconazole were shown as a, b, c, d and e that presented 8800, 880, 88, 44 and 22 μM, respectively. The concentrations of $FeSO_4$ and H_2O_2 were 1.5 and 6 mM respectively. Plus and minus signs represent the presence and absence of components, respectively.

Effect of fluconazole on DNA in the presence of copper

$CuCl_2$ without a reductive agent did not break the supercoiled pUC19 plasmid DNA while it converted the DNA to both relaxed form and linear form in the presence of ascorbic acid as a reductive agent. Fluconazole did not induce $CuCl_2$ to break the DNA and was unable to protect DNA from the breaking effect of $CuCl_2$ in the presence of ascorbic acid (Fig. 3).

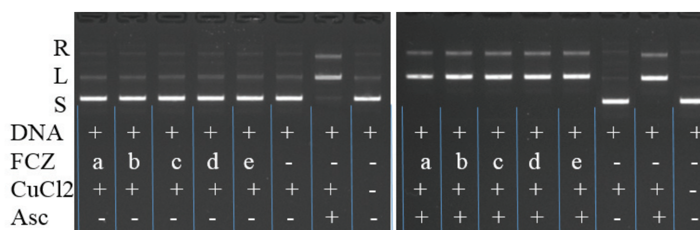

Fig. 3. Effect of fluconazole (FCZ) on DNA in the presence of copper. pUC19 plasmid DNA was used as 46 μM base pairs. The concentrations of fluconazole were shown as a, b, c, d and e that represented 8800, 880, 88, 44 and 22 μM, respectively, and the concentration of $CuCl_2$ and Asc were 60 μM. Plus and minus signs represent the presence and absence of the components, respectively.

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DNA binding study of fluconazole by UV absorption titration

In the study conducted using the UV titration method, the absorption peak wavelength of DNA did not change while the absorbance value at 260 nm was increased by fluconazole in a concentration-dependent manner (Fig. 4A). Fluconazole itself has an absorption peak at 261 nm and therefore, the absorbance value related to fluconazole was subtracted from the obtained absorption values. It was observed that the absorption value of DNA was decreased by fluconazole in the concentration range 0.000393 mol to 0.00157 mol. The decrease indicated the interaction of the drug with DNA (Fig. 4B). The free binding energy and binding constant were calculated as –4.14 kcal* mol^{-1} and 1.087×10^3 M^{-1}, respectively (Fig. 4C).

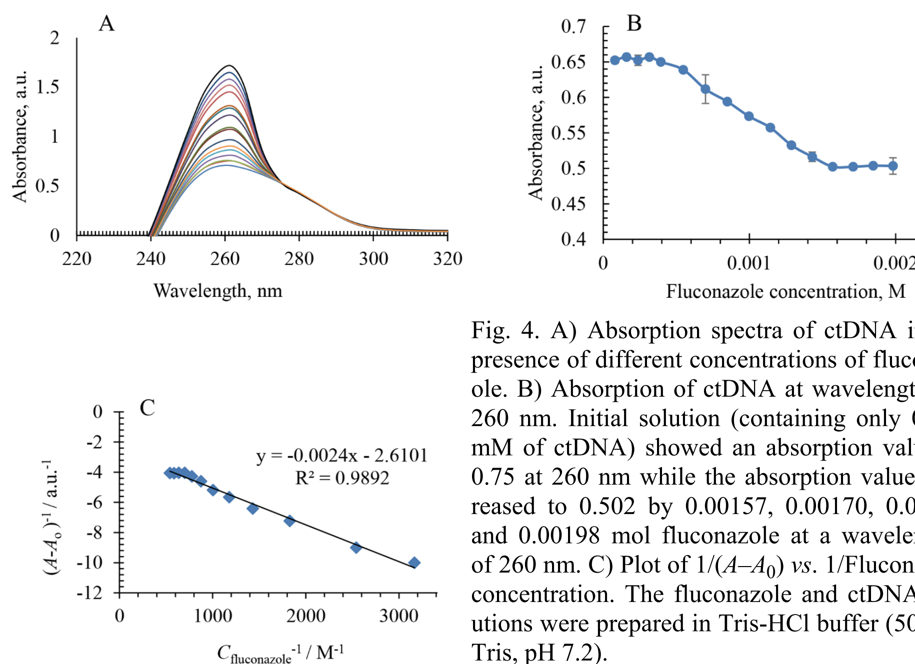

Fig. 4. A) Absorption spectra of ctDNA in the presence of different concentrations of fluconazole. B) Absorption of ctDNA at wavelengths of 260 nm. Initial solution (containing only 0.113 mM of ctDNA) showed an absorption value of 0.75 at 260 nm while the absorption value decreased to 0.502 by 0.00157, 0.00170, 0.00184 and 0.00198 mol fluconazole at a wavelengths of 260 nm. C) Plot of 1/(A–A_0) *vs*. 1/Fluconazole concentration. The fluconazole and ctDNA solutions were prepared in Tris-HCl buffer (50 mM Tris, pH 7.2).

DNA binding of fluconazole study by the molecular docking method

The docking study showed that fluconazole (ligand) binds in the minor groove of double-helix DNA. One of the ligand conformation having the lowest binding energy (–7.9 kcal mol^{-1}) made 2 hydrogen bonds including A:DG4:N2--Flu:N7 and B:DG22:N2-Flu:N7 with the bond lengths of 3.14 and 3.19 Å, respectively (Fig. 5). In addition, the drug formed hydrophobic bonds with the nucleotides including A: DA6 and B: BDC21.

* 1 kcal = 4184 J

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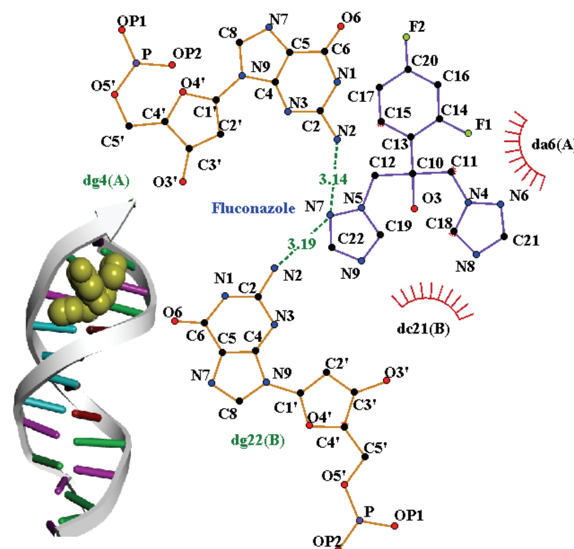

Fig. 5. Molecular modeling of the key interactions between fluconazole and double-helix DNA. In the two-dimensional binding schematics, the bonds shown by the dashed lines represent hydrogen bonds and the bonds shown by radius lines represent hydrophobic bonds. The nucleotides are shown by a one-letter code followed by the numbers showing their position in the DNA strands, A or B within parenthesis identify DNA strands (A: CGCGAATTCGCG / B: CGCGAATTCGCG). In the three-dimensional representation of the interactions, fluconazole is shown by the space fill (CPK) model in the minor groove of the double-strand DNA.

DISCUSSION

In this research, pUC19 plasmid DNA was used as the DNA sample because the native conformation of pUC19 plasmid DNA is a supercoiled form. Any breakage in one of the double strands converts the supercoiled form to an open circular (relaxed) form, while breakage at the same position on both strands makes linear plasmid DNA. The migration of the linear form through an agarose gel is slower than the supercoiled form and faster than the open circle form.[22] The concentrations of fluconazole in human plasma have been reported as 26.9 mg ml^{-1} (87.8 μM).[23] Human cells are exposed to ascorbate concentrations in the range of 10 to 70 μM.[24] Although, regarding H_2O_2 concentrations in human plasma, there are various reports such as 100 mM and 5 μM.[25,26] In this study, the fluconazole, Asc and H_2O_2 concentrations were selected considering this information. Asc was used at high concentrations (88 and 60 μM) found in human plasma and H_2O_2 was applied at two concentrations (6 μM and 6 mM), which could be considered as high concentrations. In this study, even in the presence of high concentrations of an oxidative agent (H_2O_2, 6 μM and mM) and a reducing agent (Asc, 88 μM), the DNA was not broken by fluconazole concen-

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trations higher than those in human plasma and higher than those used by the aforementioned investigators. These tests were not repeated in the presence of low concentrations of oxidative agent and reducing agent since high concentration of oxidative and reducing agent tests were negative. Human physiological plasma iron was reported to be 82 μM.[27] The iron concentration in the human brain was reported as 34 mM.[28] Considering the mentioned information, in this study, the concentration of $FeSO_4$ and H_2O_2 was selected as 1.5 and 6 mM as used by Li and coworkers.[29] Fluconazole concentration $\geq$ 88 μM could protect 46 μM of DNA against radical hydroxyl produced by the reaction between 1.5 mM of $FeSO_4$ and 6 mM of H_2O_2 while the drug concentration $\leq$ 44 μM could not provide protection. Therefore, we are of the opinion that the protection was provided by the binding to and coverage of DNA by the drug. Most of the cellular copper is gathered in the nucleus and bound to DNA bases.[30] The concentration of copper (60 μM) used in this research was within the range (0.2–80 μM) of that in human cells.[31] In the presence of ascorbate or other reducing agents, copper can lead to the production of ROS by catalyzing some reactions and damage of DNA and chromatin.[32] In this research, fluconazole did not induce copper to break DNA. In addition, fluconazole could not protect DNA against the ROS originating from the reaction between copper and ascorbic acid, while it could protect DNA against hydroxyl radicals originating from iron and H_2O_2. In our opinion, this result was because the oxidation and DNA damaging effects of copper is 50 times faster than those of iron.[32] In addition, copper ions bind strongly to DNA and disrupt the double-stranded structure,[33] thus adversely affecting drug binding to DNA. The findings of the present research were not consistent with the findings of Peng and coworkers[34] who stated that fluconazole contributed to an increase of DNA damage *in vitro* when complexed with iron or copper in the presence of hydrogen peroxide. In our opinion, the inconsistency was because they treated 0.1 pmol pBSSK plasmid DNA equal to 296400 μM bp of the plasmid with 50 μM of fluconazole. If they had used a DNA concentration lower than the fluconazole concentration, their results would be consistent with the present results.

UV–Vis spectroscopy is the most common and convenient way to study the interaction between chemicals and double stranded DNA.[35] The band at 260 nm of DNA arises due to the π–π* transitions in the DNA bases. "Hyperchromic" effect and "hypochromic" effect are spectra features of DNA due to its double helical structure. The spectral change process reflects corresponding changes in conformation and structures DNA after binding of the drug bound. Hypochromism results from contraction of DNA in the helix axis, as well as from the change in conformation on DNA while, in contrast, hyperchromism derives from damage to the DNA double helix structure.[36] In this research, on addition of fluconazole, the absorbance value of the ct-DNA solution decreased at 260 nm

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and showed a hypochromic effect due to contraction of the double-stranded ct-DNA. Similarly, molecular docking analysis showed that fluconazole can bind into the DNA minor groove. A chemical substance can increase or decrease gene expression by binding to DNA and inhibit binding of proteins involved in transcription regulation.[38] In line with the present results, previously it was reported that fluconazole concentrations caused an increase of TNF-a, and a decrease of IL-6 and IL-10 expressions in PBMCs.[13] Drug binding causes structural and conformational changes in DNA, such as DNA bending, winding and double or single-strand breaks, resulting in DNA damage.[39] Therefore the DNA-breaking effects (*e.g.* CA, SCE, and micronucleus) reported by researchers could be due to the binding of fluconazole to DNA.

CONCLUSIONS

In conclusion, fluconazole did not break double helix DNA even in the presence of H_2O_2 and ascorbic acid. According to the result of the spectrophotometric and docking analysis, the drug strongly binds to double-stranded DNA. In addition, fluconazole protects DNA against hydroxyl radicals originating from iron and H_2O_2 when its concentration is above the DNA concentration.

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ИЗВОД

IN VITRO И *IN SILICO* АНАЛИЗА ЕФЕКТА ФУКОНАЗОЛА, АНТИФУНГАЛНОГ ЛЕКА, НА ДНК

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Фуконазол је важан антифунгални лек који се широко користи за лечење перитонитиса, криптококалног менингитиса, инфекција уринарног тракта и езофагуса, као и за вагиналну кандидијазу. У овом раду је ДНК плазмида pUC19 третирана различитим концентрацијама фуконазола у присуству аскорбинске киселине, H_2O_2, гвожђа, комбинације гвожђа и H_2O_2, бакра и бакра у присуству аскорбинске киселине, након чега је следила агарозна електрофореза. Фуконазол–ДНК интеракције су праћене UV–Vis спектрофотометријском титрацијом и *in silico* методама. Двоструки ланац ДНК се није кидао више него у хуманој плазми ни у присуству оксидујућих и редукујућих агенаса у комбинацији са већим концентрацијама фуконазола. Фуконазол у концентрацији $\geq$ 88 μM је могао заштитити 46 μM ДНК од хидроксил радикала насталог у реакцији између 1,5 mM $FeSO_4$ и 6 mM H_2O_2, док ту заштиту није могао пружити у концентрацији $\leq$ 44 μM. Лек није могао заштитити ДНК од реактивних кисеоничних једињења насталих у реакцији бакра и аскорбинске киселине. Константа везивања фуконазол–ДНК, одређена UV–Vis спектрометријски и докинг анализом, је процењена на $1,087\times10^3$, односно $6,22\times10^5$ M^{-1}.

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