**Dear Editor and Reviewers,**

We highly appreciate the detailed valuable comments of the referees on our manuscript entitled "In-vitro and in-silico analysis of the effect of fluconazole, an antifungal drug, on DNA". The suggestions are quite helpful for us and we incorporate them in the revised paper. The manuscript was carefully rewritten according to the suggestions of the Reviewer. So, we hope the reviewers and the editors will be satisfied with our responses to the ‘comments’ and the revisions for the manuscript.

My student, Sumeyye has a serious need to graduate. To solve the problem we need to publish this manuscript as soon as possible. Therefore we would be grateful if you could help us in this regard.

Thanks and Best Regards

Yours Sincerely

Dr. Ibrahim Arman

30/01/2020

Reviewer A:  
  
Does the manuscript contain enough significant original material?:  
        yes  
  
Is the manuscript clearly and concisely written?:  
        yes  
  
Are the conclusions adequately supported by the data?:  
        no  
  
Does the manuscript give appropriate credit to related recent publications?:  
  
        no  
  
Are the references appropriate and free of important omissions?:  
        yes  
  
Is the length of the manuscript appropriate?:  
        yes  
  
Does the manuscript need condensation or extension?:  
        yes  
  
Is the quality of the figures (including legends and axes labelling)  
satisfactory?:  
        yes  
  
Are the nomenclature and units in accordance with SI?:  
        yes  
  
Are the English grammar and syntax satisfactory?:  
        yes  
  
ADDITIONAL COMMENTS  
Please indicate the page numbers for suggested corrections. Please, be as specific as possible if major correction by the author(s) is recommended! :  
        Presented study deals with the binding and protective effects of  
fluconazole on plasmid DNA. This is an interesting study since it seems that  
fluconazole may have both beneficial and detrimental effects on a DNA  
molecule.  However, some important issues need to be addressed before  
publication.  
  
Major concerns:  
  
Comment 1: From UV-VIS binding analysis it seems that values are taken at every 5 nm. What is the reason for this? Information on spectrophotometer used in this study is missing.

Response 1: UV-vis spectrophotometer analysis was done and repeated by both UV-vis spectrophotometer (shimadzu, UV-1800) and Optizen 2120. Scanning of the samples was done at every 1 nm wavelength to detect potential red or blue shift resulted from the interaction of fluconazole with DNA.

Comment 2: There is no mentioning if presented spectra are obtained by subtracting sample spectra form those obtained from fluconazole itself. This is very important since fluconazole absorbs at the same wavelength of 260 nm so the observed effect of absorbance increase could be just from the increased concentration of fluconazole during titration of DNA.

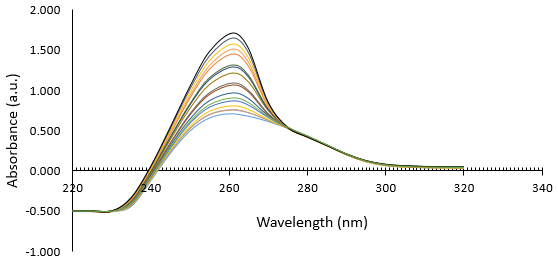
Response 2: Thank you very much for this useful comment, there was actually a mistake and we did the test again considering this comment and we revised this part of the manuscript.

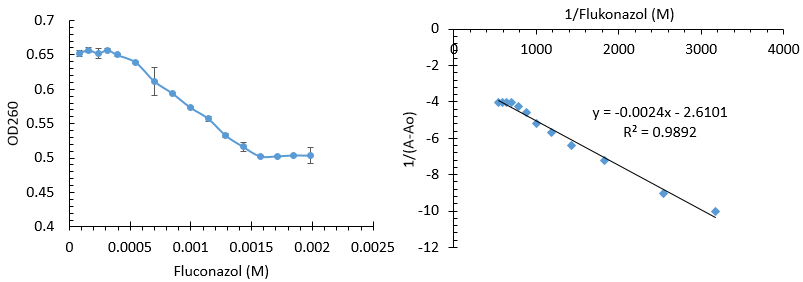
First, in order to detect potential red or blue shift, we did spectrophotometric titration and scanned at wavelengths from 220 to 320 nm. We then measured the absorbance changes of DNA after the drug addition, in order to detect hypochromic or hyperchromic, by the following ways:

1- Absorbance of the fluconazole and fluconazole-DNA were measured separately at 260 nm, then the absorbance of DNA calculated by subtracting the absorbance of the fluconazole.

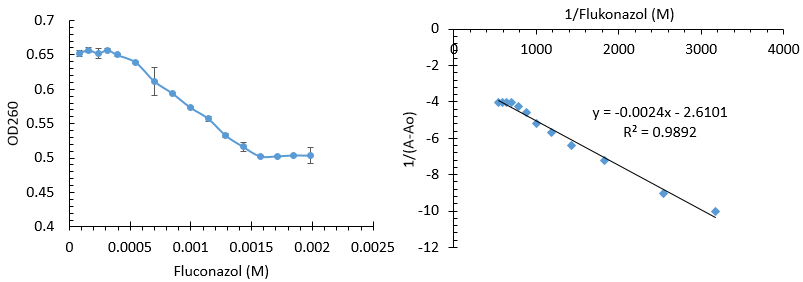
2- Absorbance of the fluconazole-DNA was done using blank containing an equal concentration of fluconazole. I mean, the test cuvette and blank cuvette had an equal concentration of fluconazole and buffer.

We obtained the same results from the two tests done by the two methods.



  
  
Comment 3: No plot obtained from the equation used to calculate binding affinity is presented. This should be added as a Figure in the manuscript. Also, there is no mentioning if the binding study is performed in triplicate, at least.

Response 3: This part was revised

  
  
Comment 4: The author state that there are no papers describing the direct effect of fluconazole on DNA. This is incorrect. For example paper by Peng et al, from 2018: Fluconazole induces ROS in Cryptococcus neoformans and contributes to DNA damage in vitro, deals with fluconazole effect on DNA. So, better literature search is needed. Furthermore, these authors stated that fluconazole causes DNA damage in vitro so additional comment about this should be provided.

Responce 4: The sentences of “there are no papers describing the direct effect of fluconazole on DNA” was deleted. According to the Journal's Author Guidelines, an article can have a maximum of 35 references, so we had to consider limitation in the written article.

We had before read the paper published by Peng but we could not use it, in our opinion the part of that paper we could discuss in the discussion of our manuscript need to revise because:

1- In Figure 3D lane12, the mixture of CuSo4 and H2O2 produces radical OH and it must have broken the DNA.1

2- EDTA is a chelator and it can chelate Fe and Cu not radical OH present in the reaction mixture therefore it cannot stop the reaction of DNA breaking and conserve DNA against radical OH present in the mixture. On the other hand, the complex of Fe-EDTA can produce radical OH from H2O2. 2

3- As it was shown in figure 3A Lane 5, FeSo4 + H2O2 produces radical hydroxyl and breaks DNA. We do not think it makes sense to use densitometer programs to compare bands on different gels. It may be useful to compare bands on the same gel.

They used 0.1 pmol pBSSK plasmid DNA equal to 296400 µM bp of the plasmid. In our opinion, if they used the DNA concentration (bp) lower than fluconazole concentration, their results would be consistent with our results. All of the studies presented in Figures 1, 2 and 3 of our research were repeated 3 times and every time, the same results were obtained. We showed only one that repeats in the manuscript.  
Comment 5: Molecular docking analysis gave two orders of magnitude higher affinity  
constant when compared to the experimental one. Some discussion is needed  
for this.

Response 5: This part of the manuscript was revised  
  
Minor concerns:  
  
1) Line 113: ... as a concentration of 24 mM should be rephrased to ...at the final concentration of 24 mM.

Response: This was corrected  
  
2) Line 144: word fluconazole at the beginning of the sentence should start with a capital letter

Response: This was corrected  
  
3) Letters that appear on figures, R, L and S should be defined somewhere in the manuscript or in Figure   Legends.  
This was corrected

------------------------------------------------------  
Reviewer B:  
  
Does the manuscript contain enough significant original material?:  
        no  
  
Is the manuscript clearly and concisely written?:  
        no  
  
Are the conclusions adequately supported by the data?:  
        no  
  
Does the manuscript give appropriate credit to related recent publications?:  
  
        no  
  
Are the references appropriate and free of important omissions?:  
        no  
  
Is the length of the manuscript appropriate?:  
        yes  
  
Does the manuscript need condensation or extension?:  
        no  
  
Is the quality of the figures (including legends and axes labelling)  
satisfactory?:  
        no  
  
Are the nomenclature and units in accordance with SI?:  
        yes  
  
Are the English grammar and syntax satisfactory?:  
        no  
  
ADDITIONAL COMMENTS  
Please indicate the page numbers for suggested corrections.  
Please, be as specific as possible if major correction by the author(s) is  
recommended! :  
        I would suggest a careful reading by a native English speaker, since many  
sentence structures and phrases are hard to follow. The manuscript requires  
substantial English language editing.  
  
REPORT:  
        The study conducted by these authors includes a different approach to assess DNA damage related to the use of fluconazole.

The statement that “there is not any research regarding the direct effect of the fluconazole on DNA” is clearly exaggerated, since the authors cite the previous reports which include the Comet Assay results.

The sentence was deleted

What is noted is the difference in signals for the untreated DNA between the presented agarose  
gels. Also, the signal for the DNA treated with H2O2  (6 µM) and FeSO4 (1.5 mM) in Figure 1 represents a smear, while when the same DNA was treated with much higher concentration of H2O2  (6 mM) and FeSO4 (1.5 mM) in Figure 2 a clear pattern of bands was detected.

Furthermore, in the same figure, the band corresponding to linear DNA seems more intense for treatments of pUC19 in the presence of both fluconazole and FeSO4 that for the DNA treated with  
just FeSO4, but no densitometric analysis was performed and also triplicates were not shown. Therefore, the illustration of the results discussed in the manuscript is relatively poor. The quality of the results shown in the second gel in Figure 1 is poor, since an additional band appeared between  
those corresponding to supercoiled and linear DNA. Still, the protective effect of fluconazole is generally well presented in the second gel in Figure 2.

The electrophoresis related to the mentioned gel (Right part of Figure 1) was done again and it was corrected in the revised version of the manuscript. Although all of the studies presented in Figures 1, 2 and 3 were repeated 3 times, we showed only one that repeats in the manuscript. In our opinion, the simplest way is to visually compare the intensity of bands to the band related to the control sample of the same gel. On the other hand, we did not any densitometer program to use.

The authors have not stated the type of the solvent used for dissolving fluconazole, which is important, since this compound is soluble in organic solvents and very poorly soluble in water.     
Response: Thank you very much for this helpful comment. The missed sentence was added as follow:

0.5% of ethanol (100% pure) was used to facilitate the dissolution of fluconazole.

The text requires substantial English editing.

Response: We did our best to edit the manuscript completely

For instance, in line 33, “drug was used” should be changed to “drug is used” (term “was” implies that it is not used any more). Various sentences need to be rephrased (“living things”, “researchers are very interested”?). In the line 78 “of” should be deleted. The abbreviation Asc was not explained in the text. In line 52, Fe+++ should be changed to Fe3+. In line 59, references are missing, since the reference numbered 12 does not include the data on the effects of resveratrol, bleomycin, etc.  
Response: All of these were corrected   
In my opinion, this manuscript should:  
        be published after major revision and additional review  
  
If manuscript is suitable for publishing, referees recommendation :  
        Original scientific paper  
  
------------------------------------------------------  
  
------------------------------------------------------  
**Reviewer C:**  
  
Does the manuscript contain enough significant original material?:  
        yes  
  
Is the manuscript clearly and concisely written?:  
        no  
  
Are the conclusions adequately supported by the data?:  
        yes  
  
Does the manuscript give appropriate credit to related recent publications?:  
  
        yes  
  
Are the references appropriate and free of important omissions?:  
        no  
  
Is the length of the manuscript appropriate?:  
        yes  
  
Does the manuscript need condensation or extension?:  
        no  
  
Is the quality of the figures (including legends and axes labelling)  
satisfactory?:  
        yes  
  
Are the nomenclature and units in accordance with SI?:  
        yes  
  
Are the English grammar and syntax satisfactory?:  
        yes  
  
ADDITIONAL COMMENTS  
Please indicate the page numbers for suggested corrections. Please, be as specific as possible if major correction by the author(s) is recommended! :  
        Line 121 – Reference is missing for Chimera program.

Response: That was corrected  
Line 123 – Reference is missing for 1BNA crystal structure.

Response: That was corrected  
Line 125 and 126 – References are missing for AutoDock and Discovery software.

Response: That was corrected  
Table 1 – Authors should omit Run column. Cluster RMSD column should be omitted as well.

Response: That was corrected  
Line 204 to 209 – Whole section regarding docking analysis should be deleted or moved to introduction.

Response: That was corrected  
Line 209 – Reference for AutoDock is wrong. Accurate reference for AutoDock4 is (AutoDock 4.2 Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S. and Olson, A. J. (2009) Autodock4 and AutoDockTools4: automated docking with selective receptor flexiblity. J. Computational Chemistry 2009, 16: 2785-91.).

Response: Docking analysis was done using Auto Dock Vina software. Related reference was added.  
Line 250 – Authors should reference conformation (in Table 1) that corresponds to stated binding constant.

Response: The table was deleted and the manuscript was revised as follows:  
Docking study showed that fluconazole (ligand) was located in the minor groove of double-helix DNA. One of the ligand conformation having lowest binding energy (-7.9 kcal/mol) and highest Kb value (6.22 × 105 M-1) made 2 hydrogen bonds including A:DG4: N2 - Flu: N7 and B:DG22: N2 - Flu: N7 with the bond length of 3.14 and 3.19 Å respectively (Fig. 5). In addition, the drug had hydrophobic bonds with the nucleotides including A: DA6 and B: BDC21.

REPORT:  
        1. Authors should consider using Auto Dock Vina software (<http://vina.scripps.edu/>) instead of AutoDock4 due to improved accuracy of new version. Experimental section should be more precise. Authors should give more detail about the experimental procedure.  For example: how the crystal structure was prepared for docking? How the DNA bind site was determined? How many docking runs were made and were they rigid of flexible? What were criteria for docking poses selection? Also, references should be given for each instance of software used.

Response: This part was revised as follow:

The crystal structure of B-DNA (PDB ID: 1BNA) was downloaded from the Protein Database(RCSB) in PDB format . The 3D structure of the drug was downloaded from the PubChem database in SDF format and converted to PDB format by Discovery Studio 3.0 software.3 Macromolecule (DNA) and ligand (drug) files in PDB format were prepared using AutoDock tools version 1.5.4 and Molecular docking studies were calculated using Autodock Vina program.4 B-DNA is used as a rigid input receptor molecule for docking, whereas semustine and lomustine are used as flexible ligands and number of active torsions are set to 6. The receptor was enclosed in a script box which had a much number of grid points in x × y × z directions of 52 × 48 × 98 and a grid spacing of 0.375 Å. The amounts of independent docking runs performed for docking simulation was set to 10. The lowest-energy docked conformer is considered to be the best result and is used to further analyze the molecular interactions of fluconazole with DNA. LIGPLOT v.4.5.3 was used to study the two dimensional (2D) structutr of the fluconazole – DNA interaction. 5

2. Authors report multiple Fluconazole docking conformations but only one of them is explained in detail (in text and Fig 5). What are the differences between conformers 1 and 37? Do all conformers share same key binding interactions?  Are there any significant key interactions differences that could lead to different binding to DNA? Importance of results presented in Table 1 need to be clarified or condensed to only few significant conformers.

Response: The table was deleted and the manuscript was revised as follows:  
Docking study showed that fluconazole (ligand) was located in the minor groove of double-helix DNA. One of the ligand conformation having lowest binding energy (-7.9 kcal/mol) and highest Kb value (6.22 × 105 M-1) made 2 hydrogen bonds including A:DG4: N2 - Flu: N7 and B:DG22: N2 - Flu: N7 with the bond length of 3.14 and 3.19 Å respectively (Fig. 5). In addition, the drug had hydrophobic bonds with the nucleotides including A: DA6 and B: BDC21.

3. Authors should consider expanding the discussion section to discuss the importance of obtained docking analysis results and observed key binding interactions. Also, authors should comment on quality of in-silico determined binding constant compared to experimental results.  
Response: In this research, by the addition of fluconazole, the absorbance value of the ct-DNA solution decreased at 260 nm and showed a hypochromic effect due to contraction of the double-stranded ct-DNA. The binding constant obtained using UV-Vis spectrophotometry analysis and docking analysis was 1.087×103 M-1 and 6.22×105 M-1 respectively. The inconsistencies of the spectrophotometry and docking analysis results may be due to the differences in the methods by that the two analyses are performed. One is based on algorithms and scoring function (theory) and analyzes the interaction between DNA and drug while the other one is an experimental method that can be affected by some other ingredients and factors such as buffer and temperature. The value of Kb for fluconazole is comparable to esculetin (an anti-cancer agent) (1.87×104 M-1) which binds to DNA.6A chemical substance can increase or decrease gene expression by binding to DNA and inhibit binding of proteins involved in transcription regulation.7 In line with our results, before it was reported that fluconazole concentrations caused to increase TNF-a, and decrease IL-6 and IL-10 expressions in PBMCs 8. Drug binding causes structural and conformational changes in the DNA such as DNA bending, winding double or single-strand breaks, resulting in DNA damage.9Therefore the DNA-breaking effects (e.g. CA, SCE, and micronucleus) reported by researchers can be due to the binding of fluconazole to DNA.

References:

1. K. Yokawa, T. Kagenishi and T. Kawano, *Biosci Biotechnol Biochem* **75**(2011)1377(10.1271/bbb.100900)

2. W. Koppenol, *J Free Radicals Biol Med* **1**(1985)281,

3. I. Accelrys, *San Diego, CA* **92121**(2011),

4. O. Trott and A. J. Olson, *J. Comput. Chem.* **31**(2010)455,

5. A. C. Wallace, R. A. Laskowski and J. M. Thornton, *Protein engineering, design and selection* **8**(1995)127,

6. T. Sarwar, M. A. Husain, S. U. Rehman, H. M. Ishqi and M. Tabish, *Mol. Biosyst.* **11**(2015)522,

7. J. M. Gottesfeld, J. M. Turner and P. B. Dervan, *Gene Expression, The Journal of Liver Research* **9**(2001)77,

8. G. S. Silva, L. Zuravski, M. Duarte, M. M. Machado and L. F. S. Oliveira, *Immunopharmacol. Immunotoxicol.* **41**(2019)123(10.1080/08923973.2019.1566357)

9. S. A. Shaikh, S. R. Ahmed and B. Jayaram, *Arch Biochem Biophys* **429**(2004)81(10.1016/j.abb.2004.05.019)