RESEARCH ARTICLE

The biological effects of vanadyl curcumin and vanadyl diacetylcurcumin complexes: the effect on structure, function and oxidative stability of the peroxidase enzyme, antibacterial activity and cytotoxic effect

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Abstract
Curcumin has multiple pharmacological effects, but it has poor stability. Complexation of curcumin with metals improves its stability. Here, the effects of vanadyl curcumin and vanadyl diacetylcurcumin on the function and structure of horseradish peroxidase enzyme were evaluated by spectroscopic techniques. Cytotoxic effect of the complexes was also assessed on MCF-7 breast cancer, bladder and LNCaP prostate carcinoma cell line. The results showed that the complexes improve catalytic activity of HRP, and also increase its tolerance against the oxidative condition. The result also indicated that the affinity of HRP for hydrogen peroxide substrate decreases, while the affinity increases for phenol substrate. Circular dichroism and fluorescence spectroscopies showed that compactness of the enzyme structure around the catalytic heme group and the distance between the heme group and tryptophan residue decreases after the binding. The antibacterial and cytotoxic results indicated that the complexes have anticancer potential, but they have no considerable antibacterial activity.

Introduction
Recently, many studies have been carried out on curcumin and curcuminoid complexes with metals, because of their potential as therapeutics in a wide variety of diseases¹–³. Curcumin (diferuloylmethane), a polyphenol, is the main active component of the food flavor, turmeric (Curcuma longa)¹–³. It has a large variety of biological activities, including neuroprotective⁴,⁵, antidiabetic⁶, cytotoxic⁷, antioxidant, antiinflammatory⁸, antitumour¹–³, antimicrobial⁹, anticancer¹–³, wound healing¹⁰ and antiarthritic¹ properties. Several metallocomplexes of curcumin¹¹–¹⁵ such as vanadyl curcumin complexes¹⁴,¹⁵ have been synthesized, characterized and their biological activities evaluated. Anticancer activity and selective cytotoxicity, antiAlzheimer’s disease activity, and antioxidative/neuroprotective effects of metal curcumin complexes have been previously reported¹⁶.

Vanadium is a ubiquitous trace element that has biological function. The total content of vanadium ranges between 50 and 200 μg in the human body. Vanadium presents in the ionic form and about 90% is bound with proteins. Vanadium showed potential therapeutic value in treating diabetes¹⁷–¹⁹. In addition, a number of complexes containing vanadium as a metal centers have reported to possess a wide spectrum of antitumor properties²⁰,²¹. Vanadium is also known to have antioxidant properties¹⁹.

There are some reports on the synthesize and characterization of curcumin and curcuminoid vanadyl complexes¹⁴,¹⁵. It has been previously reported that vanadyl curcumin is more effective for inhibiting synoviocyte proliferation, smooth muscle cell growth and mouse lymphoma cell growth than curcumin alone¹⁴. Mohammadi et al. synthesized vanadyl curcumin (VO(cur)₂) and vanadyl diacetylcurcumin (VO(DAC)₂), and investigated their chemical and biological properties¹⁵.

The aim of the present study is trying to shed some light on the effects of two vanadyl curcuminoids complexes on the peroxidase enzyme. Here, we report an experimental study aimed to evaluate the effect of vanadyl curcumin and vanadyl diacetylcurcumin on...
the horseradish peroxidase (HRP) enzyme. Curcumin has been proven to inhibit numerous enzymes and some effects of curcumin are mediated through enzyme inhibition. In this investigation, a peroxidase enzyme was chosen, because on one hand both vanadyl ion and curcumin have antioxidant properties and on the other hand, peroxidase enzymes contribute to antioxidant defense of organisms by removing hydrogen peroxide (H₂O₂). Peroxidases catalyze the breakdown of hydrogen peroxide, which is one of the reactive oxygen species and dangerous to the cell. Accordingly, it is reasonable to expect that the antioxidant properties of curcumin and its derivatives may be attributed to their effects on the peroxidase enzymes.

HRP (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7), a plant peroxidase, has been studied for more than a century. It oxidizes a wide variety of substrates by hydrogen peroxide. HRP-C used in this investigation is a monomeric glycoprotein with molecular weight of 44 kDa. It contains eight N-linked carbohydrate chains and one noncovalently bound hemin moiety. It has also four disulfide bridges and two Ca²⁺ binding sites, and an extensive hydrogen bond network.

In this study, the enzymatic parameters of HRP such as Vₘₐₓ (maximum rate of reaction), Kₘ (Michaelis constant), kₖₐₜ (turnover number), Eₐ (activation energy of the enzymatic reaction), ΔΗ° (enthalpy of activation), ΔS° (entropy of activation), and ΔG° (free energy of activation) were measured in the absence and presence of vanadyl curcumin and vanadyl diacetylcuccurmin. Furthermore, the activity of the enzyme was measured at oxidative concentrations of hydrogen peroxide to evaluate effect of the complexes on oxidative stability of the enzyme. Hydrogen peroxide as a well-known oxidizing agent can inactivate enzymes by oxidizing the cysteine (–SH) or methionine residues (–SCH₃). Further aim of this work is also to evaluate structural change of the enzyme in the presence of the complexes.

In addition to investigating the enzymatic effects of the complexes, their antibacterial activity was evaluated. Curcumin has been subjected to a variety of antimicrobial investigations due to extensive traditional uses and low side effects. Tajbakhsh et al. investigated antibacterial activity of indium curcumin complexes; their result indicated that the complexes have more antibacterial effect than curcumin itself. In this study, antibacterial activity of vanadyl curcumin complexes was assessed against two bacteria species including *Escherichia coli* and *Staphylococcus aureus* by broth microdilution method.

In this investigation, anticancer effects of vanadyl curcumin and vanadyl diacetylcucurmin complexes were also evaluated. Curcumin blocks cancer cell growth in vitro and in vivo. It inhibits tumorigenesis and cell proliferation in a variety of cancer cell lines. Mohammadi et al. evaluated cytotoxicity of vanadyl curcumin complexes by MTT (methyl thiazolyldtetrazolium) assay. Testing of cell toxicity of the compounds in mouse lymphoma cell showed that the vanadyl complexes are remarkably potent than curcumin. Here, in continuing efforts toward the assessment of the anticancer activity of vanadyl curcumin complexes, the cytotoxic effect of these complexes was assessed on MCF-7 (C135) breast cancer, bladder (C5637) and LNCaP (C439) prostate carcinoma cell lines.

**Material and methods**

**Materials**

HRP (K16937816), Mueller–Hinton medium, hydrogen peroxide, MTT and trypan blue were purchased from Merck (Darmstadt, Germany). All materials for cell culture were purchased from Gibco (Carlsbad, CA). All other materials were purchased from Sigma (St. Louis, MO). Vanadyl curcumin and vanadyl diacetylcurcumin were synthesized and purified by Mohammadi et al.

**Determination of the protein concentration and the enzymatic activity**

Concentration of the enzyme was spectrophotometrically measured using an extinction coefficient of 102 mM⁻¹ cm⁻¹ at 403 nm. 4-Aminoantipyrine and hydrogen peroxide as color-generating substrates were used for the determination of the enzymatic assay by colorimetric method. Six hundred microliters reaction mixture containing 1.25 mM 4-aminoantipyrine, 85 mM phenol and 0.85 mM hydrogen peroxide was prepared in phosphate buffer 100 mM, pH 6. Fifty microliters of the enzyme (0.001 mg/mL) was then added to the substrates solution, and after 3 s, the rate of color generation at 510 nm was measured by UV M501-Camspect spectrophotometer (UK). The rate of reaction was determined using the following equation:

\[ V(\text{unit/mg}) = \frac{\Delta A_{510/min}}{6.58 \text{ mg enzyme/mL reaction mixture}}. \] (1)

Various concentrations of H₂O₂ in the range of 0.2–85 mM and the saturated concentrations of phenol and 4-aminoantipyrine were prepared to determine Kₘ (Michaelis constant) value for H₂O₂. For determination of Kₘ of phenol, the enzyme was assayed in the range of 0.013–0.78 mM phenol and the saturated concentrations of 4-aminoantipyrine and H₂O₂.

**Determination of the activation energy and thermodynamic parameters of activation**

The rate of enzymatic reaction was measured at the temperature ranged between 10 and 30 °C to determine the activation energy and thermodynamic parameters of activation. The Arrhenius plot was constructed from experimentally determined kₖₐₜ values. The plot was used to calculate Eₐ as follows:

\[ E_a = -R \times \text{slope of the Arrhenius plot}. \] (2)

ΔG° was determined by analysis of the Eyring plot (ln(kₖₐₜ/T) versus 1/T) as follows:

\[ \Delta G^o = -R \times \text{slope of the Eyring plot}. \] (3)

The activation enthalpy ΔH° and ΔS° at temperature T were calculated using the following equations:

\[ \Delta H^o = E_a - RT, \] (4)

\[ \Delta S^o = \frac{\Delta H^o - \Delta G^o}{T}. \] (5)

**Determination of the enzyme stability under the oxidative condition**

In order to investigate the effect of oxidative condition on the enzyme activity, it was assayed at the high concentrations of H₂O₂. As noted, the optimum concentration of H₂O₂ as substrate is 0.85 mM; the higher concentrations of H₂O₂ cause to decrease in activity of the enzyme, because H₂O₂ is also an oxidizing agent. Accordingly, we assayed the enzyme in the range of 0.78–0.62 mM H₂O₂ to investigate the effect of complexes on the stability of HRP under the oxidative condition.

**Circular dichroism**

Circular dichroism measurements were performed on an Aviv Model 215 Circular dichroism Spectrometer (Lakewood, NJ) at room temperature. The spectra of near-UV and visible regions...
were recorded in a quartz cell of 1 cm path length. In all measurements, the enzyme concentration was 0.22 mg/mL. The molar ellipticity was determined as:

\[
\theta = \frac{\theta_0 \times 100 \text{MRW}}{c \times l},
\]

where \(c\) is the enzyme concentration in milligram per milliliter, \(l\) is the light path length in centimeters, MRW is the mean residue weight of HRP and \(\theta\) is the observed ellipticity in degrees.

**Fluorescence spectroscopy**

Fluorescence spectra of the protein were obtained at room temperature using a Varian Cary Eclipse fluorescence spectrophotometer (Australia) in a quartz cell of 1 cm path length in 20 mM phosphate buffer, pH 6. The excitation wavelength was separately set at 280 and 295 nm to record the intrinsic fluorescence spectra of HRP. The synchronous fluorescence spectra were obtained using \(\Delta \lambda = 60\) nm and \(\Delta \lambda = 15\) nm. Concentration of the protein was 0.05 mg/mL.

**Cell culture and cytotoxicity assay**

MCF-7 (C135) breast cancer, bladder (C5637) and LNCaP (C439) prostate carcinoma cell lines were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium, supplemented with 10% fetal bovine serum and 501U/mL penicillin and 50 \(\mu\)g/mL streptomycin in a CO\(_2\) incubator at 37\(^\circ\)C. One hundred microliters of the cell suspension containing 10\(^6\) cells/mL was added to each well of the plate. A 2 mL of the cell suspension containing 10\(^6\) cells/mL was added to each well of the plate. The desired concentrations of the complexes were then added to the cell culture medium. Next, the plate was incubated at 37\(^\circ\)C for overnight. MTT solution (5 mg/mL) was then added to each well and the plates incubated for 4 h at 37\(^\circ\)C. Absorbance of the samples was measured at 630 nm with enzyme-linked immunosorbent assay (ELISA) plate reader, after the plate was spun down for 15 min at low speed, for dissolution of formazan crystals.

**Antibacterial susceptibility testing**

Antibacterial activity of the complexes was investigated using dilution test method. One of the bacterial colonies was selected and grown in 2 mL Mueller-Hinton broth (MHB) medium at 37\(^\circ\)C until the turbidity of 0.5 McFarland was achieved. Then, the stock solution of the complex was prepared in 20 mM phosphate buffer and poured into the sterile tube. Next, twofold serial dilutions were carried out. After that, the 0.5 McFarland standards was diluted 1000 times and 100 \(\mu\)L of the diluted suspension was poured into the sterilized tubes and all the tubes were incubated for 1 h at 25\(^\circ\)C. Finally, 100 \(\mu\)L sterile MHB medium was poured into each sterile tube and all the tubes were incubated overnight (12–16 h) at 35–37 \(^\circ\)C. In each experiment, one positive control consisted of inoculums, buffer and MHB and one negative control consisted of buffer and MHB also were incubated. Minimal inhibitory concentration (MIC) was determined as the lowest concentration of the complexes displaying no visible growth in the tubes.

**Results and discussion**

**Circular dichroism**

Circular dichroism and fluorescence spectroscopy were used to evaluate structural changes of HRP contributing to interaction with the complexes. Near-UV CD spectra give information on the tertiary structure of proteins. In the visible region, the polypeptide chain has no absorbance, although heme, the catalytic group of HRP, absorbs visible light. Therefore, visible CD spectra of HRP give information on the tertiary structure of HRP around the heme catalytic center. It is noteworthy that free form of heme is optically inactive compound; while in asymmetric environment of the protein it becomes optically active.

Near-UV and visible CD spectra of HRP in the absence and presence of 1:1 molar ratio of the complexes to the enzyme are presented in Figure 1. As shown, HRP has a negative peak at 290 nm and a positive peak at 260 nm. Intensity of the negative peak remarkably increases in the presence of vanadyl diacetylcurcin, probably indicating that the complex increases rigidity of the environment in vicinity of (some) Trp and Tyr residues. In addition, the negative peak shifts to the lower wavelengths; this blue shift may result from a decrease in polarity of the microenvironment around (some) Trp and Tyr residues.

As shown in Figure 1(A), the negative peak, intensity of the positive one at 260 nm decreases, probably indicating that rigidity of the environment in vicinity of (some) Phe residues increases in the presence of VO(DAC)\(_2\). As shown in Figure 1(B), in the presence of another complex, vanadyl diacetylcurcin, the near UV CD spectrum of the protein is relatively monotonous implying considerable changes in the environmental asymmetry of aromatic amino acid residues.

Circular dichroism spectra of the enzyme in the visible region are shown in Figure 1(B). Obviously, intensity of the enzyme spectrum remarkably decreases upon interaction of the enzyme with vanadyl diacetylcurcin. A slight decrease and meaningful red shift is also shown after binding of the enzyme to vanadyl curcin. These changes indicate that the complexes influence on the heme microenvironment of HRP. It is more likely that an increase in the polarity and/or a decrease in rigidity of the microenvironment in the vicinity of the heme catalytic center occur in the presence of the complexes.

**Fluorescence spectroscopy**

Fluorescence spectroscopy is a sensitive technique capable of giving information about various aspects of the structure and dynamics of proteins. Intensity of intrinsic fluorescence...
of HRP is dependent on the structural change and/or the fluorescence energy transfer from tryptophan to heme. Heme quenches emission of Trp residue. In the fluorescence measurements, HRP was excited at 280 and 295 nm. The fluorescence emission of the protein excited at 295 nm arises solely from the only one tryptophan (Trp117) residue of HRP, but the emission of the protein excited at 280 nm arises from Trp and Tyr residues.

Fluorescence spectra of HRP excited at 280 and 295 nm were obtained at the fixed concentration of the enzyme (1 \( \mu \)M) and various concentrations of vanadyl diacetylcurcumin (Figure 2) and vanadyl curcumin (Figure 3) in the range of 0–20 \( \mu \)M. As shown in Figure 2(A), fluorescence intensity of the enzyme excited at 280 nm decreases with increasing concentration of vanadyl diacetylcurcumin. This change is also shown, when the enzyme is excited at 295 nm (Figure 2B). The intrinsic emission spectra of the enzyme in the presence of vanadyl curcumin are shown in Figure 3. The quenching effect is observed again at the both exciting wavelengths, although the spectra are not quenched uninterruptedly upon binding to vanadyl curcumin; obviously, emission of the spectra is quenched till 7 \( \mu \)M vanadyl curcumin, intensity of the spectra then increases and \( \lambda_{\text{max}} \) shifts to the lower wavelengths. The blue shift is more obvious, when the only Trp residue of HRP is selectively excited at 295 nm. The decrease in intensity of the spectra may occur as a consequence of the quenching effect of heme group. When the distance between the Trp residue and heme group reduces, the quenching effect of heme on the Trp emission is strengthened, causing to a decrease in the intensity of the spectra. The blue shift in \( \lambda_{\text{max}} \) may result from the exposure of the Trp and/or Tyr residues to a more hydrophobic environment.

The change in conformation of HRP can be also demonstrated by synchronous fluorescence spectra. Synchronous fluorescence spectroscopy gives information about the structural changes of proteins, when either tyrosine or tryptophan present in polypeptide chain. The synchronous fluorescence gives the characteristic information of tyrosine and tryptophan residues at \( \Delta \lambda = 15 \) nm and \( \Delta \lambda = 60 \) nm, respectively.

Synchronous spectra for the excitation of tryptophan and tyrosine in the presence of vanadyl curcumin (Supplementary data, Figure 1S) showed a gradual decrease in the intensity upon addition of 1–7 \( \mu \)M vanadyl curcumin to the protein solution. From this point forward, the intensity of emission increases. At \( \Delta \lambda = 15 \), the change of intensity is concomitant with remarkable red shift, while at \( \Delta \lambda = 60 \), no meaningful shift is observed. This result agrees with the result of intrinsic fluorescence of the enzyme at the excitation of 280 and 295 nm; similar to the intrinsic fluorescence, the change in the synchronous spectra can be explained by the distance between heme group and Trp residue and the change in the microenvironment of Tyr and Trp residues. Synchronous spectra for vanadyl diacetylcurcumin (Supplementary data, Figure 2S), another complex, show that the intensity of synchronous spectra of both Trp and Tyr residues decreases after addition of the complex. A red shift is also observed for the spectra of Tyr residues, while no shift is shown in the spectra of Trp residue. This change probably indicates that the average environment of Tyr residues become relatively more hydrophilic after the enzyme binds to vanadyl diacetylcurcumin. It is also possible that the average accessible surface area of these residues increases after the interaction with vanadyl diacetylcurcumin.

**Effect of VO(cur)\(_2\) and VO(DAC)\(_2\) on the catalytic activity of HRP**

In order to investigate the effect of complexes on the activity of HRP, the complex was mixed with the oxidizing substrate (phenol + 4-aminoantipyrine), hydrogen peroxide as the other substrate was then added and the rate of the enzymatic reaction was measured. Concentration of the complexes varied in the range of 0–10 \( \mu \)M. All experiments were carried out at phosphate buffer 100 mM, pH 6. This pH value was chosen for the experiments, because curcumin is unstable at neutral and basic pH and
decomposes to the other compounds, but under acidic conditions, the degradation of curcumin is much slower\textsuperscript{22,23}. It is noteworthy that the optimum pH of HRP is also in the range of 6–6.5\textsuperscript{50}. The percentage of the enzyme activity at the various concentrations of the complexes is shown in Figure 4. The enzyme activity without the complexes was considered 100\%. Obviously, the activity of the enzyme significantly increases with increasing the concentrations of the both complexes. The result also indicates that vanadyl diacetylcurcumin is almost two times more effective than vanadyl curcumin; for example, the activity increases 23\% and 40\% at 1\(\mu\)M concentration of vanadyl curcumin and vanadyl diacetylcurcumin, respectively.

**The effect of VO(cur)\(_{2}\) and VO(DAC)\(_{2}\) on the stability of HRP under the oxidative condition**

In order to evaluate the stability of HRP under the oxidative condition, the enzyme was assayed at various concentrations of \(\text{H}_2\text{O}_2\) as an oxidative agent. \(\text{H}_2\text{O}_2\) is also one of the substrate of HRP and reduced to \(\text{H}_2\text{O}\) by the enzyme. As reported in the "Methods" section, the optimum concentration of \(\text{H}_2\text{O}_2\) is 0.85\(\mu\)M for the enzymatic assay of HRP. Our previous result demonstrated that the activity of the enzyme decreases above the optimum concentration of \(\text{H}_2\text{O}_2\)\textsuperscript{39}. It has been reported that \(\text{H}_2\text{O}_2\) can oxidize the cysteine (–SH) or methionine residues (–SCH\(_3\)), and inactivate enzymes by oxidizing their thiol groups\textsuperscript{31}. Accordingly, we measured activity of the enzyme at the optimum concentration of \(\text{H}_2\text{O}_2\) (0.78\(\mu\)M) as the control and the higher concentrations of \(\text{H}_2\text{O}_2\) in the range of 1.5–6.2\(\mu\)M. The rate of the enzyme reaction versus concentration of \(\text{H}_2\text{O}_2\) is presented in Figure 5. The rate of reaction was considered as 100\% at the optimum concentration of \(\text{H}_2\text{O}_2\). As shown, activities of all samples exponentially decrease with increasing concentration of \(\text{H}_2\text{O}_2\), although in the presence of the complexes, the enzyme is more stable under the oxidizing condition. Obviously, the remaining activity of the enzyme in the presence of the different concentrations of vanadyl curcumin (Figure 5A) and vanadyl diacetylcurcumin (Figure 5B) is more than that in the absence of the complexes.

In order to evaluate the stability of the enzyme under the oxidative condition, the rate of enzymatic reaction was also measured at different concentrations of the both complexes in the range of 0–10\(\mu\)M. In this experiment, oxidizing concentration of hydrogen peroxide was 4\(X\) (\(X\) is the optimum concentration of \(\text{H}_2\text{O}_2\) for the enzymatic assay). The result is shown in Figure 6.

Percentage of decrease in activity was determined as:

\[
\text{Decrease in activity (\%) = } \frac{V_X - V_{4X}}{V_0} \times 100,
\]

where \(V_{4X}\) and \(V_X\) are the rate of enzymatic reaction at 4\(X\) and \(X\) concentration of \(\text{H}_2\text{O}_2\) at each concentration of the complexes and \(V_0\) is the rate of enzymatic reaction in the absence of the complex. As shown, the activity of the enzyme decreases in the absence and presence of the complexes under the oxidative condition, but the complex reduces percentage of the decrease in activity of the enzyme under this condition.

\(\text{IC}_{50}\) [half minimal (50\%) inhibitory concentration (IC)] of hydrogen peroxide was also calculated for quantitative analysis of the oxidative stability. It indicates how much of hydrogen peroxide is needed to inhibit the enzyme by half. The values of \(\text{IC}_{50}\) are presented in Table 1. As shown, the presence of the both complexes leads to increase in \(\text{IC}_{50}\) values.

**The effect of VO(cur)\(_{2}\) and VO(DAC)\(_{2}\) on the kinetic parameters of the enzymatic reaction**

The enzymatic parameters including \(V_{\text{max}}\), \(k_{\text{cat}}\) and \(K_m\) for the both substrates were measured in order to evaluate the effect of complexes on the kinetics of the enzymatic reaction. In order to calculate \(K_m\), the experimental data are plotted on Eadie–Hofstee plots (Supplementary data, Figures 1S and 2S)\textsuperscript{51}. The values of \(V_{\text{max}}\), \(k_{\text{cat}}\) and \(K_m\) at 0, 0.5 and 3\(\mu\)M concentration of vanadyl curcumin and vanadyl diacetylcurcumin are, respectively, summarized in Tables 2 and 3. As presented, the rate of enzymatic reaction increases upon interaction of the enzyme with the both complexes. For example, \(V_{\text{max}}\) and \(k_{\text{cat}}\) values at 3\(\mu\)M vanadyl curcumin and vanadyl diacetylcurcumin are, respectively, 1.4-and 1.7-fold higher than that of the enzyme without the complex. The
$K_m$ values for hydrogen peroxide substrate increase suggesting that the affinity of the enzyme to hydrogen peroxide decreases due to its interaction with the complexes\(^3\). As shown in Tables 2 and 3, unlike the hydrogen peroxide case, the $K_m$ value for phenol decreases due to the interaction of vanadyl curcumin and vanadyl diacetylcurcumin with the enzyme indicating higher affinity for the substrate towards the enzyme\(^3\). As shown in tables, catalytic efficiency for hydrogen peroxide decreases, while it increases for phenol in the presence of the both complexes.

The effect of VO(cur)\(_2\) and VO(DAC)\(_2\) on the activation energy of enzymatic reaction and the enthalpy, entropy and free energy of activation

The thermodynamic parameters for the enzymatic reaction were calculated\(^3\) by measuring activities at different temperatures in the range of 10–30 °C. The Arrhenius and Eyring plots were plotted to calculate activation energy of the enzymatic reaction and free energy of activation (Supplementary data, Figure 5S). Activation energy of enzymatic reaction ($E_a$), free energy ($\Delta G^a$), enthalpy ($\Delta H^a$) and entropy ($\Delta S^a$) of activation are summarized in Table 4. As shown, $E_a$ was found to be 57.1 kJ mol\(^{-1}\) without the complexes and 39.9 and 51.6 kJ mol\(^{-1}\) in the presence of vanadyl curcumin and vanadyl diacetylcurcumin, respectively. The values indicate that $E_a$ decreases upon interaction of the enzyme with both complexes; obviously, unlike vanadyl curcumin, vanadyl diacetylcurcumin has slight effect on the activation energy of the enzyme. The result demonstrates that the complexes accelerate velocity of the chemical reactions by stabilizing the transition state of the reaction\(^3\). Therefore, the increase in the rate of enzymatic reaction ($V_{max}$) observed in the presence of the complexes can be explained by the change of activation energy. The change in $\Delta G^a$ and $\Delta H^a$ is similar to $E_a$; a slight decrease upon the interaction with vanadyl diacetylcurcumin and noticeable decrease upon interaction with another vanadyl complex. The lower enthalpy value of the enzyme in the presence of the complexes especially vanadyl curcumin describes that formation of the transition state or activated complex between the enzyme and substrate is more efficient. Likewise, lower $\Delta G^a$ value suggests that conversion of its transition complex into products is more spontaneous after the enzyme binds to the complexes. As shown in Table 4, the change in $\Delta S^a$ is negligible, so the reaction is enthalpy driven.

Antibacterial activity and cytotoxicity of VO(cur)\(_2\) and VO(DAC)\(_2\)

The cytotoxicity of vanadyl curcumin and vanadyl diacetylcurcumin toward MCF-7 (C135) breast cancer, bladder (C5637) and LNCaP (C439) prostate carcinoma cell lines was assayed using MTT assay. The percentage of viable cells at different concentrations of vanadyl curcumin and vanadyl diacetylcurcumin is

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**Figure 6.** Percentage of decrease in activity of HRP on the oxidative condition (at 3.12 μM concentration of H\(_2\)O\(_2\) that is four-fold more than the optimum concentration of H\(_2\)O\(_2\) for the enzymatic assay of HRP) at various concentrations of vanadyl curcumin (VO(cur)\(_2\)) and vanadyl diacetylcurcumin (VO(DAC)\(_2\)).

**Table 1.** IC\(_{50}\) values (μM) of hydrogen peroxide in absence (control) and presence of 1, 2 and 3 μM vanadyl curcumin (VO(cur)\(_2\)) and vanadyl diacetylcurcumin (VO(DAC)\(_2\)).

|          | VO(cur)\(_2\) | VO(DAC)\(_2\) |
|----------|---------------|---------------|
| Control  | 2.8           | 3             |
| 1 μM     | 5.2           | 3.2           |
| 2 μM     | 6.7           | 4.1           |
| 3 μM     | 6.7           | 4             |

**Table 2.** Michaelis constant for H\(_2\)O\(_2\) substrate ($^1K_m$) and phenol substrate ($^2K_m$), maximum rate of the enzymatic reaction ($V_{max}$), catalytic rate constant ($k_{cat}$) and catalytic efficiency ($k_{cat}/K_m$) of HRP in the absence (control) and presence of 0.5 and 3 μM vanadyl curcumin (VO(cur)\(_2\)).

| VO(Cur)\(_2\) | $^1K_m$ (mM) | $^2K_m$ (mM) | $V_{max}$ (U/mg) | $k_{cat}$ (s\(^{-1}\)) | $k_{cat}/^1K_m$ (s\(^{-1}\)mM\(^{-1}\)) | $k_{cat}/^2K_m$ (s\(^{-1}\)mM\(^{-1}\)) |
|--------------|-------------|-------------|-----------------|-------------------------|---------------------------------|---------------------------------|
| 0 μM         | 0.11 ± 0.01 | 5.6 ± 0.8   | 395 ± 23        | 288 ± 17               | 2618                            | 51                              |
| 0.5 μM       | 0.24 ± 0.03 | 4.6 ± 0.4   | 450 ± 57        | 328 ± 42               | 1366                            | 71                              |
| 3 μM         | 0.36 ± 0.03 | 3.5 ± 0.5   | 560 ± 24        | 408 ± 17               | 1133                            | 116                             |

**Table 3.** Michaelis constant for H\(_2\)O\(_2\) substrate ($^1K_m$) and phenol substrate ($^2K_m$), maximum rate of the enzymatic reaction ($V_{max}$), catalytic rate constant ($k_{cat}$) and catalytic efficiency ($k_{cat}/K_m$) of HRP in the absence (control) and presence of 0.5 and 3 μM vanadyl diacetylcurcumin (VO(DAC)\(_2\)).

| VO(DAC)\(_2\) | $^1K_m$ (mM) | $^2K_m$ (mM) | $V_{max}$ (U/mg) | $k_{cat}$ (s\(^{-1}\)) | $k_{cat}/^1K_m$ (s\(^{-1}\)mM\(^{-1}\)) | $k_{cat}/^2K_m$ (s\(^{-1}\)mM\(^{-1}\)) |
|---------------|-------------|-------------|-----------------|-------------------------|---------------------------------|---------------------------------|
| 0 μM          | 0.120 ± 0.006 | 5.15 ± 0.20 | 386 ± 21        | 282 ± 15               | 2350                            | 55                              |
| 0.5 μM        | 0.290 ± 0.007 | 3.80 ± 0.35 | 501 ± 28        | 367 ± 20               | 1262                            | 96                              |
| 3 μM          | 0.46 ± 0.02  | 3.95 ± 0.5  | 560 ± 14        | 409 ± 10               | 889                             | 225                             |
depicted in Figures 7 and 8, respectively. Microscopic images of the three cell lines after treatment with vanadyl curcumin are also shown in Supplementary data (Figures 6S, 7S and 8S). Obviously, the both complexes can effectively inhibit proliferation of the cell lines. There is also no substantial difference between cytotoxic effect of vanadyl curcumin and vanadyldiacetyl curcumin. The both complexes, in general, induce a substantial loss of viability of bladder cell line.

As noted, the antibacterial activity of the complexes was evaluated using dilution test method. Experiments were performed using Gram-negative and Gram-positive organisms, namely, *E. coli* (KCTC-1924) and *S. aureus* (KCTC-1916), respectively. The bacterial growth was estimated by measuring the turbidity (OD at 630 nm) of the samples (Supplementary data, Figures 9S and 10S). In addition, a standard loop was dipped into the tubes containing the samples; a loopful of the suspension was removed, subcultured to a Mueller-Hinton Agar plate and incubated at 37°C for 24 h (Supplementary data, Figures 11S, 12S and 13S). The results indicate that vanadyl curcumin inhibits *E. coli* and *S. aureus* growth, but vanadyldiacetyl curcumin only inhibits *E. coli* growth in a dose-dependent manner; obviously, vanadyl diacetylcurcumin has no antibacterial activity against *S. aureus*. MIC values of vanadyl curcumin for the both organisms and vanadyl diacetylcurcumin for the Gram-negative one were found as 200 μM. Bacterial colonies were shown in the plates at 200 μM concentrations of the complexes indicating MBC is more than 200 μM. As is well known, MIC was defined as the lowest concentration that completely inhibited visible bacterial growth and the minimum bactericidal concentration (MBC) is defined as the lowest concentration of antibacterial agent at which no visible bacterial growth (colony) is observed after subculturing.

**Conclusion**

In this investigation, the effect of vanadyl curcumin and vanadyl diacetyl curcumin on the structure and function of a peroxidase enzyme was evaluated. The results indicated the ability of these compounds to not only activate peroxidation reaction in HRP, but also increase oxidative stability of the enzyme. Therefore, it can be concluded that the complexes on one hand improve the antioxidant activity of the peroxidase enzyme and therefore help the enzyme eliminate hydrogen peroxide more efficiently and on the other hand the complexes increase the activity under oxidative condition, so they prevent the loss of the enzyme activity even at oxidative concentrations of hydrogen peroxide. The both effects result in increasing the antioxidant efficiency of the peroxidase enzyme.

We carried out the experiments on HRP; whether vanadyl curcumin complexes can influence on the other peroxidase enzyme as well remains to be determined.

This investigation also presents the possibility that the complexes might serve as potential antitumor agents especially for bladder cancers, but they are not good antibacterial compounds, because their MIC values are too high.

**Declaration of interest**

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