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Effect of verapamil on *Enterococcus faecalis*, *Escherichia coli* and on DNA damage induced by copper and iron

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Verapamil is commonly used oral drug for treatment of high blood pressure and heart diseases worldwide. Here, we investigated the effect of verapamil on two of human intestinal flora bacterial strains *Enterococcus faecalis* and *Escherichia coli* using the broth medium dilution method and agar disc diffusion method. In addition, DNA was treated with verapamil, verapamil plus H_2O_2, verapamil plus ascorbic acid, verapamil plus iron and verapamil plus copper and analyzed by agarose gel electrophoresis. The interaction of verapamil with DNA was investigated by UV-Vis spectrophotometer and *in silico* methods. Verapamil inhibited the growth of both *E. faecalis* and *E. coli* up to 90% at concentrations of 9, 18 and 25 mM. The concentration of 9 mM (480 mg dose) of the drug was found as both MIC and MBC value for the two bacterial strains. The drug did not show any breaking or protective effect on the supercoiled plasmid DNA. In the UV-Vis spectrophotometer analysis, verapamil did not show any interaction with the DNA. In contrast, *in silico* analysis showed the drug to bind to a minor groove of double helix DNA by hydrogen bonds and hydrophobic interactions with the binding energy of –7.3 kcal/mol and binding constant (K_b) value of 2.3×10^5 M^{-1}.

Keywords: Binding energy, Docking, Drug-DNA interactions, Gut flora, High blood pressure, Hypertension, Intestinal flora bacteria

Verapamil is a calcium channel blocker used to treat hypertension, and it has been reported as an inhibitor of P-gp and some CYP enzymes[1]. It is used for the treatment, control and prevention of high blood pressure, heart diseases and migraine diseases. Since antihypertensive drugs show heterogeneity as a chemical, they have recently attracted the attention of many researchers to study the drugs in terms of side effects[2]. Al-harthy and coworkers[3] reported that addition of verapamil enhances the cytotoxic effect of doxorubicin against the growth of MCF-7 cells. Jaferiyan and his co-workers[4] showed that verapamil enhances the cytotoxic effects of docetaxel and vinblastine combined therapy against non-small cell lung cancer cell lines. Milosevic *et al.*[5] who investigated the effect of verapamil hydrochloride on human lymphocytes using the Micronucleus test and the Fish method, showed that 100 µM of verapamil had a significant cytotoxic effect. Verapamil is commercially available as capsules, tablets and injectable forms (https://www.drug bank.ca/drugs/DB00661).

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DNA (Deoxyribonucleic acid) is an important material in maintaining cell life. The study of Drug-DNA interactions is an interesting and important issue both in understanding the interaction mechanism and in the design of new drugs. The minor groove of DNA is sensitive to the attacks of small drug molecules. Depending on the chemical structure of drugs, they show selectivity for some nucleophilic parts of DNA[6]. Cells have many endogenous antioxidant systems to ensure a balance between ROS and antioxidants[7]. Increased levels of the reactive oxygen, decreased levels of antioxidant enzymes and insufficient DNA repair mechanisms lead to oxidative DNA damages including single and double strands breaks and base modifications[8]. Therefore, the primary cause of cell death can result from oxidative DNA damages. The exogenous sources of free radicals are drugs, metal ions, pollutants and radiation. Metal ions react with H_2O_2 in biological systems to produce highly reactive radicals such as hydroxyl radicals resulting in oxidative DNA damage[9]. In a study, it was observed that the interaction of pyrimidine, β-amino alanine and β-hydroxy imidazole with Fe (II) in the *Bleomycin* caused DNA damage[6].

There is an ecological balance between the human host and microorganisms that colonize mucous

surfaces. The normal microflora represents a complex and important ecological system and alterations in the flora may give rise to serious clinical implications. Administration of antimicrobial agents causes disturbances in the ecological balance between host and microorganisms[10]. Since verapamil is administered orally to cardiac patients, it is in direct contact with microbiota in the gastrointestinal tract for a long time[11]. Verapamil has been shown to have an antimicrobial potentiating effect in the treatment of *Mycobacterium tuberculosis*[12]. Also, verapamil at high concentrations (400-800 µg/mL level) has been reported to produce inhibitory action against some bacterial strains, such as *Bacillus leicheniformis* 10341, *Staphylococcus aureus* NCTC 6571 and *Escherichia coli* K12Row[13]. In this study, we investigated the effect of verapamil on DNA strand breakage induced by copper and iron and also the effect of the drug on the growth of *Escherichia coli* and *Enterococcus faecalis* commonly found in the human intestine.

Materials and Methods

In this study, verapamil hydrochloride (catalog No. 2811, Acros Organics) and *E. coli* ATCC-25922 and *E. faecalis* ATCC-29212 were used.

Preparation of plasmid DNA

pUC19 plasmid DNA that had been previously transferred to the *Escherichia coli* NEB5α strain was purified. Plasmid purification was carried out following the EZ-10 Spin-Column Plasmid DNA Miniprep Kit (bio basic) protocol. Beer-Lambert's law and agarose gel (1%) electrophoresis were used to analyze the amount and purity of the DNA.

Effect of verapamil hydrochloride on DNA

To determine the DNA damaging potential of verapamil hydrochloride, 18.5 µM base pairs (bp) of pUC19 plasmid DNA was treated with 1850, 185, 18.5, 1.85 and 0.185 µM of verapamil hydrochloride at 37°C for 45 min. The test was repeated in the presence of H_2O_2 (6 mM) as an oxidative agent and ascorbic acid (Asc) (60 µM) as a reducing agent. After incubation, the reaction mixtures were run in agarose gel (1%) electrophoresis at 55 volts for 55 min, followed by staining with ethidium bromide. All of the tests were performed in phosphate saline buffer (PBS) (pH = 7.5)[14].

Effect of verapamil on iron-mediated DNA breakage

In this part, 18.5 µM bp of pUC19 plasmid DNA were treated with 30 µM and 1.5 mM of $FeSO_4$ plus different concentrations of verapamil hydrochloride (1850, 185 and 18.5 µM) using the procedure used by Li *et al*. [15]. It was then incubated at 37°C for 1 h. A tube containing all of the ingredients except verapamil hydrochloride and another containing just untreated plasmid DNA were used as control samples. To examine the protective effects of verapamil hydrochloride on DNA against the oxidative agent, the pUC19 plasmid DNA was treated with 30 µM $FeSO_4$ plus 30 µM H_2O_2 plus different concentrations of verapamil hydrochloride (1850, 185, 18.5, 1.85 and 0.185 µM) and followed by incubation at 37°C for 1 h. After that, the reaction mixture was subjected to analysis on agarose gel (1%) electrophoresis for 55 min at 90 volts. All tests were performed in PBS (pH, 7.4) buffer.

Effect of verapamil on copper mediated DNA breakage

In order to study the effect of verapamil hydrochloride on DNA-copper interaction, 18.5 µM bp of pUC19 plasmid DNA were treated with 60 µM of $CuCl_2$ plus different concentrations (1850, 185, 18.5, 1.85 and 0.185 µM) of the drug at 37°C for 45 min. The reaction mixture was then subjected to analysis on a 1% agarose gel electrophoresis for 55 min at 90 volts. The tests were repeated by all of the ingredients plus Asc (60 µM). A plasmid DNA treated with no substance was used as a control sample. All tests were performed in PBS (pH, 7.4) buffer[16].

Binding activity of verapamil on DNA

The interaction between verapamil hydrochloride and DNA was investigated by UV-Vis spectrophotometer and docking methods. At first, 87 µM of calf thymus DNA that was dissolved in Tris-EDTA buffer (0.5 molar EDTA, and 50 mM Tris, pH 7.2) was titrated with 2 µL of verapamil hydrochloride (1.6 mM) and analyzed by absorption spectra recording in the range of 225-300 nm. After each verapamil hydrochloride addition, drug-DNA solutions were allowed to incubate for 5 min. The intrinsic DNA binding constant (K_b) was obtained using the following equation[17,18]:

$$\frac{1}{A-A0} = \frac{1}{A\infty - A0} + \frac{1}{\mathrm{K}(A\infty - A0)}\frac{1}{[Vera]}$$

where A0 is the absorption of DNA at 260 nm in the absence of verapamil hydrochloride; A∞ is the ultimate absorption in the presence of verapamil hydrochloride. A is the absorbance of verapamil hydrochloride-DNA. While the calf thymus DNA concentration was kept constant at 3.3 mM at pH 7.4,

the verapamil hydrochloride concentration varied between 0.16 mM and 1.6 mM. To study the verapamil -DNA interaction with docking method, the DNA sequence (PDB ID: 1BNA) was obtained from the Protein Database (https://www.rcsb.org/) in a 3D form and 3D form of verapamil was retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) database. Auto Dock Tools was used to prepare the DNA and verapamil hydrochloride files. The complex system was enclosed in a box with a number of grid points in x×y×z directions, (50×50×100), and a grid spacing of 0.375 Å. The output from AutoDock was rendered with Discovery Studio tool[19].

Antibacterial effect of verapamil hydrochloride

In this part, two flora bacterial strains (*E. faecalis* as a Gram positive strain and *E. coli* as a Gram negative strain) were inoculated into Mueller Hinton broth medium containing different concentrations of verapamil hydrochloride (0.75, 1.5, 9, 18 and 25 mM) followed by adjusting to standard turbidity of 0.5 McFarland. After that, the cultures were incubated overnight at 37°C at 150 rpm. The growth of the strains was evaluated by UV-visible spectrophotometry (Optizen 2120 UV) and CFU (colony forming units) counting. The lowest drug concentration that inhibited the growth of the strains was determined as MIC (minimum inhibition concentration) and the lowest drug concentration that killed the strains was determined as MBC (minimum bactericidal concentration). In addition, the bacterial strains of *E. coli*, *E. faecalis* were cultured on Mueller Hinton agar plats by spread plate technique. After that sterile blank discs containing 25, 18, 9, 1.5 and 0.75 mM of verapamil hydrochloride were placed on the culture before incubation. Then the cultures were incubated at 37°C for an overnight and the antibacterial effect of the verapamil tried to assay by the zone diameter formed around the colonies[20].

Results and Discussion

Effect of verapamil on DNA

In the supercoiled plasmid DNA that was treated with different concentrations of verapamil hydrochloride, verapamil hydrochloride plus Asc and verapamil hydrochloride plus hydrogen peroxide were not shown any breakages in agarose gel electrophoresis (Fig. 1A).

Effect of verapamil on iron mediated DNA damage

Different concentrations of verapamil hydrochloride did not show any breaking activity on supercoiled plasmid DNA in the presence of iron. In addition, they could not show any protective effect against radical hydroxide originated by Fenton reaction (Fig. 1B).

Effect of verapamil on copper mediated DNA damage

Different concentrations of verapamil hydrochloride did not show any breaking activity on supercoiled plasmid DNA in the presence of copper. In addition, they could not protect supercoiled plasmid DNA against the damaging activity of copper plus Asc (Fig. 1C).

DNA binding activity of verapamil

At 260 nm, the initial absorbance value (0.575) of the DNA solution did not change significantly after titration with verapamil hydrochloride. As shown in Fig. 2, the verapamil hydrochloride-free DNA solution showed an absorbance peak at 260 nm, while

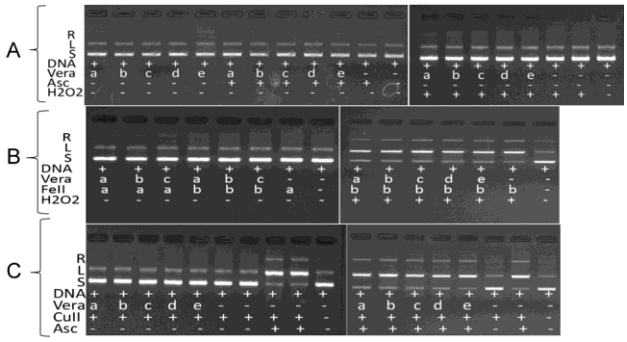

Fig. 1 — Effect of verapamil hydrochloride on DNA in the presence of (A) reductive and oxidative agenet (Ascorbic acid (Asc) concentration was 60 µM and H_2O_2 concentration was 6 mM); (B) iron (concentrations of $FeSO_4$ were shown as a and b that presented 1.5 mM and 30 µM, respectively. The concentration of H_2O_2 was 30 µM); and (C) copper ($CuCl_2$ and Asc were 60 µM). [pUC19 plasmid DNA was used as 18.5 µM bp. Different concentrations of verapamil hydrochloride are shown as a, b, c, d and e that representing 1850, 185, 18.5, 1.85 and 0.185 µM, respectively]

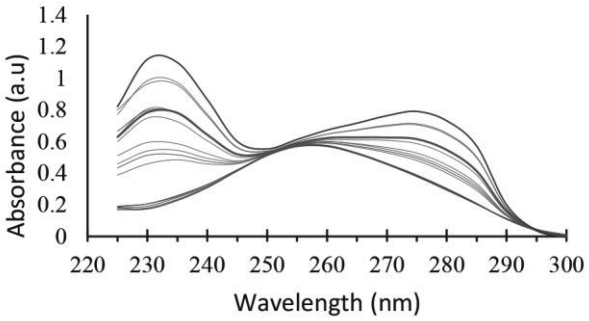

Fig. 2 — Ultra violet-Vis DNA-verapamil hydrochloride. The spectra have been recorded at wavelengths ranging 225-300 nm.

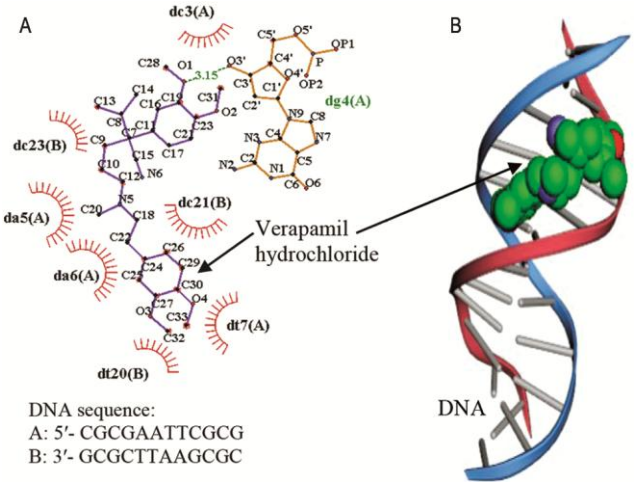

Fig. 3 — Molecular modeling of the hydrogen bonding interaction between verapamil and double helix DNA. The bonds shown by dashed lines represent hydrogenic bonds and the bonds shown by radius lines represent hydrophobic bonds.

Table 1 — Docking summary of verapamil hydrochloride with DNA

Rank	Run	Binding energy (kcal/mol)	Cluster RMSD	K_b (M^{-1})
1	79	–7.32	0	2.34×10^5
2	57	–7.22	0	1.97×10^5
3	96	–6.69	0	8.06×10^4
4	10	–6.1	0	2.98×10^4
5	45	–6.07	0	2.83×10^4
6	52	–5.96	0	2.35×10^4
7	18	–5.92	0	2.20×10^4
68	44	–2.09	0	3.41×10^4

the solution showed two absorbance peaks (at 235 and 275 nm) after the addition of 165 µM verapamil hydrochloride. Verapamil hydrochloride itself has a maximum of UV absorption at 232 and 278 nm.

Docking study showed that in the conformation with lowest ΔG, verapamil hydrochloride was located in the minor groove of double-helix DNA by making one hydrogen bond including DG4: O3 - Vera: O1, with the bond length of 3.15 Å, and hydrophobic bonds with some nucleotides (C3, G4, A5, A6 and T7 from A chain and C8, C10 and T11 from B chain) (Fig. 3). The ligand conformer with the lowest binding energy and the one with the highest binding energy showed –7.32 and -2.09 kcal/mol, respectively (Table 1).

Antibacterial effect of verapamil hydrochloride

As it was shown in Figs 4 & 5 the concentration of 9 mM of verapamil hydrochloride was determined as MIC (minimum inhibition concentration) and MBC (minimum bactericidal concentration). The concentrations of 1.5 and 0.75 mM of verapamil

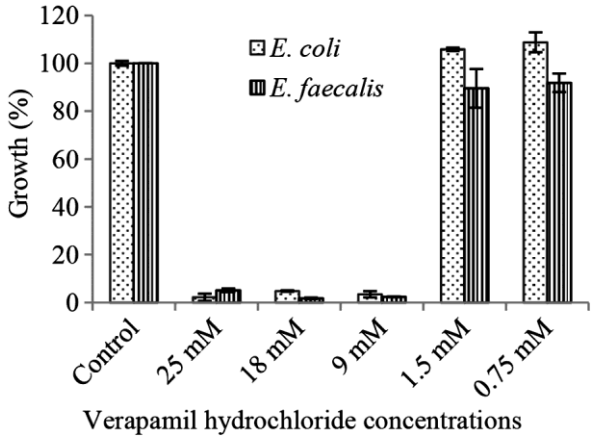

Fig. 4 — Relative growth of *E. faecalis* and *E. coli* in medium containing various concentrations of verapamil hydrochloride. The CFU numbers of the control samples for *E. faecalis* and *E. coli* that were 9.88×10^{21} and 9.118×10^{14} were selected as 100 %. All tests were performed in triplicate.

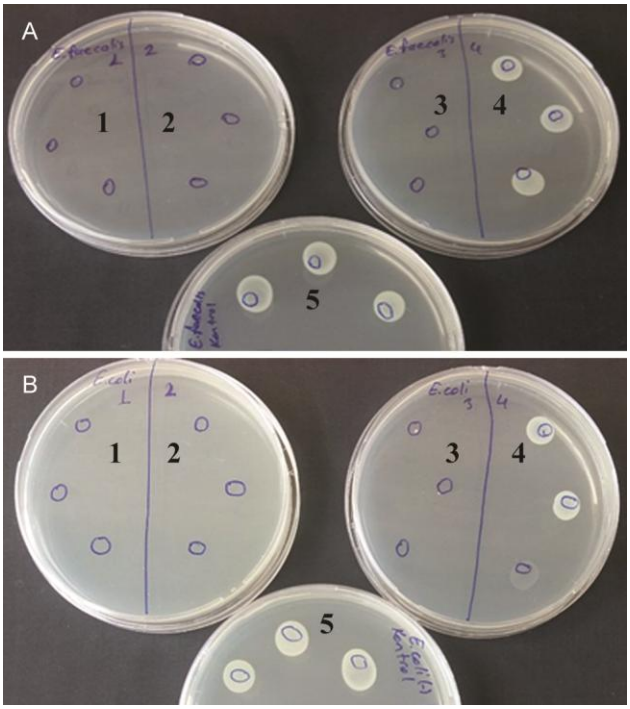

Fig. 5 — MBC analysis of verapamil hydrochloride. a and b represent *E. faecalis* and *E. coli*, respectively, also the number of 1, 2, 3, 4 and 5 represent 25, 18, 9, 1.5 and 0.75 mM of verapamil hydrochloride, respectively

hydrochloride caused to increase the growth of *E. coli* and *E. faecalis* as 6 and 9%, respectively.

Discussion

Damage to cellular DNA is involved in mutagenesis and the development of cancer. In order to explore DNA damage caused by chemicals, one of

the inexpensive, rapid, and valuable method is the incubation of a supercoiled plasmid DNA with the selected chemical followed by agarose gel electrophoresis[21]. DNA breakage can be analyzed by study conversion of supercoiled pUC19 plasmid DNA into relaxed circular and linear forms of DNA. The intact pUC19 plasmid DNA is found in supercoiled form while pUC19 plasmid DNA with a single strand break is in relaxed circular form and finally, plasmid DNA with breaks in both strands is found in linear form. Using this information, the breaking potential of verapamil hydrochloride on DNA was determined by gel electrophoresis analysis of pUC19 after treatment with different concentrations of verapamil hydrochloride. Human physiological plasma iron and copper total concentrations (free and bound) range between (11-31 µM) and (14-19 µM), respectively[22]. Copper is one of the most redox-active metal ions found in cells and is closely associated with chromatin [23]. The concentration of verapamil in human plasma had been reported to be as almost 0.055-0.355 mg/mL[24] and 10-500 ng/mL[25]. Blood level data are important to study the toxic effect of drugs and chemicals[24]. Therefore, iron, copper and verapamil hydrochloride concentrations have been selected considering their concentrations in human plasma. In this study, the supercoiled pUC19 plasmid DNA was not broken by verapamil hydrochloride even at present H_2O_2 or Asc. In contrast, some drugs such as resveratrol[26] and chloramphenicol[27] were reported to beak DNA. In addition, verapamil hydrochloride could not protect the DNA against damaging hydroxyl radicals generated by Fenton reactions. UV-visible spectroscopy is the most common method to study drug-DNA interaction. After binding of a drug to DNA, in the spectrum of DNA hypochromic effects occurred due to changes in the conformation and structure of DNA[20]. A hyperchromic effect is due to the binding of chemicals to DNA which might result in the unwinding of DNA helix[28]. In UV-Vis spectrophotometry analysis, the absorbance of ctDNA did not show any changes by the addition of verapamil hydrochloride, in other words, verapamil hydrochloride did not interact with ctDNA. On the other hand in docking study, verapamil hydrochloride showed to make one hydrogen bond (DG4: O3 - Vera: O1) and some hydrophobic bonds with DNA. In this study, PBS was used as a solvent buffer, therefore we think that the result of UV-Vis spectrophotometry analysis was because of the NaCl present in PBS buffer that decreased hydrophobicity of the drug. Grujicic *et al*. [29] showed that the rate of micronucleus in peripheral blood lymphocytes of pregnant women treated with verapamil hydrochloride was increased.

In this study, the three high concentrations of verapamil hydrochloride showed a significant cytotoxic effect on *E. faecalis* and *E. coli*. Considering the mean fluid volume in the small intestine (105±72 mL and 54±41 mL, P <0.01)[30], the oral doses of verapamil hydrochloride (40-400 mg) make the concentration of 0.75, 1.5, 9, 18 and 25 mM that were used in this research to study the drug antibacterial effects. The clinical manifestations of arthritis were appeared by the expression of beta-galactosidase by *E. coli*. In addition decrease of *E. coli* strain in the intestinal of the patients with ulcerative colitis cause to increase in the amount of non-normal flora bacteria even pathogenic strains in the intestinal tract[31]. Because colicin proteins expressed by *E. coli* inhibit bacterial growth of other species[32]. The growth of *E. faecalis*, Gram positive strain, was affected by all of the concentrations of verapamil hydrochloride. The drug affected the growth processes of these two bacterial strains, leading to disruption of intestinal homeostasis. This can lead to disbalance in the gastrointestinal tract, causing dysbiosis, change or deterioration in the structure of the microbiota, resulting in serious metabolic and inflammatory disorders[33].

Conclusion

Verapamil hydrochloride showed a significantly high antibacterial effect on the bacterial strains *Enterococcus faecalis* and *Escherichia coli* from human intestinal flora. The results of this study have shown that the drug did not break double helix DNA even in the presence of H_2O_2 and ascorbic acid. Further, the drug did not show any protective effect on the supercoiled plasmid DNA against damaging hydroxyl radicals. In addition, the drug made remarkable hydrophobic bonds with double strand DNA. Therefore, the cytotoxic and genotoxic effects of the drug can be attributed not to its direct effect on DNA, but to the metabolite of the drug.

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Conflict of interest

Authors declare no competing interests.

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